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Impact of seawater acidification on shell property of the Manila clam *Ruditapes philippinarum* grown within and without sediment*

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Abstract Although the impact of ocean acidification on marine bivalves has been previously investigated under mainly controlled laboratory conditions, it is still unclear whether the impact of acidification on sediment-burrowing species differs between those within or without sediment. In order to fill this gap in our knowledge, we compared shell properties of the infaunal Manila clam (*Ruditapes philippinarum*) exposed to three pH concentrations (7.4, 7.7, and 8.0), within and without sediments. In the first experiment (140 d), clams were exposed to seawater in an acidification system without sediment. A decrease in shell weight corresponding to the increase in dissolution rate was observed in the group of pH 7.4, at which shell color disappeared or whitened. SEM observations confirmed the changes of the external shell surface. In the second experiment (170 d), sediment was placed at the bottom of each exposure chamber. The effects were found obvious in shell dissolution rate and shell color in the shell specimens exposed to overlying seawater but not found in the shell specimens exposed to sediment. Although the experimental period was longer in the second experiment, shell specimens in the first experiment were more seriously damaged than those in the second experiment under acidic seawater conditions. Our results, in relation to the defense function of the shell, show that marine bivalves in burrowing behavior are more adaptable to seawater acidification than those who do not burrow into sediment.

Keyword: ocean acidification; sediment; Manila clam (*Ruditapes philippinarum*); shell properties

1 INTRODUCTION

Since the industrial revolution, atmospheric carbon dioxide (CO_2) concentrations have increased due to extensive anthropogenic activities, an increase that is projected to continue over the next century (Houghton et al., 2001; Raven et al., 2005; Solomon et al., 2007; Doney et al., 2009). Approximately one-third of the atmospheric CO_2 dissolves in the surface of the oceans (Raven et al., 2005), causing an increase in partial pressure of CO_2 (pCO_2), an increase in hydrogen ion (H^+) concentrations, and a decrease in carbonate ion (CO_3^{2-}) concentrations in the seawater. These changes result in a decline in seawater pH and a state of calcium carbonate ($CaCO_3$) saturation (Raven et al., 2005).

It is generally accepted that the pH of the open ocean ranges from -0.14 unit with RCP 2.6 (421×10^{-6} CO_2) to -0.43 unit with RCP 8.5 (936×10^{-6} CO_2) by 2100 (Hoegh-Guldberg et al., 2014). Furthermore, increasing atmospheric CO_2 levels may cause a further decline of 0.7 pH units by 2300 (Caldeira and Wickett, 2003). The implication of ocean acidification to seawater chemistry is reasonably well understood; however, the ecological implication for marine organisms is hard to predict due to the

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diversity of organisms and their complex ecosystems (Fabry et al., 2008; Widdicombe and Spicer, 2008). Acidification of the oceans is considered a serious environmental problem facing marine organisms, particularly to calcifying organisms such as coral, coralline algae, coccolithophores, and molluscs (Feely et al., 2004; Orr et al., 2005; Doney et al., 2009; Albright et al., 2018).

Molluscs, an ecologically important and economically valuable species, can determinate and maintain ecosystem health by controlling the abundance of primary producers and contributing to the inorganic carbon cycle in coastal marine ecosystems (Gazeau et al., 2013; Parker et al., 2013). Therefore, predicting the response of molluscs to ocean acidification has implications to the future development of these species and the marine ecological system. Previous studies have shown that ocean acidification can have a detrimental effect on molluscs. It may decrease the growth and survival (Berge et al., 2006; Beniash et al., 2010; Dickinson et al., 2012) and alter the immune response, physiological response, and gene expression (Berge et al., 2006; Bibby et al., 2008; Cummings et al., 2011; Liu and He, 2012). For example, Beniash et al. (2010) found that high CO_2 levels (pH ~7.5, pCO_2 ~3 500 μatm) caused a significant increase in mortality rates for juvenile eastern oysters (*Crassostrea virginica*) and inhibited both shell and soft-body growth compared to control samples; high CO_2 levels also resulted in changes to the ultrastructure and mechanical properties of their shells. However, previous studies have also reported that the mortality of some molluscs exhibited no significant response to high pCO_2 (Range et al., 2011; Fernández-Reiriz et al., 2012), such as *Saccostrea glomerata* (Amaral et al., 2012) and *Mytilus galloprovincialis* (Range et al., 2012). In addition, Range et al. (2011) showed that there were no effects of pH decrease (-0.4 and -0.7) on calcification, shell and soft tissue growth for juvenile clams *Ruditapes decussatu* after exposure for 75 days, and that survival significantly increased, thereby suggesting that acidification of ocean water can increase survival. These results suggest that different marine mollusc species exhibit different responses to high pCO_2. It is therefore important to increase the number of species in a culture system to investigate the effects of ocean acidification.

