Acta Oceanol. Sin., 2016, Vol. 35, No. 4, P. 51–57
DOI: 10.1007/s13131-016-0838-5
http://www.hyxb.org.cn
E-mail: hyxbe@263.net

Combined effects of temperature and copper ion concentration on the superoxide dismutase activity in *Crassostrea ariakensis*

WANG Hui[1], YANG Hongshuai[2], LIU Jiahui[2], LI Yanhong[2], LIU Zhigang[2]*

[1] School of Life Sciences, Huaiyin Normal University, Huaian 223300, China

[2] Fisheries College, Guangdong Ocean University, Zhanjiang 524088, China

Received 30 November 2014; accepted 14 September 2015

Abstract

Superoxide dismutase (SOD) is a crucial antioxidant enzyme playing the first defense line in antioxidant pathways against reactive oxygen species in various organisms including marine invertebrates. There exist mainly two specific forms, Cu/Zn-SOD (SOD1) and Mn-SOD (SOD2), in eukaryotes. SODs are known to be concurrently modulated by a variety of environmental stressors. By using central composite experimental design and response surface method, the joint effects of water temperature (18–34°C) and copper ion concentration (0.1–1.5 mg/L) on the total SOD activity in the digestive gland of *Crassostrea ariakensis* were studied. The results showed that the linear effect of temperature was highly significant (P<0.01), the quadratic effect of temperature was significant (P<0.05); the linear effect of copper ion concentration was not significant (P>0.05), while the quadratic effect of copper ion concentration was highly significant (P<0.01); the interactive effect of temperature and copper ion concentration was not significant (P>0.05); the effect of temperature was greater than that of copper ion concentration. The model equation of digestive gland SOD enzyme activity towards the two factors of interest was established, with R^2 and predictive R^2 as high as 0.961 6 and 0.820 7, respectively, suggesting that the goodness-of-fit to experimental data be very satisfactory, and could be applied to prediction of digestive gland SOD activity in *C. ariakensis* under the conditions of the experiment. Our results would be conducive to addressing the health of aquatic animals and/or to detecting environmental problems by taking SOD as a potential bioindicator.

Key words: *Crassostrea ariakensis*, superoxide dismutase, temperature, copper ion concentration, combined effect

Citation: Wang Hui, Yang Hongshuai, Liu Jiahui, Li Yanhong, Liu Zhigang. 2016. Combined effects of temperature and copper ion concentration on the superoxide dismutase activity in *Crassostrea ariakensis*. Acta Oceanologica Sinica, 35(4): 51–57, doi: 10.1007/s13131-016-0838-5

1 Introduction

Crassostrea ariakensis, which belongs in phylum Mollusca, class Bivalvia, order Pterioidae, family Ostreidae, is a eurythermic and euryhaline marine bivalve mollusk. Because of its palatable taste and high nutritional value, it has become one of the principal commercial aquaculture species along the south coast of China (Wang et al., 2014).

Commercial mollusks are mainly cultured in coastal waters and are deeply influenced by the environmental factors, of which temperature and heavy metals matter very much (Bayne, 1965). Temperature impacts on the growth, reproduction and metabolism of shellfish by modulating in vivo biological and biochemical reactions (Bougrier et al., 1995; Fearman and Moltschaniwskyj, 2010). The territory of shellfish is very limited, thereby heavy metals are easily enriched in them (Coglianese and Martin, 1981). Copper is the most toxic heavy metal to oysters, and it can retard the development of embryos and larvae (Coglianese and Martin, 1981; Jiang and Niu, 2006). The fluctuations in temperature and copper ion concentration can cause oxidative stress to varying degrees in shellfish, induce the excess production of reactive oxygen species (ROS) and give rise to cellular damage and alteration of antioxidative enzyme activity, in turn doing harm to the health of organisms (Lushchak, 2011; Maria and Bebianno, 2011).

ROS is essential products of oxygen-consuming process in organisms (Kim et al., 2007). Moderate amount of ROS can stimulate the signal transduction pathway (Pantano et al., 2006), and plays an important role in cellular growth, death and removal of pathogens (Lesser, 2006; Hooper et al., 2007; Li et al., 2010). But excess ROS can result in lipid oxidation, denaturation of proteins and nucleic acids in organisms (Lesser, 2006). In normal cases, ROS is held at a roughly identical level through a dynamic equilibrium mechanism in aquatic animals. Clearance of the excessive ROS that is caused by the sharp change in environmental stressors mainly depends on some small reductive molecules (glutathione, ascorbic acid, etc.) and a series of antioxidases (superoxide dismutase, catalase, etc.) (Livingstone, 2001). Superoxide dismutase (SOD, EC 1.15.1.1) is a crucial type of metalloenzyme that can catalyze excessive ROS into oxygen and hydrogen peroxide through the disproportionation reaction (Umasuthan et

Foundation item: The Guangdong Province Education Department under contract No. GCZX-A0909; the Guangdong Province Ocean and Fisheries Science & Technology Extension Project under contract No. 20120980; the Guangdong Province Industry-University-Science Partnership Project under contract No. 20110908; the Science & Technology Project of Huaiyin Normal University under contract No.WH0031.

