RESEARCH REPORT

Combined toxicity of cadmium and lead on early life stages of the Pacific oyster, *Crassostrea gigas*

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Abstract

Trace metals cause toxic effect on the early development period of marine animals, however only few studies address the toxic interactions of trace metals on bivalves. In the present study, the individual and combined toxicities of dissolved cadmium (Cd) and lead (Pb) on early life stages of the Pacific oyster *Crassostrea gigas* have been investigated. Embryotoxicity, larvae mortality and genotoxicity were measured under single and combined exposure of the two tested metals. For embryotoxicity, the median effective concentration (EC_{50}) values for individual Cd, Pb and their mixture were of 272.2 µg/L, 660.3 µg/L and 373.1 µg/L, respectively. The median lethal concentrations (LC_{50}) for 96 h larval mortality were determined to be 353.3 µg/L, 699.5 µg/L and 205.5 µg/L for individual Cd, Pb and their mixture, respectively. Moreover, the Marking-Dawson additive toxicity indices were 0.10 for embryogenesis and 1.40 for larval mortality indicating, respectively, an additive effect and a trend to synergism for the Cd and Pb combination. Furthermore, DNA strand breaks were observed in oyster embryos after individual Cd, Pb and their mixture exposure, and a significant positive correlation was demonstrated between embryotoxicity and genotoxicty. The current study suggests the toxicity of Cd is higher than that of Pb, and the Cd-Pb mixture is slightly more toxic than individual Cd or Pb to the Pacific oyster. These data will be helpful to predict the toxicity of metal mixtures, and provide biological criteria for the implementation of marine water quality standards to protect these marine organisms.

Key Words: embryotoxicity; larval mortality; genotoxicity; trace metals; mixture; *Crassostrea gigas*

Introduction

Aquatic organisms are constantly exposed to complex mixtures of chemical pollutants dissolved in the water and tolerate their potential toxic impacts.

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Among the chemical pollutants, trace metals (*e.g.*, cadmium and lead) represent one of the most widespread and serious forms of environmental contamination (Devi *et al.,* 1996; Zaki *et al.,* 2016). Both Cd and Pb are abundant, non-essential elements that are continuously accumulated in the environment as a result of industrial activities. They are classified as human and animal carcinogens by the US EPA and International Agency for Research on Cancer (IARC) (Tchounwou *et al.*, 2012).

Bivalve species, particularly their early life stages, are widely used for aquatic environmental bioassays because of their high susceptibility and ecological relevance to chemical toxic pollutants (His *et al.*, 1999a; Beiras *et al.*, 2003). Therefore, the early life stages of the Pacific oyster *Crassostrea gigas* have been widely used to assess the toxicity of pollutants, such as trace metals, pesticides,

herbicides, PAHs and synthetic estrogenic hormones (Bellas, 2006; Wessel *et al.*, 2007; Akcha *et al.*, 2012; Mai *et al.*, 2012, 2014; Gamain *et al.,* 2016). Although many studies have demonstrated the toxicities of Cd and/or Pb to invertebrate embryos and larvae such as sea urchins (Fernandez and Beiras, 2001), mussels (Beiras and Albentosa, 2004; Nadella *et al.*, 2009), clams (Wang *et al.*, 2009; Fathallah *et al.*, 2013), ascidians (Bellas *et al.*, 2001) and crab (Bakker *et al.,* 2017), the toxic effects of Cd and Pb on the early life stages of the Pacific oyster are not reported.

In fact, the marine environments are normally affected by more than one trace metal, and information on their interactions provides a more realistic assessment of their toxicity to marine organisms. The toxicity of a chemical can be enhanced (positive interaction or synergism), reduced (negative interaction or antagonism), or unaffected (no interaction) by the presence of another toxicant (Marking and Dawson, 1975). If two chemicals in a mixture do not affect the toxicity of each other, simple joint action is present. Therefore, the combined toxicities of two chemicals are additive, which means their toxicities may be predicted by the summation of toxic potencies measured in toxicity units [TU]. Previous studies showed that the combination of metals such as Cu-Ag/Zn, Mn-Mo, Hg-Cd/Pb/Cu, Cd-Pb and Zn-Cd had significantly toxic effect on aquatic organisms (Macinnes *et al.*, 1981; Coglianese and Martin, 1981; Morgan *et al.*, 1986; Fernandez and Beiras, 2001; Prato and Biandolino, 2007; Fathallah *et al.*, 2013; Gamain *et al.,* 2016). However, to our knowledge, there is no literature about combined effects of Cd and Pb on early life stages of Pacific oyster.

The Bohai Sea is a nearly enclosed interior sea located in northeast China and is a well-known area for bivalves farming such as clams, oysters and scallops. However, contaminations by metals (such as As, Cd, Cr, Cu, Ni, Pb and Zn) have been reported in coastal areas and estuaries of Bohai Sea (Gao *et al.*, 2014). The concentration of Cd in seawater of Bohai Sea ranged from 0.007 to 5.0 µg/L, and the concentration for Pb was 0.058~27.17 µg/L (Table 1). Moreover, the Cd and Pb concentrations were 0.02~1463 µg/g and 0.62~1828 µg/g respectively in sediments. In order to compare the toxic effect of individual and combined metals to early life stages of *Crassostrea gigas*, the embryotoxicity (percentages of abnormal larvae), genotoxicity (DNA damage) and larval mortality of the oyster were investigated. In addition, the correlation between genotoxicity and embryotoxicity data was also analyzed.