Molluscs have important benefits for benthic communities through shell production. Mollusc shells are not only an important substratum for the attachment of epibionts, they also provide refuges from predation, physical or physiological stress (Gutiérrez et al., 2003). Recently, the number of studies examining the use of shells (for example the color, thickness, weight and density of the shell) to evaluate the effects of ocean acidification have increased (for example Ries et al., 2016; Clements et al., 2017, 2018; Onitsuka et al., 2018). The results have confirmed that ocean acidification can increase shell dissolution rates and shell damage (McClintock et al., 2009; Waldbusser et al., 2011; Busch et al., 2014).

Although it is commonly known that many molluscs inhabit seawater sediment, few studies have addressed the effects of sediment on the molluscs in relation to acidification of the overlying seawater. Kitidis et al. (2011) suggested the water column ammonia oxidation rates were decreased under acidic conditions, but sediment ammonia oxidation rates were not affected by reduced pH. Gazeau et al. (2014) found the parameters and processes (i.e. mineralization, denitrification) had no relationship with the overlying seawater pH, the ocean acidification will have limited impacts on the associated sediment-water fluxes. However, Clements et al. (2018) showed that sediment pH is generally lower than the water column pH due to microbial activity in the sediment. A study suggests that extreme levels but not current and projected near-future levels of acidification (ΔpH ~1 unit) can reduce the susceptibility of eastern oyster shells to *P. websteri* infections (Clements et al., 2017). Green et al. (2004) showed the significant mortality rates for the *Mercenaria mercenaria* (0.2, 0.3, and 1 mm) in experimental under-saturated sediments ($\Omega_{aragonite}$=~3.0), and dissolution-induced mortality may help explain the exponential losses of juvenile bivalves following their transition from the pelagic larval phase to the benthic juvenile phase. Green et al. (2009) showed the significant mortality for the juvenile clams *Mercenaria mercenaria* (0.2, 0.4, and 0.6 mm) occurred under the most under-saturated conditions ($\Omega_{aragonite}$=0.4), and the death by dissolution was an important size-dependent mortality factor for juvenile bivalves. Understanding whether sediment can affect the shell of organisms with burrowing behavior in the ocean acidification is therefore important, the results of which may provide a key to estimating the impact of ocean acidification on molluscs.

The Manila clam *Ruditapes philippinarum* is indigenous to estuarine and coastal waters of the