*Corresponding author, E-mail: Liuzg919@126.com

al., 2012). In eukaryotes there are chiefly two forms of SOD, i.e., Cu/Zn-SOD (SOD1) and Mn-SOD (SOD2). The former exists in cytoplasm, whereas the latter exists in mitochondria. SOD functions in conjunction with catalase to maintain the oxidation-reduction equilibrium in organisms (Fridovich, 1995; An and Choi, 2010). So far the effects of only single abiotic or biotic environmental factor such as dissolved oxygen (Chen et al., 2007a), salinity (Jo et al., 2008), bacteria (Zhou et al., 2011) and virus (Tang et al., 2010; Luo et al., 2014) on the total SOD activity have been studied. Due to the effect of only single factor being investigated, the interaction between factors cannot be examined; additionally, neither were the quadratic effects examined, nor were the predictive models established in these studies.

Accommodating the fact that lots of factors act on organisms in concert rather than singly, it is necessary to examine the combined effects of some important environmental stimuli. The objective of this study was to investigate the combined effects of environmentally designed stresses (temperature and copper ion concentration) on the total SOD activity in *C. ariakensis* using the central composite design and response surface method. We have examined how the two factors affect the digestive gland SOD activity, established the reliable model of the SOD activity towards the two factors.

2 Materials and methods

2.1 *Experimental subjects*

Experimental subjects, which were provided by the oyster culture farm in Potou District, Zhanjiang, Guangdong Province, were all healthy adults of the same age with a mean shell length 6.5(±0.57) cm. After removing those exterior biofoulings, they were acclimated for 14 d in sand-filtered sea water whose heavy metal concentration met State Grade I sea water standards. Temperature and salinity during the acclimation were 25(±0.1)°C and 27(±0.3), respectively. Mixed algae (ratio 1: 1) of *Platymonas subcordiformis* and *Chlorella* sp. were fed to these subjects each day. Culture water was exchanged once daily and aerated consecutively.

2.2 *Experimental design*

In our study, the central composite design (face-centered) was employed to examine the joint effects of water temperature and copper ion centration on the digestive gland SOD activity in *C. ariakensis*. According to the results of preliminary trials, temperature was set to range from 18°C to 34°C, and copper ion concentration from 0.1 mg/L to 1.5 mg/L. When this form of central composite design is used, each factor has three coded levels in factor space, i.e., –1, 0, +1, and the star arm $|\alpha|=1$. To check the model adequacy, the number of center points was set to 3. There were 11 experimental runs (combination of the two factors). Each factorial and axial point was replicated once. The order of these experimental runs was randomized to eliminate systematic errors (Montgomery, 2005).

2.3 *Experimental management*

According to the design (Table 1), 20 oysters were cultured for each factorial and axial replicate which was carried out in plastic bucket with 50 L sand-filtered sea water. Temperature was automatically controlled by electronic heaters (EHEIM), salinity and pH were held at 25(±0.2) and 7.8(±0.1), respectively. Each day water exchange was undertaken once with its two-factor setups consistent with those corresponding regimes in Table 1. The whole experiment lasted for 30 d.

2.4 *Enzyme fluid preparation*

After the experiment ended, five oysters were taken from each replicate and shells cleared. The soft tissues were rinsed several times using distilled water. The digestive gland was separated and put in tissue homogenizer, then 9-fold volume precooled 0.9% physiological saline water (m/v) was added and ice-homogenized. The homogenized tissue fluid was decanted into centrifuge tube for 10 min centrifugation at 10°C and 3 000 r/min. The supernatant was transferred into new centrifuge tube and maintained at 4°C. Data measurement was performed within 6 h (Sun and Li, 2000).

2.6 *Enzyme activity measurement*

The Coomassie Brilliant Blue method was used to mensurate the protein contents contained in above enzyme fluid. Enzyme activity was gauged using SOD visible light reagent kit made by Nanjing Jiancheng Bioengineering Institute. All operations were carried out in accordance with the specifications written on the reagent kit numbered A001-1. As shown on this reagent kit, only the total SOD activity would be checked.

One tissue SOD unit (U) is defined as the quantity of SOD when SOD inhibition rate reaches 50% for 1 mg tissue protein in 1 mL reaction solution (Jiang and Niu, 2006; Kim et al., 2007).