Materials and Methods

Chemicals and seawater

Reference toxicants (CdCl$_2$ and Pb(NO$_3$)$_2$), formalin and DMSO (dimethyl sulfoxide) were purchased from Sinopharm Chemical Reagent (analytical grade, China). Dispase, Triton X-100, low melting point (LMP) agarose, normal melting point (NMP) agarose, and MEM-alpha (Minimum Essential Medium) were purchased from Solarbio (Beijing Solarbio Science & Technology Co., Ltd,

Table 1 The concentrations of tested metals in Bohai Sea and this study (µg/L)

	Cadmium	Lead
Bohai Sea	0.007~5.0	0.058~27.17
This study	0.35	1.32

Concentrations (µg/L) of cadmium and lead in Bohai Sea reported by Gao *et al.* (2014), and measured in Yantai in May 2015 (*present study*).

China). Seawater was collected from the coastal area of Yantai (China) that is populated by natural oysters. Then the seawater was filtered using membrane filter of 0.2 µm to eliminate debris and microorganisms.

Chemicals exposure and analysis

Metal stock solutions (100 mg/L) were prepared by dissolving CdCl$_2$ and Pb(NO$_3$)$_2$ into double distilled water. For the exposure experiments, these working solutions were then diluted to reach the final tested concentrations. In the test of single toxicity, five concentrations of each chemical in geometric progression were prepared. For the combined exposure experiment, five treatments were prepared by addition of equal fractions of the chemical concentrations tested in the individual series. A preliminary study was conducted to define the lowest exposure concentration. Briefly, according to the average concentration of Cd and Pb in Bohai Sea, the embryos and D-shaped larvae were exposed to 0.02, 0.2, 2, 20 and 200 µg/L Cd and Pb, respectively. Then the percentage of normal D-shaped larvae and mortality were calculated and the lowest exposure concentration was determined as 20 µg/L for Cd and Pb. Actual concentrations of Cd and Pb in the experimental solutions were measured according to the method of "The specification for marine monitoring" (GB17378 4-2007) by anodic stripping voltammetry (ASV). The ASV measurements were carried out using a voltammeter (VA 797 Computrace, Metrohm Inc.). The recoveries were 97 %~102 %, 90 %~98 % for Cd and Pb, respectively. All analyses were carried out thrice. The concentrations of metals tested in the individual and mixed series were summarized in Table 2.

Embryotoxicity assay

The oysters were purchased from a local aquaculture farm in Yantai (Shandong, China). Mature male and female oysters were stripped to get the gonad. Spermatozoa and oocytes were collected from 10 individuals and sieved separately through a 50 µm and 100 µm meshes, respectively. The eggs were fertilized with sperms in a ratio of 1:10 in filtered seawater. Fertilization success was verified under microscope and embryos were placed in contaminated seawater and transferred to beakers for embryotoxicity and genotoxicity assays. Each experiment was replicated three times using three different batches of oysters. For embryotoxicity assay,
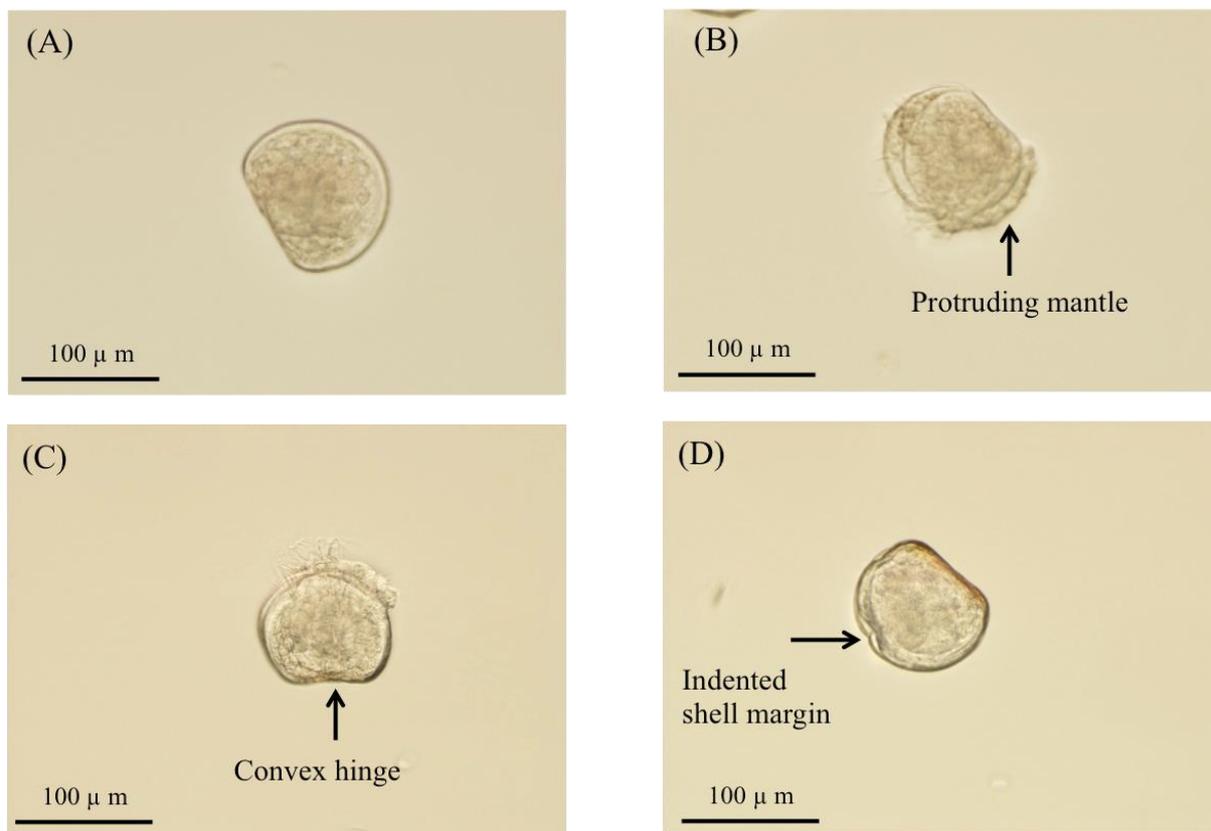
Fig. 1 Oyster D-shaped larvae at 24 h post of fertilization. Normal (A); Abnormal: protruding mantle (B), convex hinge (C), indented shell margin (D).