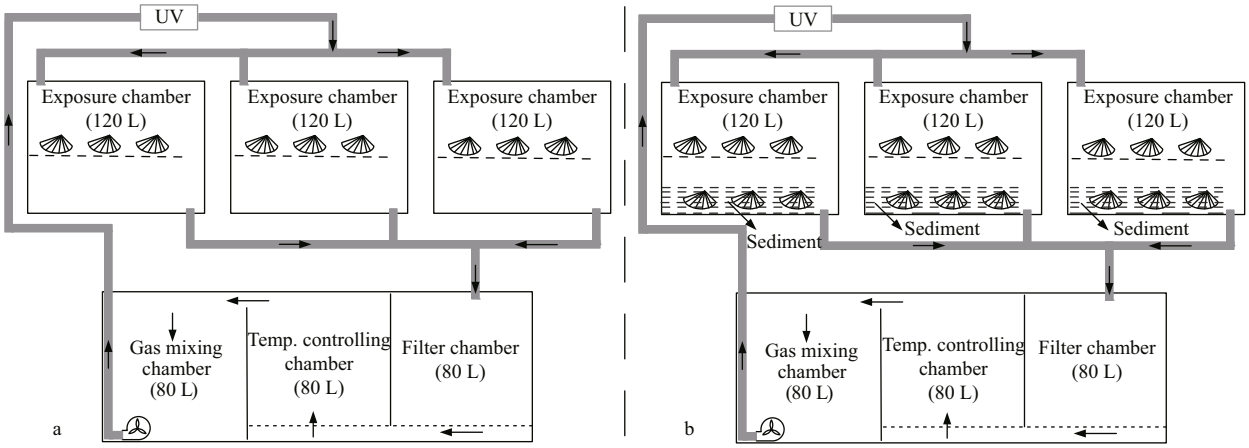

Fig.1 Experimental setup used in this study

We had three sets of experimental setups in our experiment, each of them corresponding to one pH treatment. The first experiment (2014, 140 d): the adult clams, laid upon a rigid plastic grid, were suspended in each exposure chamber, and the acidification system without sediment (a); the second experiment (2015, 170 d): the sediment was placed on the bottom of each exposure chamber, and the adult clams were split into two treatments, one treatment was exposed to seawater, another treatment was exposed to sediment (b).

Indo-Pacific region living in sediment in these areas. Due to its high nutritive value and delicate flavor, *R. philippinarum* represents one of the most important aquaculture species in China, Japan and Korea (Uddin et al., 2012), and also in Europe (Spain, Italy, etc.) and North America. Due to the economic and ecological value of this species, a number of studies have been undertaken using this species as a preferred indicator for estuarine and coastal monitoring purposes (Ji et al., 2006; Yang et al., 2013; Zhao et al., 2014). Xu et al. (2016) reported the effects of seawater acidification on *R. philippinarum*, and found increased excretion rates, and decreased clearance rates, respiration rates, the scope for growth, and spawning rates in 70-day exposure to highly acidified seawater. It is important to note, however, that there is a lack of understanding to the impacts of long-term ocean acidification on the shell of on bivalves with and without sediment. In this study, we simulated conditions using predicted oceanic pH levels (7.7 and 7.4) in order to assess the impact of seawater acidification on mortality and shells of adult *R. philippinarum* under acidified conditions with and without sediment.

2 MATERIAL AND METHOD

2.1 Experimental animals

Adult Manila clams (32.2±2 mm shell length) from the same wild population were collected from an intertidal zone in Liangshui Bay, Liaodong Peninsula, NE China (39°04′14.41″N, 122°01′47.70″E). Samples were collected in April 2014 (seawater: 8.6°C; salinity: 32.4; pH: 8.18) and in April 2015 (seawater: 9.2°C; salinity: 32.6; pH: 8.12), respectively. The gonads were underdeveloped. Before the onset of the experiment, all specimens were acclimatized for two weeks in tanks with sand-filtered seawater sourced from the coastal zone (38°51′49.02″N, 121°33′03.98″E) and aerated. The volume of the tank was 750 L and the corresponding density was 0.6 clam/L in Experiment 1, and 0.8 clam/L in Experiment 2. Bivalves were fed three times daily with four algae (*Chlorella vulgaris*, *Isochrysis galban*, *Chaetoceros muelleri*, and *Nitzschia closterium*). The density of the algae in the tank was 8×10^4 cells/L every time. As the specific levels of different nutrients required for the reproductive development of clams were unknown, the four phytoplankton species were selected to provide adequate nutrition. In consideration of the effects of high pH of algal nutrient solution on seawater pH, cultured algae were centrifuged and collected.

2.2 Seawater acidification system and experimental design

The natural seawater acidification system (Fig.1) used in our study is described in detail by Xu et al. (2016). In brief, each seawater acidification system consisted of three exposure chambers (i.e., three replicates), a filter chamber, a temperature-controlling chamber, and a gas mixing chamber. We selected 10 clams from each of the 3 replicates (30 clams) randomly but did not distinguish the clams from different replicates. The replicates were not

statistically independent. It was categorized as pseudo-replication (Cornwall and Hurd, 2016). Using predicted ocean surface pH levels for 2100 and 2300 (Caldeira and Wickett, 2003), the recirculating aquaculture systems were adjusted to pH levels of 7.7 and 7.4 by bubbling pure CO_2 through the seawater. The atmospheric air was diffused into the seawater in every treatment.