2.7 *Data analysis*

Following quadratic regression model of digestive gland SOD activity in *C. ariakensis* was assumed, with temperature and copper ion concentration as the two explanatory variables in this model:

$$Y=\beta_0+\beta_1 T+\beta_2\times[Cu^{2+}]+\beta_3 T\times[Cu^{2+}]+\beta_4 T^2+\beta_5\times[Cu^{2+}]^2+\varepsilon,$$

where Y is the response, i.e., SOD activity or its transformation; β_0 is intercept; β_1, β_2 are linear effects of temperature and Cu^{2+} concentration, respectively; β_3 is the interactive effect between temperature and Cu^{2+} concentration; β_4, β_5 are quadratic effects of temperature and Cu^{2+} concentration, respectively; $[Cu^{2+}]$ is Cu^{2+} concentration; ε is random error, assuming that it conforms to the normal distribution with a mean of zero. All above effects were estimated using the least squares method, and were tested by F statistic; the model adequacy was indicated using the lack-of-fit test (F test). Statistical analysis of the experimental data was undertaken utilizing the software SAS (V9.13), and response surface and corresponding contour plots were given to show how the two factors, temperature and copper ion concentration, affect the response of interest.

3 Results

3.1 *Several explanations about results*

The SOD activity data corresponding to each experimental run are presented in Table 1. Since each factorial point (Runs 2, 6, 7, 10) and axial point (Runs 1, 3, 4, 5) were replicated, standard errors were provided with their SOD activity data. Center points (Runs 8, 9, 11) were listed separately to evaluate the model adequacy using lack-of-fit test, thereby their standard errors were not given. At the close of the experiment, death only occurred in Runs 4 and 7, and mortality was 10% and 5%, respectively. Prior to ANOVA, the Levene test showed that error variances were homoscedastic, thereby experimental data were not transformed using Box-Cox approach.

Table 1. Face-centered central composite experimental design and results

Run	Coded		Actual		Digestive gland SOD activity/U·mg^{-1}
	T	Cu^{2+}	T/°C	Cu^{2+}/mg·L^{-1}	
1	0	$-\alpha$	26	0.1	50.57±4.89
2	1	–1	34	0.1	71.66±14.62
3	$-\alpha$	0	18	0.8	70.66±6.20
4	α	0	34	0.8	81.33±9.94
5	0	α	26	1.5	54.3±3.74
6	–1	–1	18	0.1	47.69±5.27
7	1	1	34	1.5	67.48±2.92
8	0	0	26	0.8	73.68
9	0	0	26	0.8	75.65
10	–1	1	18	1.5	57.81±2.69
11	0	0	26	0.8	74.85

Notes: (1) α is the star arm, and $|\alpha|$=1 for this experimental design; (2) Two factors: temperature (T) and copper (Cu^{2+}) concentration.

Table 2. ANOVA for the model of SOD in the digestive gland of *C. ariakensis*

Source	Sum of squares	*df*	Mean square	*F* value	*P* value
Model	1 248.80	5	249.76	25.04	<0.01
Residual	49.88	5	9.98		
Lack of fit	47.92	3	15.97	16.27	0.058
Pure error	1.96	2	0.98		

Notes: R^2=0.961 6, Adj. R^2=0.923 2, Pred. R^2=0.820 7.

3.2 *Model adequacy checking*

ANOVA results for the model adequacy were listed in Table 2. It can be seen clearly that the model P<0.01, showing that the model of digestive gland SOD activity was of high statistical significance. The P value for the model lack-of-fit test was 0.058, >0.05, demonstrating that the model assumed in our study was adequate.

3.3 *Model establishment*

Model coefficients were given in Table 3. The linear effect of temperature was highly significant (P<0.01); the linear effect of copper ion concentration and the interaction between the two factors were nonsignificant (P>0.05); the quadratic effect of temperature was significant (P<0.05) and the quadratic effect of copper ion concentration was highly significant (P<0.01). In order to directly compare the magnitude of the effects of two factors, all coefficients were given in terms of coded factors. It can be seen clearly in Table 3 that the effect of temperature was far greater than that of copper ion concentration. Following model equation of the digestive gland SOD activity towards temperature and copper ion concentration was obtained:

$$Y = 64.89 - 2.785T + 78.87\left[Cu^{2+}\right] - 0.638T \times \left[Cu^{2+}\right] + 0.08T^2 - 37.48\left[Cu^{2+}\right]^2.$$

Unadjusted, adjusted and predictive coefficients of determination for the above model equation were 0.961 6, 0.923 2 and 0.820 7, respectively. Additionally, because the form of composite design used in this study has orthogonality, those nonsignificant terms can be directly deleted from the equation.

3.4 *Visualization of the combined effects of temperature and copper ion concentration on the SOD activity in the digestive gland of C. ariakensis*

Visualizations of how the two factors affected the digestive gland SOD activity in *C. ariakensis* were given in Fig. 1. It can be found distinctly in Fig. 1 that the SOD activity varied with temperature and copper ion concentration in a curvilinear manner. When temperature was held at a fixed level, SOD activity increased and then declined as copper ion concentration increased, with the inflection point occurred at ca. 0.9 mg/L. When copper ion concentration was fixed at a certain level, the SOD activity increased with increasing temperature, and was found to still increase when temperature beyond 34°C, the upper-limit temperature set up in this study; minimum SOD activity occurred at around 20°C.