approximately five hundreds of embryos were incubated for 24 h (at 24 °C in the dark) in beakers containing 200 mL of filtered seawater. There were three replicates for each contamination exposed group and control. This incubation time enables the embryos to develop to the D-shaped larvae stage. Following the exposure, the larvae of each beaker were fixed using 8 % formalin (0.5 ml/beaker) and the percentage of abnormal oyster larvae was recorded. For each assay beaker, three hundreds of larvae were directly observed under an inverted microscope (Olympus, magnification ×200) to determinate the percentage of developmental arrests (stages blastula, gastrula or trocophore), normal and abnormal D-shaped larvae, such as incomplete shell, indented shell margin, protruding mantle and convex hinge (Fig. 1), according to the criteria described by His *et al.* (1999a) and Wessel *et al.* (2007). The median effective concentration (EC$_{50}$) defined as the metals concentration that resulted in a 50 % reduction in normal D-shaped larvae number for each individual pollutant and the mixture. The calculation of EC$_{50}$ was normalized to the mean percentage of larval abnormality in the control group using Abbot's formula (Emmens, 1948), $p = [(Pe-Pc)/(100-Pc)] \times 100$, where P_c and P_e are control and experimental percentage response, respectively. The EC$_{50s}$ values and the lowest observed effective concentration (LOEC) were calculated by the probit method (Newman, 1995) with SPSS 16.0 statistical software.
Comet assay

For genotoxicity assays, embryos were incubated in 250 mL beakers at 24 °C for 16 h in the dark. This incubation time enables the embryos to reach unshelled larvae that can be enzymatically digested for comet assay. Three replicates were performed per treatment, and each replicate contained a total of 20 ×10^4 larvae. Cell isolation was performed by the method described by Wessel *et al.* (2007) with slight modifications. Following 16 h of exposure, the embryos were recovered by sieving with a 50 μm mesh. One milliliter of embryo suspension (about 2000 embryos) was incubated with 1 mL of collagenase solution at concentration of 1 g/L for 20 min at 37 °C with gentle shaking (150 rpm). The reaction was stopped by centrifugation for 10 min at 1300 rpm (4 °C). The cell pellet was then suspended in 1 mL of minimum essential medium (MEM) at a final cell density of about 10×10^4 cells/mL. Cell viability was determined for each sample by trypan-blue exclusion test (Wessel *et al.*, 2007). Comet analysis was only carried out with cell suspension with good cell viability (trypan blue exclusion test > 80 %). The comet assay was performed on isolated cells following the method proposed by Akcha *et al.* (2003), with some modifications. Briefly, 50 μL of cell suspension (about 5000 cells) was added to 100 μL of 1 % low melting point (LMP) agarose solution, and then 85 μL of this mixture was deposited on a frosted microscope slide

Table 2 Nominal and measured concentrations of metals in the embryotoxicity, larval mortality and genotoxicity assays

Cd (µg/L)					
Nominal	20	100	500	2500	12500
Measured	23.24 ± 0.59	106.01 ± 7.55	480.04 ± 31.46	2129.55 ± 328.77	11566.71 ± 957.34
Pb (µg/L)					
Nominal	20	100	500	2500	12500
Measured	19.61 ± 0.98	96.68 ± 1.86	389.95 ± 37.30	1784.86 ± 49.17	8948.38 ± 588.19
Cd + Pb mixture					
Nominal	10 + 10	50 + 50	250 + 250	1250 + 1250	6250 + 6250
Measured	9.49 ± 1.17 (Cd) 10.54 ± 0.95 (Pb)	45.75 ± 2.78 (Cd) 46.14 ± 0.45 (Pb)	253.31 ± 29.73 (Cd) 236.75 ± 12.30 (Pb)	910.61 ± 68.73 (Cd) 1087.56 ± 45.31 (Pb)	6170.22 ± 279.77 (Cd) 5318.94 ± 239.26 (Pb)
Embryotoxicity (TU)	0.05	0.24	1.29	4.99	30.72
Larval mortality (TU)	0.04	0.20	1.06	4.13	25.07

Data are presented as mean ± standard deviations (n = 3).

pre-coated with 1 % normal melting point (NMP) agarose. The slides were kept at 4 °C for 5 min to allow the agarose to solidify. Then 90 µL aliquot of 1 % LMP were pipetted on the pre-coated slide. After the solidification of agarose, the slides were placed in ice-cold lysing solution in the dark at 4 °C for 1 h. Alkaline treatment was performed for 20 min to allow DNA unwinding in a freshly prepared electrophoresis buffer. Electrophoresis was carried out at 25 V, 300 mA, for 20 min. Slides were analyzed at 400× magnification using an optical fluorescence microscope (Olympus BX 51) and an image analysis system (Komet 5.5, Kinetic Imaging Ltd.). DNA damage was expressed as the percentage of total DNA that has migrated from the head (Tail DNA %). A hundred of randomly selected nucleoids was analyzed on two replicate gels.