For this study, two experiments were conducted. The first experiment was conducted from April to September, 2014 in 140 days, in which 150 adult clams were suspended in each exposure chamber in the acidification system without sediment (Fig.1a). The second experiment was done from April to October 2015 in 170 days, in which a rigid plastic grid was hung 20 cm underwater and sediment was placed on the bottom for each exposure chamber, 100 adult clams were laid upon the rigid plastic grid, and 100 clams were exposed to sediment in each exposure chamber (Fig.1b). The depth of the sediment was about 15 cm. The sediment and the clams were collected in the same place simultaneously.

In both experiments, clams were exposed to two pH treatments (7.7 and 7.4) and a control (nominally 8.0) after acclimatization. Seawater pH levels in the systems were measured daily with a water quality analyzer (YSI Pro plus), calibrated it daily in pH 7, 10 buffers. The pH scale was measured on NBS. The mass flow controllers (MFC) were used to control CO_2 flow to ensuring pH stability. As surface temperatures for the Yellow Sea naturally range from 3.2°C in February to 25.2°C in August, and the thermal optimum for *R. philippinarum* is 20°C (Han et al., 2008), after the clams were exposed to the different pH treatments, we manipulated temperature increases from 10 to 20°C within 30 days (ca. 0.3°C), after which we maintained a constant temperature of 20°C in order to mimic natural temperature conditions. Temperature and salinity were measured daily in each experimental system. Total alkalinity (T_A) was measured using an alkalinity titrator. The pH, temperature, salinity and T_A data were used to calculate the rest of the carbonate system parameters using the CO2SYS software Pierrot et al. (2006).

2.3 Clam measurement

We observed that adult clams were spawning in all treatments of both experiments on the 115th day and 110th day, respectively, and it should be noted that the increase of mortality observed in the latter stages of the experiments was affected by spawning. At the end of both experiments, we recorded shell color using a camera and did a qualitative evaluation, measured shell weight, shell density, and shell dissolution rate. The structure of the shell surface was observed using a scanning electron microscope (SEM).

Mortality was measured by recording the number of dead clams every day and pick out the dead clams. As dead clams cumulatively increased during the experimental period, we estimated average mortality per day to assure comparability between both experiments. Average mortality (%/d) was calculated using cumulative mortality (%) divided by experiment time (d).

Shell weight measurements were made at the beginning and end of the experiments. In order to calculate the average shell weight at the beginning of each experiment, we randomly selected 30 clams and weighed their empty shells. At the end of the experiment period, 30 clams were randomly selected from the exposure chamber (10 clams from each of the 3 replicates) in the first experiment. In the second experiment, we randomly selected 30 clams exposed to overlying seawater and 30 clams exposed to sediment (10 clams from each of the 3 replicates). The selected clams were dissected and their shells were oven dried at 60°C for 48 h to obtain dry weight.

Shell dissolution rate, estimated using shell weight at the beginning and at the end of the experiment (Duarte et al., 2015) was calculated as:

$$v=(W_1-W_0)/T, \quad (1)$$

where, v is the shell dissolution rate (μg per clam/d); W_0 and W_1 are shell weights (g) at the beginning and the end of the experiment, respectively; and T is experiment time (d).

Shell density was measured using the following procedure: (1) a beaker containing pure water was weighed using an electronic balance, the weight being recorded as W_2 (g); and (2) the shell, which was weighed at the end of the experiment (W_1), was suspended in pure water by a filament and kept away the wall and bottom of the beaker. When the shell was motionless, weight W_3 (g) was recorded using an electronic balance. The density of the pure water was recorded as ρ_0 (g/cm^3) and shell density ρ (g/cm^3) was calculated using the following equation:

$$\rho=W_1\times\rho_0/(W_3-W_2). \quad (2)$$

We used a JEOL JSM-7800F SEM to observe the structure of the external surface of the shell. In order to not impact the vacuum environment inside the SEM, shells were initially dried.