4 Discussion

4.1 *Effect of water temperature on SOD activity*

It can be seen clearly in Table 3 that the linear effect of temperature on the digestive gland SOD activity in *C. ariakensis* was highly significant (P<0.01); the SOD activity gradually increased with increasing temperature over the range set up in this study (Fig. 1). There have been no reports on the effect of temperature on the SOD activity in *C.ariakensis* so far, fewer studies are found only in some other bivalves, for example, *Chlamys farreri* (Chen et al., 2007b), *Perna viridis* (Verlecar et al., 2007) and *Scapharca broughtonii* (Anand Cheol, 2010). They all reported similar findings to our results as to the effect of temperature on the SOD activity. Abele et al. (2001) pointed out that in marine mollusks thermal stress is often accompanied by oxidative stress, which can be embodied through the change in SOD activity. In suitable range of temperature, aquatic animals adapt themselves to the environmental changes through their own physiological and biochemical mechanism (Hochachka and Somero, 1984). Change of the water temperature enhances the respiration of organisms, intensifies the production of ROS and oxidative stress, in turn increasing the SOD activity (Verlecar et al., 2007; An and Choi, 2010). However, the change of temperature does not always result in increased SOD activity. For instance, when studying the ef-

Table 3. Coefficient estimates in the regression model of digestive gland SOD activity in *C. ariakensis* (95% confidence intervals (C. I.) and standard errors were also given)

Factor	Coefficient estimate (±SE)	*P* value	95% C. I.	
			Low	High
Intercept	73.16 (±1.62)	–	68.99	77.32
T	7.39 (±1.29)	0.002 3	4.07	10.70
$[Cu^{2+}]$	1.61 (±1.29)	0.266 7	–1.70	4.93
$T\times[Cu^{2+}]$	–3.58 (±1.58)	0.073 0	–7.63	0.48
T^2	5.19 (±1.98)	0.047 3	0.09	10.29
$[Cu^{2+}]^2$	–18.37 (±1.98)	0.000 2	–23.47	–13.27

Notes: The coefficient estimates were given in terms of coded factors.

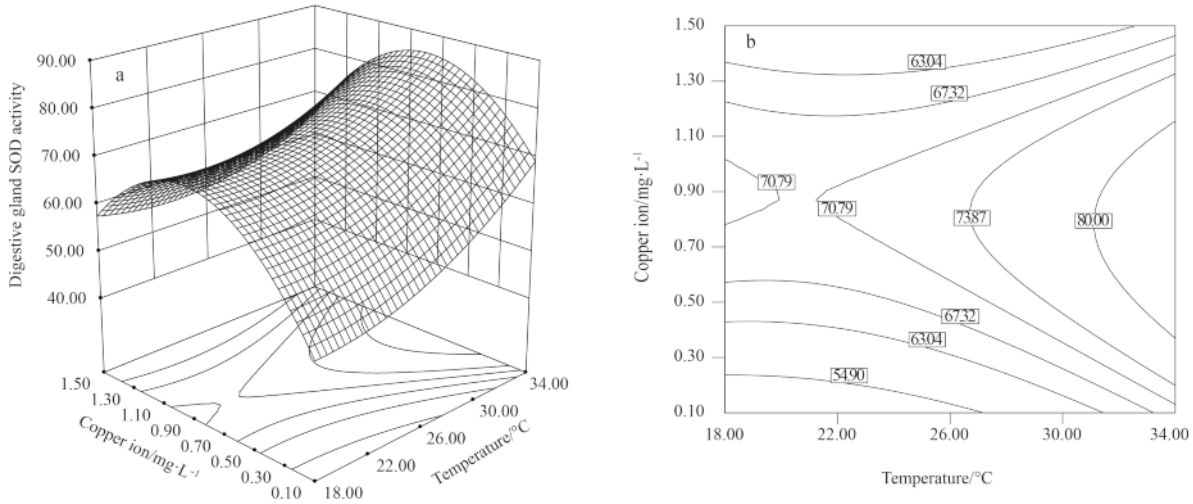

Fig. 1. Response surface (a) and contour plot (b) for the combined effects of temperature and Cu^{2+} concentration on the SOD activity in the digestive gland of *C. ariakensis*.

fect of water temperature on the immunity of *Chamelea gallina*, Monari et al. (2007) found that the SOD activities at 30°C and 20°C were lower than at 25°C after 7 d of temperature stimulation. This finding is distinct from ours. This may be caused by the difference in species, tissue and age (size), etc. Although in our study the linear effect of temperature was found significant, it was not enough to describe its effect on the SOD activity. The quadratic effect of temperature on the SOD activity was also significant (Table 3), showing that there is a peak value for the influence of temperature on the SOD activity, i.e., at a certain point beyond 34°C the SOD activity will arrive at its maximum and subsequently begin to decline because temperature has lied out of the optimal range for this species. The SOD activity still increases at temperatures beyond 34°C, the upper limit set up in our study. The thermal stability of serum SOD in *Chlamys farreri* was shown to be very high, and its activity was still strong when kept at 80°C for 30 min. But as temperature continued to increase, the SOD activity no longer lasted (Sun and Li, 2000). The increase of antioxidase activity resulting from high temperatures to scavenge excess ROS in organisms is short-lived, and the activity of antioxidases will gradually decrease over time (Parihar et al., 1997). Wang et al. (2012) found that the SOD activity of *C. farreri* under high temperature exposure increased notably at 48 h, but decreased markedly at 96 h.