Larval mortality

After fertilization, the zygotes were filtered with a 50 µm mesh and gently washed three times with filtered seawater, and then re-suspended in filtered seawater (FSW) at 24 °C. After 24 h of incubation, the D-shaped larvae were obtained and re-suspended in 250 mL glass beakers (approximately 2×10^3 larvae/beaker), each containing 200 mL of different concentrations of trace metal solution (Table 2). There were three replicates for the control and contamination exposed groups. The D-shaped larvae were fed with *Isochrysis* spp at a concentration of 1 - 10×10^4 cells/mL three times daily. After exposure for 96 h, the larvae were sampled and anaesthetized using magnesium chloride solution to make them static. Then the percentage of larval mortality was then assessed under inverted microscope, and three replicates tests were performed for each sample. The LC_{50}, defined as the metal concentration that resulted in half maximal larval mortality compared to the control group, and their 95 % confidence intervals (CIs) were calculated by the probit method (Newman, 1995).

Interaction analysis

To identify the type of interaction in binary mixtures of Cd and Pb, the additive toxicity index (ATI) of Marking and Dawson (1975) and its 95 % CI were calculated. The effective contributions of two chemical (A and B) in a mixture are represented by the formula: $S = A_m/A_i + B_m/B_i$, where A and B are chemicals, m and i are the toxicities (EC_{50}'s/LC_{50}'s) of the individual chemicals and the mixtures, respectively. S is the sum of effective concentrations which is modified by one of two procedures: either the additive index = $1/S - 1$ for $S \le 1.0$ (greater than additive toxicity) or $S(-1) + 1$ for $S \ge 1.0$ (less than additive toxicity), and additive effects are demonstrated when S = 1. Additive, synergism and antagonism effects are indicated by zero, positive, and negative values of this index, respectively. The inclusion of zero within the 95 % CI suggests a lack of significant deviation from additivity, while 95 % CI lying above or below zero indicates significant synergism or antagonism, respectively. The toxicity unit (TU) for combined pollutants was calculated using the formula: $TU = C_i/EC_{50i}$ or $TU = C_i/LC_{50i}$, C_i is the concentration of each toxicant in the mixture and EC_{50i}/LC_{50i} is the half maximal efficient concentration for each individual toxicant (Sprague and Ramsay, 1965).
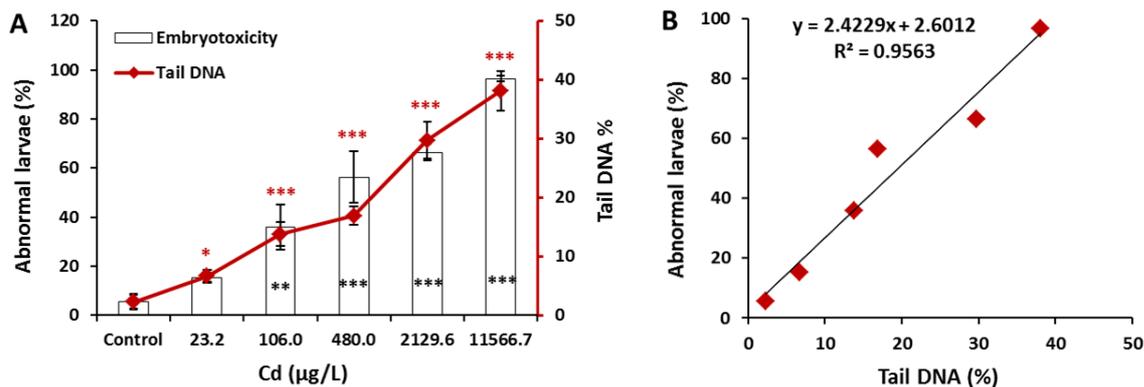
Fig. 2 (A) Percentages (Mean ± S.D., n = 3) of abnormal D-shaped larvae and tail DNA following oyster embryos exposed to different concentrations of cadmium. Asterisks indicate significant differences between exposed and control treatment ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). (B) Relationship between tail DNA and D-shaped larvae abnormalities in oyster ($p = 0.0007$).

Data analysis

Regression linear analysis (Microsoft Excel, 2010) was used to assess relationships between DNA damage and the percentage of abnormal oyster after pollutant exposure. SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for data analysis. Percentage data were transformed (arcsine of the square root) before one-way ANOVA, and presented in figures as non-transformed percentages. Homogeneity of variance was tested using Levene's test. The data were analyzed by one-way ANOVA and Tukey's test was used to compare the results between the control group and the treated groups, and the differences between the treated groups. Statistical significance was accepted when $p < 0.05$.

Results

Chemical analysis

The actual Cd and Pb concentrations were determined in the exposure solutions and summarized in Table 2. Measured concentrations ranged from 71.4% to 116.2 % of the nominal concentrations. Significant differences ($p < 0.05$)

were observed between the nominal and measured concentrations. Therefore, measured concentrations were used for the presentation and calculation of toxicity parameters.