Table 1 Overlying water carbonate system parameters during the experimental period

Carbonate system parameter		Experimental treatment					
		The first experiment (2014, 140 d)			The second experiment (2015, 170 d)		
		Control	pH 7.7	pH 7.4	Control	pH 7.7	pH 7.4
Temperature (°C)	0–30 d	10–20	10–20	10–20	10–20	10–20	10–20
	30–end	20.3±0.2	20.3±0.3	20.4±0.2	20.6±0.4	20.4±0.4	20.5±0.4
Salinity		32.0±0.4	32.0±0.4	32.0±0.4	32.2±0.5	32.2±0.5	32.2±0.5
pH_T		7.95±0.04	7.69±0.03	7.41±0.03	7.84±0.06	7.69±0.06	7.42±0.08
T_A (μmol/kg)		2 229±116	2 266±70	2 242±51	2 316±275	2 250±87	2 775±709
pCO_2 (μatm)		516±119	1 001±206	1 987±422	740±127	1 021±85	2 385±969
DIC (μmol/ kg)		2 043±123	2 137±132	2 223±142	2267±293	2 198±80	2 730±973
Ω_{ca}		3.20±1.36	1.87±0.79	1.03±0.45	2.77±1.12	1.89±0.61	1.33±0.81
Ω_{ar}		2.05±0.91	1.20±0.52	0.66±0.30	1.78±0.74	1.21±0.41	0.85±0.54

Overlying water temperature, salinity, pH_T and total alkalinity (T_A) were measured as well as were used to calculate partial pressure of CO_2 (pCO_2), dissolved inorganic carbon (DIC), saturation stage of omega aragonite (Ω_{ca}) and calcite (Ω_{ar}) using the CO2SYS software (Pierrot et al. (2006)). The porewater carbonate system parameters were unmeasured in the second experiment.

2.4 Statistical analysis

All data were analyzed using Microsoft Excel (2010) and Statistical Package for Social Sciences (SPSS) 20.0. Use the ANOVA first, the pH as the independent Variable and the results (dissolution, density, shell weight, and live weight) as the Dependent Variables. If the result of the ANOVA is significant, then perform Tukey analysis. Differences were considered significant at $P<0.05$.

3 RESULT

3.1 Seawater chemistry, mortality and live weight

The parameters of the overlying seawater are presented in Table 1. For both experiments water temperature was controlled according to the protocol outlined in the methods: water temperature increased from 10 to 20°C during the first 30 days (ca. 0.3°C), after which it was maintained at the constant temperature of 20°C. The pH target levels were successfully attained; however, the average pH value of the control treatment in the second experiment was 7.84, this being slightly lower than the average value in the first experiment. Salinity remained stable and the average value was maintained at 32 throughout both experiments. Average T_A values showed no clear change between treatments (2 229–2 316 μmol/kg), except for the pH 7.4 treatment in the second experiment (2 775 μmol/kg). Average pCO_2 corresponding to the control, pH 7.7 treatment, and pH 7.4 treatment was 516, 1 001, and 1 987 μatm in the first experiment and 740, 1 021, and 2 385 μatm in the second experiment, respectively. The saturation state of aragonite (Ω_{ca}) and calcite (Ω_{ar}) decreased with a decline in the pH value; Ω_{ca} and Ω_{ar} values for the pH 7.4 treatment in the second experiment were higher than those recorded in the first experiment.

Table 2 Average mortality of *Ruditapes philippinarum* in both experiments

Experimental treatment	Average mortality per day (%)					
	A		B1		B2	
	0–70 d	0–140 d	0–100 d	0–170 d	0–100 d	0–170 d
Control	0.06	0.13	0.22	0.41	0.17	0.33
pH 7.7	0.06	0.14	0.25	0.48	0.24	0.39
pH7.4	0.07	0.19	0.24	0.36	0.20	0.32

In the first experiment, the initial clam number at each treatment was 450, A: clams were exposed to seawater in the acidification system without sediment (140 d); in the second experiment, the initial clam number at each treatment was 300; B1: clams were exposed to seawater in the acidification system with sediment (170 d); B2: clams were exposed to sediment in the acidification system (170 d).

Average mortality results of *R. philippinarum* for both experiments are shown in Table 2. It can be seen that average mortality showed no clear difference between the control and treatments in the first experiment after 70 days of exposure, whereas average mortality for the pH 7.4 treatment (0.19 %/d) was higher than that recorded for the pH 7.7 treatment (0.14 %/d) and the control (0.13 %/d) after 140 days in the first experiment. In the second experiment, average mortalities of clams exposed to seawater were higher than clams exposed to sediment under the

same pH conditions. For example, average mortality of clams in the pH 7.7 system (170 d) was 0.48 %/d (seawater) and 0.39 %/d (sediment).