4.2 *Effect of copper ion concentration on SOD activity*

So far in terms of the effect of copper ion on the shellfish SOD activity, studies are mostly to investigate the temporal change of SOD activity with copper ion held at a specific concentration (Isani et al., 2003; Vlahogianni and Valavanidis, 2007; Gomes et al., 2012), little is known about the change of shellfish SOD activity with varying copper ion concentrations. In the present study, the linear effect of copper ion concentration on the digestive gland SOD activity in *C. ariakensis* was found to be nonsignificant ($P>0.05$), but the quadratic effect was highly significant ($P<0.01$) (Table 3), indicating that in the range of copper ion concentration set in this study the digestive gland SOD activity varies with copper ion concentration in a curvilinear fashion, this also can be distinctly seen in Fig. 1. Analogous results have been reported in *Crassostrea rivularis* (Jiang and Niu, 2006), *Pinctada fucata* (Jing et al., 2006, 2007), *Macrobrachium rosenbergii* (Li et al., 2008) and *Mytilus coruscus* (Li et al., 2012). Lower-concentration heavy metal ions in water can evoke the production of lots of reactive oxygen species in shellfish, and the oxidative stress induced will be eliminated by increased SOD activity (Koutsogiannaki et al., 2014). When the concentration of water heavy metal ions is higher, DNA structure may be damaged by the excess reactive oxygen species from enhanced oxidative stress in shellfish, thus resulting in decrease of the SOD activity (Li and Fang, 1993; Luo et al., 2014). In addition, the variation of the SOD activity with copper ion concentration is tissue-specific. For example, Li et al. (2012) found that the SOD activity in *M. coruscus* varied with such tissue as sex gland, gill and adductor muscle. The quadratic effect of copper ion concentration on the SOD activity was highly significant (Table 3), demonstrating that there exist a maximum for the SOD activity. In Fig. 1 the copper ion concentration that corresponds to the maximal SOD activity is ca. 0.9 mg/L. Due to the stimulation from low-concentration copper ions (hormesis ef-

fect), excess ROS are produced, which results in increased SOD activity (Stebbing, 1982). As copper ion concentration increases, copper ions also accumulate in tissues of shellfish (Wu et al., 2005). As ROS-producing catalyzer (Chan et al., 1982), coupled with the oxidative stress induced, free copper ions combine with the thiol groups of cysteine, thereby leading to the decreased SOD protein activity (Isani et al., 2003). Li et al. (2008) reported that there prevalently exist the acidic isoforms of SOD (SOD-3) in *M. rosenbergii*. Although bioactive, SOD-3 is subject to inactivation resulting from the oxidation of ROS.

4.3 *Interactive effect of temperature and copper ion concentration on SOD activity*

The effect of single factor on the SOD activity in shellfish has been mostly studied thus far (Chen et al., 2007a, b; Jo et al., 2008; Zhou et al., 2011; Luo et al., 2014). Demerits of these monofactor studies are their inability to examine the quadratic effects of, and the interactive effects between multiple factors or stimuli, and their inability to construct the reliable and predictable models. Temperature impinges on the absorption of heavy metals into mollusks by changing the chemical and dynamic characteristics of water body (Mubiana and Blust, 2007). The shellfish SOD activity also varies with season and salinity in heavy metals-polluted sea areas (Vlahogianni et al., 2007). This shows that the between-factors interaction should not be ignored and they are of great significance (Montgomery, 2005). In our study the interactive effect between temperature and copper ion concentration on the SOD activity was statistically tested and found to be nonsignificant (P>0.05) (Table 3), indicating that the two factors affect the digestive gland SOD activity of *C. ariakensis* in an independent fashion or no factorial synergism or antagonism occurs within the set factor ranges. However, according to Verlecar et al. (2007), temperature does change the active oxygen metabolism by adjusting antioxidant enzyme activities in *Perna viridis*, which can be employed as biomarker to detect sublethal effects of pollution. Maria and Bebianno (2011) found the significant interaction between copper ion concentration and benzo(a)pyrene (BaP) on the antioxidase activity in *M. galloprovincialis*. Wang et al. (2012) also reported that the complicated interactions among host, pathogen and environment were the main causes for the mass mortality of cultured *Chlamys farreri* during summer period. Although Geret et al. (2002), Jing et al. (2007), Banni et al. (2014) investigated the effects of two factors on the SOD activity in shellfish, no interactions were examined, nor were models given in these studies. Additionally, the expression of SOD genes was also influenced by the interaction between biotic factors such as bacteria and virus (De Zoysa et al., 2011).