Embryotoxicity and genotoxicity

Effect of individual Cd on embryotoxicity is illustrated in the concentration-response curves (Fig. 2A). Cd could significantly affect the embryogenesis of the oyster at a concentration of 106.0 µg/L ($p < 0.01$), at which the inhibition was up to a 35.9 % decrease in number of normal D-shaped larvae compared to the control. At the highest tested concentration (11566.7 µg/L), the larvae abnormalities reached 96.5 %. The level of DNA damage was significantly ($p < 0.05$) increased from 6.6 % to 38.1 % with increasing Cd concentration. Cd inhibited the embryonic development of *C. gigas* with an EC_{50} value of 272.2 µg/L (Table 3), and the LOEC value of Cd for embryogenesis was 106.0 µg/L. In addition, a strong positive correlation ($R^2 = 0.956$, $p = 0.0007$) was observed between the DNA damage level and the percentage of abnormal D-shaped larvae after Cd exposure (Fig. 2B).

Table 3 Median effective concentrations (EC_{50}), median lethal concentration (LC_{50}), and lowest observed effect concentration (LOEC) for cadmium, lead and cadmium-lead mixture in embryotoxicity and larval mortality assays

	Embryotoxicity EC_{50} µg/L (95% CI)	Larval mortality 96 h LC_{50} µg/L (95% CI)	LOEC µg/L
Cd	272.2 (170.5~444.6)	353.3 (213.2~570.4)	106.0
Pb	660.3 (453.5~1062.4)	699.5 (508.4~983.9)	96.7
Cd + Pb	373.1 (270.2~539.3)	205.5 (138.3~293.1)	91.9
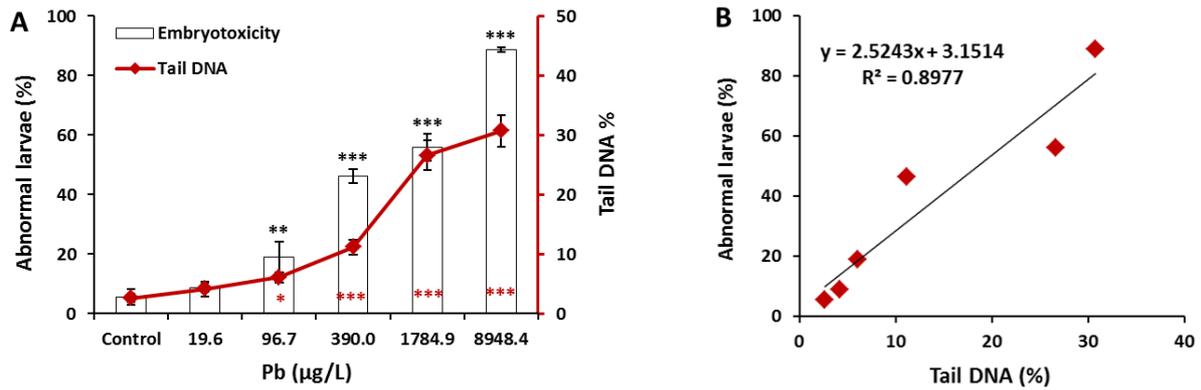
Fig. 3 (A) Percentages (Mean ± S.D., n = 3) of abnormal D-shaped larvae and tail DNA following oyster embryos exposed to different concentrations of lead. Asterisks indicate significant differences between exposed and control treatment ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). (B) Relationship between tail DNA and D-shaped larvae abnormalities in oyster ($p = 0.0041$).

A significant increase of abnormal D-shaped larvae was observed at a concentration as low as 96.7 µg/L for Pb ($p < 0.01$), and the percentage of abnormal larvae reached 88.7 % at the highest tested concentration (8948.4 µg/L) (Fig. 3A). The level of tail DNA significantly increased from 6.1 % to 30.8 % with the increase of Pb concentration ($p < 0.001$). A significant positive correlation ($R^2 = 0.898$, $p = 0.0041$) was also observed between the level of DNA damage and the percentage of abnormal D-shell larvae for Pb (Fig. 3B). The EC_{50} and LOEC values for Pb was shown in Table 3.

Figure 4 presents the percentage of abnormal D-shaped larvae and the level of DNA damage after exposure to the mixture of Cd and Pb. Significant increase of DNA damage level was observed at 0.24 TU ($p < 0.001$), corresponding to 91.9 µg/L (45.8 µg/L