At the end of the second experiment, the live weight of specimens in seawater was higher in the control treatment than in the two low pH treatments (df=2, F=2.827, P=0.066). Results for specimens in sediment recorded no obvious difference between the control treatment and the two low pH treatments (df=2, F=0.198, P=0.821) (Fig.2).

3.2 Shell color and veins

Results for shell color (Fig.3) indicated a general whitened occurred during the course of the experiment (Fig.3). At the end of the first experiment, we observed a gradient in the bleaching of shell color depending on the pH level (Fig.3a, b, c). The treated specimens exposed to pH 7.4 had blanched considerably, and those exposed to pH 7.7 had an intermediate shade. Similarly, the pH gradient descent resulted in a progressive decline of shell veins, especially shell veins in the pH 7.4 treatment which were found to have almost disappeared (Fig.4).

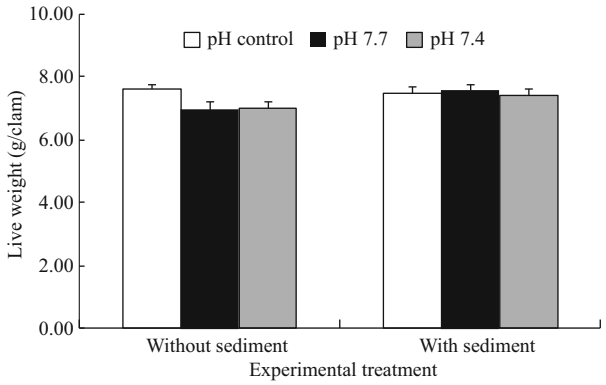

Fig.2 Live weight of *Ruditapes philippinarum* at the end of the second experiment

Data are presented in mean±SD (n=30).

The second experiment was undertaken in order to understand the effect of seawater acidification with sediment on clam shells. As per the results from the first experiment, results from the second experiment indicated that shell color whitened depending on the seawater pH (Fig.3d, e, f). Although the duration of the second experiment (170 d) was longer than the first experiment (140 d), shell color bleaching of treated specimens from the pH 7.4 seawater treatment was found to be more serious in the first experiment than in the second experiment (Fig.3c, f). In addition, we observed that the shell color of treated specimens from the sediment experiment showed no clear difference between the control and the treatments (Fig.3g, h, i). In contrast to treated specimens from sediment in the second experiment, the shell color of treated specimens from acidified seawater was whiter (Fig.3e, f, h, i).

Fig.3 Shells color of *Ruditapes philippinarum* at the end of both experiments

The treated specimens in the first row (a, b, and c) were exposed to seawater in the acidification system without sediment (140 d), a: control; b: pH 7.7; c: pH 7.4. The treated specimens in the second row (d, e, and f) were exposed to seawater in the acidification system (170 d); d: control; e: pH 7.7; f: pH 7.4. The treated specimens in the third row (g, h, and i) were exposed to sediment in the acidification system (170 d); g: control; h: pH 7.7; i: pH 7.4.

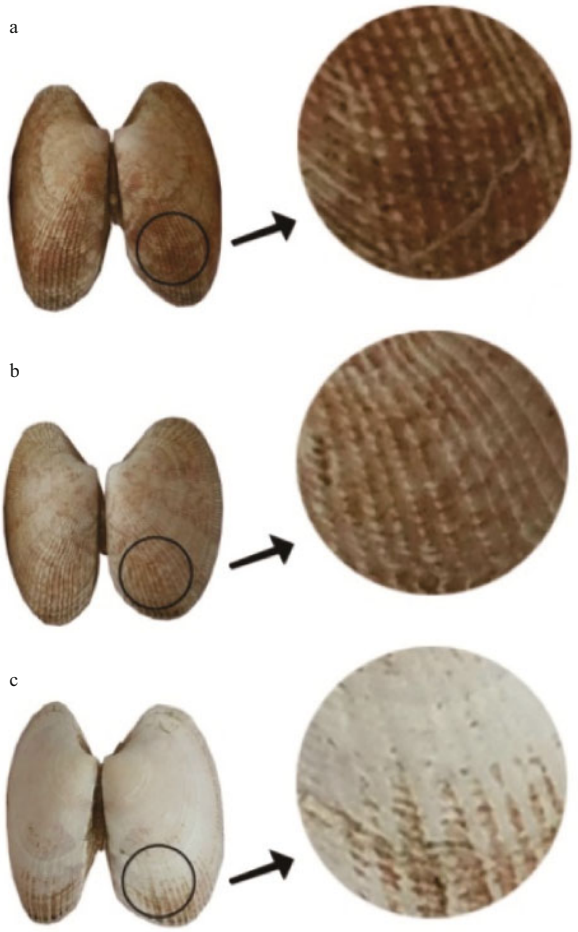

Fig.4 Shell stria of *Ruditapes philippinarum* at the end of the first experiment