4.4 *Model establishment*

In the present study the model of digestive gland SOD activity towards water temperature and copper ion concentration in *C. ariakensis* was established, with the unadjusted and adjusted coefficients of determination as high as 0.961 6 and 0.923 2, respectively, showing that the model has excellent goodness of fit to experimental data. Although interactions were examined in those studies by Verlecar et al. (2007), Maria and Bebianno (2011) and Wang et al. (2012), models were not built. In our study the predictive coefficient of determination was 0.820 7, this shows that the model can be used for practical projection of digestive gland SOD activity in *C. ariakensis* under the conditions in this study. It is obvious that the model built in this study lends itself to testing the environmental pollution by utilizing digestive gland SOD activity as an indicator and to the healthy culture of *C. ariakensis*.

Some complicated actions such as antagonism or synergism among heavy metal ions may occur for their accumulation in shellfish in natural water environment (Kargin and Çoğun, 1999). For instance, Solé et al. (1995) reported that not only were the seasonal change of oxidative enzymes in *M. galloprovincialis* influenced by the pollutants in sea area, but also was associated with some ambient parameters such as salinity, dissolved oxygen and suspended matters. In this sense the SOD activity in *C. ariakensis* should be affected by other ambient factors simultaneously than temperature and copper ion concentration in the culture environment of this species. It is not unnecessary for the combined effects of more factors on the antioxidase activity in *C. ariakensis* to be further investigated.

According to the specifications given on the reagent kit (numbered A001-1) produced by Nanjing Jiancheng Bioengineering Institute, only the total activity of digestive gland SOD was analyzed using this type of reagent kit. As is well known that SODs can be typically divided into two types in eukaryotes, i.e., SOD1 and SOD2, the former exists in cytoplasm, whereas the latter exists in mitochondria (Li et al., 2010; Umasuthan et al., 2012). Therefore, in our study the total activity of digestive gland SOD is that of SOD1 and SOD2 combined. The activity of a specific form such as SOD1 or SOD2 can be checked using the reagent kit numbered SOD A001-2. However, this is open to further study.

5 Conclusions

In the present study, we examined the joint effects of water temperature and copper ion concentration on the total SOD activity in digestive gland of *C. ariakensis* using the central composite experimental design and response surface method. The results showed that the simultaneous variation of temperature and copper ion concentration does alter the activity of antioxidant enzyme SOD by modulating active oxygen species metabolism, and the digestive gland SOD activity in *C. ariakensis* varies significantly with temperature and aqueous copper in a curvilinear fashion. The effect of temperature was far greater than that of copper ion concentration. No presence of the interaction between the two factors indicates that temperature does not modify the effect of copper on the digestive gland SOD activity. The reliable model equation of SOD activity in the digestive gland of *C. ariakensis* towards the two factors of interest could be used to predict the degree of oxidative stress caused by aqueous copper under the conditions of the study. It must be pointed out, however, that only enzymatic indicator might not suffice to mirror the change at the whole organismic level. A more general conclusion should be predicated upon the comprehensive examination of the combined impacts of more environmental stressors at varying levels of organisms.

Acknowledgements

All the biological materials used in this experiment were provided by the Potou Oyster Culture Farm of Zhanjiang City, Guangdong Province. Several technicians in the aquafarm afforded some good suggestions as to the culture during the acclimation of this species. The authors are indebted to them for their support herein.

References

Abele D, Tesch C, Wencke P, et al. 2001. How does oxidative stress re-

late to thermal tolerance in the Antarctic bivalve *Yoldia eightsi*?. Antarctic Science, 13(2): 111–118

An M I, Choi C Y. 2010. Activity of antioxidant enzymes and physiological responses in ark shell, *Scapharca broughtonii*, exposed to thermal and osmotic stress:effects on hemolymph and biochemical parameters. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 155(1): 34–42

Banni M, Hajer A, Sforzini S, et al. 2014. Transcriptional expression levels and biochemical markers of oxidative stress in *Mytilus galloprovincialis* exposed to nickel and heat stress. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 160: 23–29

Bayne B L. 1965. Growth and the delay of metamorphosis of the larvae of *Mytilus edulis* (L.). Ophelia, 2(1): 1–47

Bougrier S, Geairon P, Deslous-Paoli J M, et al. 1995. Allometric relationships and effects of temperature on clearance and oxygen consumption rates of *Crassostrea gigas* (Thunberg). Aquaculture, 134(1–2): 143–154

Chan P C, Peller O G, Kesner L. 1982. Copper (Ⅱ)-catalyzed lipid peroxidation in liposomes and erythrocyte membranes. Lipids, 17(5): 331–337

Chen Jinghua, Mai Kangsen, Ma Hongming, et al. 2007a. Effects of dissolved oxygen on survival and immune responses of scallop (*Chlamys farreri* Jones et Preston). Fish & Shellfish Immunology, 22(3): 272–281