Cd + 46.1 µg/L Pb) for the mixture. A dose-dependent manner on the genotoxic effect was observed after exposure to the mixture of Cd and Pb, and the percentage of tail DNA increased from 5.1 % to 42.6 % with the increase of mixture concentration. The percentage of abnormal D-shaped larvae was also significantly ($p < 0.001$) increased from the dose of 0.24 TU mixture compared to that of the control. After exposed to the highest concentration of 30.72 TU mixture, the abnormalities of D-shaped larvae reached 91.1 % (Fig. 4A). There was also a strong positive correlation ($R^2 = 0.975$, $p = 0.0002$) between level of DNA damage and the percentage of abnormal D-shell larvae after the mixture exposure (Fig. 4B). The EC_{50} and LOEC value for metal mixture (Cd + Pb) was shown in Table 3.
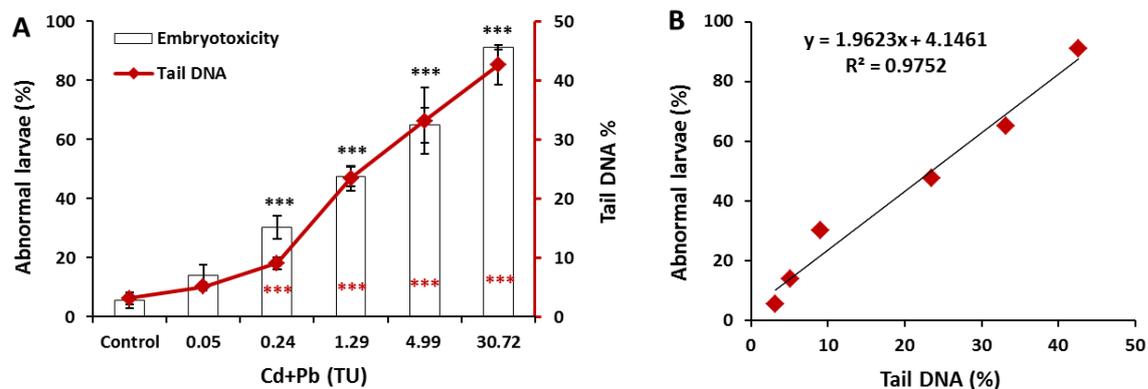
Fig. 4 (A) Percentages (Mean ± S.D., n = 3) of abnormal D-shaped larvae and tail DNA following oyster embryos exposed to different concentrations of the mixture of two metals (Cd + Pb). The X axis shows the toxicity units (TU, TU = concentration/EC_{50}). Asterisks indicate significant differences between exposed and control treatment ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). (B) Relationship between Tail DNA and D-shaped larvae abnormalities in oyster ($p = 0.0002$).
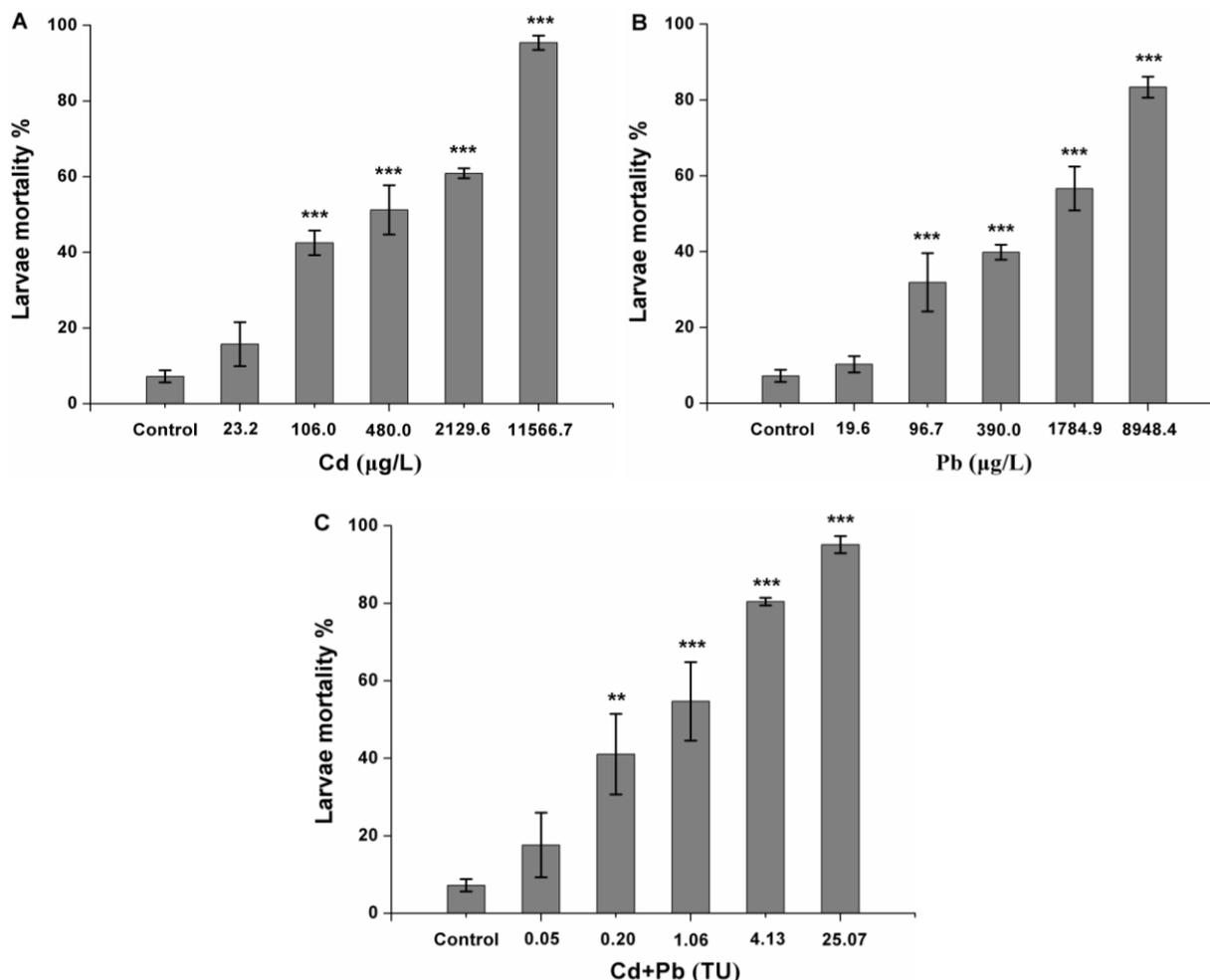
Fig. 5 The mortality (Mean ± S.D., n = 3) of D-shaped larvae after 96 h exposure to different concentrations of cadmium (A), lead (B) and the mixture of two metals (Cd + Pb) (C). The X axis shows the toxicity units (TU, TU= concentration/EC_{50}). Asterisks indicate significant differences between exposed and control treatment (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Larval mortality