Chen Muyan, Yang Hongsheng, Delaporte M, et al. 2007b. Immune condition of *Chlamys farreri* in response to acute temperature challenge. Aquaculture, 271(1–4): 479–487

Coglianese M P, Martin M. 1981. Individual and interactive effects of environmental stress on the embryonic development of the Pacific oyster, *Crassostrea gigas*: I. The toxicity of copper and silver. Marine Environmental Research, 5(1): 13–27

De Zoysa M, Whang I, Nikapitiya C, et al. 2011. Transcriptional analysis of disk abalone (*Haliotis discus discus*) antioxidant enzymes against marine bacteria and virus challenge. Fish & Shellfish Immunology, 31(1): 155–160

Fearman J, Moltschaniwskyj N A. 2010. Warmer temperatures reduce rates of gametogenesis in temperate mussels, *Mytilus galloprovincialis*. Aquaculture, 305(1–4): 20–25

Fridovich I. 1995. Superoxide radical and superoxide dismutases. Annual Review of Biochemistry, 64(1): 97–112

Geret F, Serafim A, Barreira L, et al. 2002. Response of antioxidant systems to copper in the gills of the clam *Ruditapes decussatus*. Marine Environmental Research, 54(3–5): 413–417

Gomes T, Pereira C G, Cardoso C, et al. 2012. Accumulation and toxicity of copper oxide nanoparticles in the digestive gland of *Mytilus galloprovincialis*. Aquatic Toxicology, 118–119: 72–79

Hochachka P W, Somero G N. 1984. Biochemical Adaptation. Princeton, NJ: Princeton University Press, 356–449

Hooper C, Day R, Slocombe R, et al. 2007. Stress and immune responses in abalone:limitations in current knowledge and investigative methods based on other models. Fish & Shellfish Immunology, 22(4): 63–79

Isani G, Monari M, Andreani G, et al. 2003. Effect of copper exposure on the antioxidant enzymes in bivalve *mollusc scapharca inaequivalvis*. Veterinary Research Communications, 27(S1): 691–693

Jiang Tiamjiu, Niu Tao. 2006. Effects of heavy metals on superoxide dismutase (SOD) of *Crassostrea rivularis*. Ecology and Environment (in Chinese), 15(2): 289–294

Jing Gu, Li Yu, Xie Liping, et al. 2006. Metal accumulation and enzyme activities in gills and digestive gland of pearl oyster (*Pinctada fucata*) exposed to copper. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 144(2): 184–190

Jing Gu, Li Yu, Xie Liping, et al. 2007. Different effects of Pb^{2+} and Cu^{2+} on immune and antioxidant enzyme activities in the mantle of *Pinctada fucata*. Environmental Toxicology and Pharmacology, 24(2): 122–128

Jo P G, An K W, Park M S, et al. 2008. mRNA expression of HSP90 and SOD, and physiological responses to thermal and osmotic stress in the Pacific oyster, *Crassostrea gigas*. Molluscan Research, 28: 158–164

Kargin F, Çoğun H Y. 1999. Metal interactions during accumulation and elimination of zinc and cadmium in tissues of the freshwater fish *Tilapia nilotica*. Bulletin of Environmental Contamination and Toxicology, 63(4): 511–519

Kim K Y, Lee S Y, Cho Y S, et al. 2007. Molecular characterization and mRNA expression during metal exposure and thermal stress of copper/zinc-and manganese superoxide dismutases in disk abalone, *Haliotis discus discus*. Fish & Shellfish Immunology, 23(5): 1043–1059

Koutsogiannaki S, Franzellitti S, Fabbri E, et al. 2014. Oxidative stress parameters induced by exposure to either cadmium or 17 β-estradiol on *Mytilus galloprovincialis* hemocytes. The role of signaling molecules. Aquatic Toxicology, 146: 186–195

Lesser M P. 2006. Oxidative stress in marine environments: biochemistry and physiological ecology. Annual Review of Physiology, 68(1): 253–278

Li Peifeng, Fang Yunzhong. 1993. Effect of hydrogen peroxide on the activity and physicochemical properties of yak superoxide dismutase. Chinese Journal of Biochemical (in Chinese), 9(4): 411–416

Li Yifeng, Gu Zhongqi, Liu Hong, et al. 2012. Biochemical response of the mussel *Mytilus coruscus* (Mytiloida:Mytilidae) exposed to in vivo sub-lethal copper concentrations. Chinese Journal of Oceanology and Limnology, 30(5): 738–745

Li Chenghua H, Sun Huili, Chen Aiqin, et al. 2010. Identification and characterization of an intracellular Cu, Zn-superoxide dismutase (icCu/Zn-SOD) gene from clam *Venerupis philippinarum*. Fish & Shellfish Immunology, 28(3): 499–503

Li Na, Zhao Yunlong, Yang Jian. 2008. Effects of water-borne copper on digestive and metabolic enzymes of the giant freshwater prawn *Macrobrachium rosenbergii*. Archives of Environmental Contamination and Toxicology, 55(1): 86–93