For both metals, no significant difference in larval number was observed at the lowest concentration (23.2 µg/L of Cd, 19.6 µg/L of Pb) compared to that of the control. Cd induced a significant increase ($p < 0.001$) in larval mortality at the concentration of 106.0 µg/L (Fig. 5A). For Pb exposure, the larval mortality reached 83.4% at the highest concentration of 8948.4 µg/L (Fig. 5B). The LC_{50} values and LOEC values for Cd and Pb were shown in Table 3.

Similarly, the mortality of the larvae ranged from 17.6 % to 95.2 % at the value of lowest (0.04 TU) to highest metal mixture (25.1 TU) (Fig. 5C). The LC_{50} and LOEC values for the metal combination were shown in Table 3.

Interactions

Table 4 shows the additive toxicity index (ATI) and 95 % CI of combination (Cd + Pb) for embryotoxicity and larval mortality. The result of ATI for embryotoxicity is 0.10 (- 1.13, 1.55), and the zero value is contained within the 95 % CI for the index. Therefore, the results suggested a simple additive

effect of the mixture. Whereas, ATI and 95 % CI for larval mortality was 1.40 (0.07, 4.54), indicating a significant positive interaction (synergism) between these two metals (Cd + Pb).

Discussion

The contamination of coastal ecosystems by trace metals has gained increasing attention in recent decades (His *et al.*, 1999a; Shahidul Islam and Tanaka, 2004; Pan and Wang, 2012). Chemical analyses of pollutants in seawater allow a determination of the degree and nature of pollution, but they are not able to provide evidence as to the biological consequences, and evaluate potential risks for living organisms (Chapman *et al.*, 1987). Biological monitoring using marine organisms, particularly in their early life stage, has been shown to be a sensitive approach to predict the potential risk of persistent pollutants like trace metals. Although the effects of single metals on marine species have been well documented in terms of their toxicity and bioaccumulation (Novelli *et al.*, 2003; Reichelt-Brushett and Harrison, 2005; Fitzpatrick *et al.*, 2008;

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Gopalakrishnan *et al.*, 2008), the literature about combined toxicity of cadmium and lead on early life stages of the Pacific oyster is still scarce. The present study aimed to investigate the embryotoxicity, genotoxicity and larval mortality of the Pacific oyster under individual and combined metal (cadmium and lead) exposures.

Embryotoxicity and genotoxicity

The embryo development of oysters was strongly affected by cadmium and lead at the tested concentrations. The results of EC_{50s} (Table 2) demonstrated that Cd was more toxic to embryo-larval stages than Pb. These results are in concordance with that shown in *Meretrix meretrix* (Wang *et al.*, 2009). In the current work, EC_{50} value of Cd for the Pacific oyster was 272.2 µg/L, which was similar to the EC_{50} (212.3 µg/L) value for this oyster (Mai *et al.*, 2012) and another oyster *Crassostrea rhizophorae* (211.8 - 316.2 µg/L) (da Cruz *et al.*, 2007). Similarly, EC_{50} value of Cd on embryotoxicity was 502 µg/L for the mussel *Mytilus trossolus* (Nadella *et al.*, 2009), 1925 µg/L for *Mytilus gallporovincialis* (Beiras and Albentosa, 2004), 1014 µg/L for clams *Meretrix meretrix* (Wang *et al.*, 2009) and 570.9 µg/L for *Ruditapes decussatus* (Fathallah *et al.*, 2013). For Pb, the EC_{50} for inhibition of embryogenesis was 660.3 µg/L in this study. However, the EC_{50s} for sea urchin *Paracentrotus lividus* were determined to be 482 µg/L (His *et al.*, 1999b) and 509 µg/L (Fernandez and Beiras, 2001). It was also reported an EC_{50} value of 297 µg/L for clams *M. meretrix* (Wang *et al.*, 2009), and 256.5 µg/L for *R. decussatus* (Fathallah *et al.*, 2013). The embryos of the Pacific oyster seem to be more resistant to Pb exposure than the other reported marine bivalves probably due to the sensitivity of different species and the use of different methods for testing (different trace metal salts, or pool of gametes). The toxicity of Cd in combination with Pb was also investigated in this study. The EC_{50} value for the mixture (Cd + Pb) was 373.1 µg/L, which was comparable with the EC_{50} value (355.4 µg/L) of (Cd + Pb) to *R. decussatus* (Fathallah *et al.*, 2013).

In addition, trace metals also induced DNA damage in Pacific oyster embryos after 16 h exposure. For Cd, the lowest concentration (23.2 µg/L) significantly increased DNA damage ($p < 0.01$). Mai *et al.* (2012) also reported that 10 µg/L Cd increased DNA damage level significantly ($p < 0.05$). Cd-induced DNA damage was probably due to the increase of lipid peroxidation in various tissues (Stohs and Bagchi, 1995). Previous studies have also reported that cadmium could directly induce DNA damage through breaking the single strand and inhibiting the reparation of DNA (Hassoun and Stohs, 1996; Beyersmann and Hechtenberg, 1997). Our study showed that 96.7 µg/L of Pb exposure was able to induce 6.1 % of DNA damage in oyster embryos. It was reported that Pb could cause DNA damage by the production of ROS, which initiated DNA oxidation and subsequent damage (Hsu and Guo, 2002). Oyster embryos exposed to the metal mixture also showed a significant increase of DNA damage level (9.1 %) at the concentration of 0.24 TU (corresponding to 45.8 µg/L Cd + 46.1 µg/L Pb).

In present study, significant positive correlation

Table 4 The additive toxicity index (ATI) and 95% confidence intervals (±CI 95%) for binary combinations of cadmium and lead

	ATI	- CI 95%	+ CI 95%
Embryotoxicity	0.10	-1.13	1.55
Larval mortality	1.40	0.07	4.54

between embryotoxicity and genotoxicity was demonstrated after individual Cd and the mixture exposure, whereas the correlation was lower for Pb exposure. Mai *et al.* (2012) also found a strong positive correlation between embryotoxicity and genotoxicity after metal (Cu and Cd) and pesticide (metolachlor) exposure. Similarly, relatively high correlation between embryotoxicity and genotoxicity was reported in oyster embryos after exposure to benzo(a)pyrene, 17α-ethinylestradiol and endosulfan (Wessel *et al.*, 2007).

Larval mortality

In the present study, the 96 h LC_{50} values of larval mortality for Cd, Pb and their mixture were 353.3 µg/L, 699.5 µg/L and 205.5 µg/L, respectively. The LC_{50s} for *R. decussatus* were 453.6 µg/L, 877.8 µg/L and 565.6 µg/L (for Cd, Pb and their mixture) (Fathallah *et al.*, 2013), and 68 µg/L and 353 µg/L (for Cd and Pb) for clams *M. meretrix* (Wang *et al.*, 2009). In individual Cd and Pb exposure treatments, it was evident that the susceptibility of embryos were slightly higher than larvae. The findings from this study were consistent with those in other bivalves such as *Crassostrea virginica* (Calabrese *et al.*, 1977), *C. gigas* (Beiras and Albentosa, 2004), *M. meretrix* (Wang *et al.*, 2009) and *R. decussatus* (Fathallah *et al.*, 2013).

Interactions

Additive effects have been observed in bivalves under mixtures of cadmium and lead exposure. The additive indices of the metal combination were 0.10 and 1.40 for embryo toxicity and 96 h larval mortality respectively. The combined toxicity in embryogenesis was slightly higher (10 %) than predicted by the additive model. However, the 96 h larval mortality bioassay showed a significant higher toxicity index, which would be considered as synergistic following the Marking-Dawson method (Marking and Dawson, 1975). In present study, we had observed additive or synergistic effects for combinations of Cd and Pb. Additive effects have also been observed in bivalves for mixtures of Cu-Ag (Coglianese and Martin, 1981), Mn-Mo (Morgan *et al.*, 1986), Hg-Cd, Hg-Cu and Cd-Cu (Fernandez and Beiras, 2001), Hg-Cu, Hg-Cd and Cu-Cd (Prato and Biandolino, 2007), Cd-Pb (Fathallah *et al.*, 2013) and Cu-Ag (Boukadida *et al.,* 2016). However, other authors propose alternative views. Hayes (1991) proposed that combinations yielding additive indices between 0.5 and 2 (corresponding to the range from -1 to 1 for the Marking-Dawson index)

should not be considered as significantly different from additivity. Following this criterion, the interactions studied in this study would all be considered to be additive.

Cadmium and lead pollution in coastal area of China and environmental risk to native bivalves

The coastal and estuarine areas in China are facing increasing metal pollution pressures. In seawater, the concentrations of Cd and Pb are as high as 5.0 µg/L and 27.17 µg/L respectively in Bohai Sea (Gao *et al.*, 2014), and 0.89 µg/L and 7.7 µg/L respectively in South China Sea (Zhang *et al.*, 2015). Moreover, high levels of metal concentrations are also found in sediments along the coastal and estuary areas in China. It has been reported that Cd and Pb concentrations were as high as 488.2 µg/g and 1828 µg/g respectively in sediments of Jinzhou Bay (Fan *et al.*, 2006; Zhang *et al.*, 2008), and 22.69 µg/g and 145.5 µg/g respectively in sediments of Dagu Drainage canal (Zhang *et al.*, 2012). According to the concentrations in seawater, current Cd and Pb pollutions have no adverse effect on early life stages of oyster and other native bivalves. However, the release of metals from highly polluted sediments may increase the potential risk of metal pollution. In addition, other highly toxic metals such as copper, mercury may coexist in the polluted environment. Thus, it could not be ruled out that combined metal pollution may have an adverse impact on recruitment of native bivalves.

Conclusion

In conclusion, the results indicated that oyster embryo was highly susceptible to Cd and Pb exposure, and combined metal exposure induced a positive interaction synergism effect on larval mortality of *C. gigas*. This study also suggested that Cd and Pb pollution in most coastal areas of China will not affect the recruitment of wild or cultivated oysters. However, the complex mixtures of different classes of contaminants may cause potential toxic effect on oyster embryos and larvae, especially at high-concentration areas. Therefore, to predict the impact of combined contamination on marine organisms, it would be necessary to test not only binary mixtures, but also combinations of three or more metals, or even combinations of metals with organic pollutants.

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