Livingstone D R. 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Marine Pollution Bulletin, 42(8): 656–666

Luo Lianzhong, Ke Caihuan, Guo Xiaoyu, et al. 2014. Metal accumulation and differentially expressed proteins in gill of oyster (*Crassostrea hongkongensis*) exposed to long-term heavy metal-contaminated estuary. Fish & Shellfish Immunology, 38(2): 318–329

Lushchak V I. 2011. Environmentally induced oxidative stress in aquatic animals. Aquatic Toxicology, 101(1): 13–30

Maria V L, Bebianno M J. 2011. Antioxidant and lipid peroxidation responses in *Mytilus galloprovincialis* exposed to mixtures of benzo(a)pyrene and copper. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 154(1): 56–63

Monari M, Matozzo V, Foschi J, et al. 2007. Effects of high temperatures on functional responses of haemocytes in the clam *Chamelea gallina*. Fish & Shellfish Immunology, 22(1–2): 98–114

Montgomery D C. 2005. Design and Analysis of Experiments. 6th ed. New York: John Wiley & Sons, Inc., 405–439

Mubiana V K, Blust R. 2007. Effects of temperature on scope for growth and accumulation of Cd, Co, Cu and Pb by the marine bivalve *Mytilus edulis*. Marine Environmental Research, 63(3): 219–235

Pantano C, Reynaert N L, Vliet A V D, et al. 2006. Redox-sensitive kinases of the nuclear factor-κB signaling pathway. Antioxidants & Redox Signaling, 8(9–10): 1791–1806

Parihar M S, Javeri T, Hemnani T, et al. 1997. Responses of superoxide dismutase, glutathione peroxidase and reduced glutathione antioxidant defenses in gills of the freshwater catfish (*Heteropneustes fossilis*) to short-term elevated temperature. Journal of Thermal Biology, 22(2): 151–156

Solé M, Porte C, Albaigés J. 1995. Seasonal variation in the mixed-function oxygenase system and antioxidant enzymes of the mussel *Mytilus galloprovincialis*. Environmental Toxicology and Chemistry, 14(1): 157–164

Stebbing A R D. 1982. Hormesis-the stimulation of growth by low levels of inhibitors. Science of the Total Environment, 22(3): 213–234

Sun Hushan, Li Guangyou. 2000. Activities and properties of superoxide dismutase and catalase in the haemolymph of *Chlamys farreri*. Oceanologia et Limnologia Sinica (in Chinese), 31(3): 259–265

Tang Baojun, Liu Baozhong, Wang Xiaomei, et al. 2010. Physiological and immune responses of Zhikong scallop *Chlamys farreri* to the acute viral necrobiotic virus infection. Fish & Shellfish Immunology, 29(1): 42–48

Umasuthan N, Bathige S D N K, Revathy K S, et al. 2012. A manganese superoxide dismutase (MnSOD) from *Ruditapes philippinarum*: comparative structural-and expressional-analysis with copper/zinc superoxide dismutase (Cu/Zn SOD) and biochemical analysis of its antioxidant activities. Fish & Shellfish Immunology, 33(4): 753–765

Verlecar X N, Jena K B, Chainy G B N. 2007. Biochemical markers of oxidative stress in *Perna viridis* exposed to mercury and temperature. Chemico-Biological Interactions, 167(3): 219–226

Vlahogianni T, Dassenakis M, Scoullos M J, et al. 2007. Integrated use of biomarkers (superoxide dismutase, catalase and lipid peroxidation) in mussels *Mytilus galloprovincialis* for assessing heavy metals' pollution in coastal areas from the Saronikos Gulf of Greece. Marine Pollution Bulletin, 54(9): 1361–1371

Vlahogianni T H, Valavanidis A. 2007. Heavy-metal effects on lipid peroxidation and antioxidant defence enzymes in mussels *Mytilus galloprovincialis*. Chemistry and Ecology, 23(5): 361–371

Wang Hui, Liu Jiahui, Yang Hongshuai, et al. 2014. Effect of simultaneous variation in temperature and ammonia concentration on percent fertilization and hatching in *Crassostrea ariakensis*. Journal of Thermal Biology, 41: 43–49

Wang Xingqiang, Wang Lingling, Zhang Huan, et al. 2012. Immune response and energy metabolism of *Chlamys farreri* under *Vibrio anguillarum* challenge and high temperature exposure. Fish & Shellfish Immunology, 33(4): 1016–1026

Wu Yichun, Lv Xin, Wang Fan, et al. 2005. Accumulation of Copper in *Chlamys farreri* tissues and its effect on catalase activity. Chinese Journal of Applied Environmental Biology, 11(5): 559–562

Zhou Zhi, Wang Lingling, Shi Xiaowei, et al. 2011. The modulation of catecholamines to the immune response against bacteria *Vibrio anguillarum* challenge in scallop *Chlamys farreri*. Fish & Shellfish Immunology, 31(6): 1065–1071