The treated specimens (a, b, and c) were exposed to seawater in the acidification system without sediment (140 d), a: control; b: pH 7.7; c: pH 7.4. a. the shell was cover with complete and protruding ribs in control group on day 140; b. the shell striae were still complete in the mild acidification group (pH 7.7); c. the shell striae were damaged seriously in the acidification group (pH 7.4).

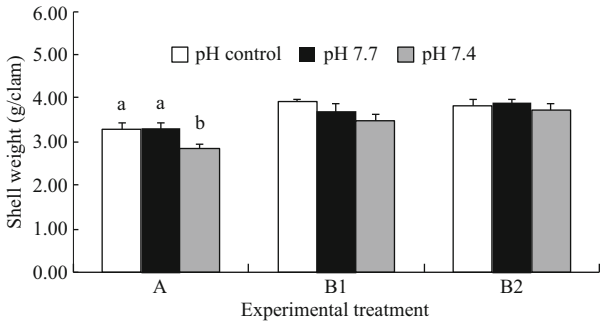

Fig.5 Shell weight of *Ruditapes philippinarum* at the end of both experiments

A: treated specimens were exposed to seawater in the acidification system without sediment (140 d); B1: treated specimens were exposed to seawater in the acidification system (170 d); B2: treated specimens were burrowed in sediment in the acidification system (170 d). Data were presented in mean±SD (n=30).

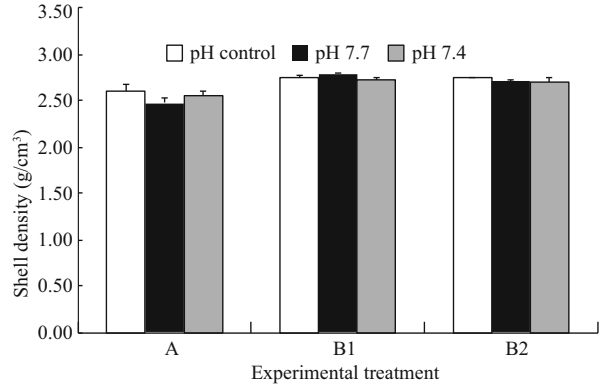

Fig.6 Shell density of *Ruditapes philippinarum* at the end of both experiments

A: treated specimens were exposed to seawater in the acidification system without sediment (140 d); B1: treated specimens were exposed to seawater in the acidification system (170 d); B2: treated specimens were burrowed in sediment in the acidification system (170 d). Data were presented in mean±SD (n=30).

3.3 Shell weight, density, and dissolution rate

Results of shell weight of *R. philippinarum* at the end of both experiments (Fig.5) indicated a difference at the end of the first experiment. Shell weight of clams exposed to seawater with a pH 7.4 was lower than those in the control and in the pH 7.7 treatment (df=2, F=4.236, P=0.019). In the second experiment, shell weight of clams exposed to seawater with a pH of 7.4 and 7.7 recorded a declined compared to the control (df=2, F=2.258, P=0.112). Shell weight showed no clear difference between the control and low pH treatments exposed to sediment in the second experiment after 170 days of exposure (df=2, F=0.338, P=0.714). As shown in Fig.6, shell density showed no significant difference between the control and the low pH treatments in the first experiment; results for the second experiment were in accordance with this result.

Shell dissolution rates of *R. philippinarum* at the end of both experiments are shown in Fig.7. Shell dissolution rate in the first experiment was affected by pH, and the shell dissolution of clams exposed to pH 7.4 (2.89 mg/clam/d) seawater was higher than the control (0.51 mg/clam/d) and the pH 7.7 (0.59 mg/clam/d) treatment (df=2, F=3.066, P=0.063). Shell dissolution rates of clams exposed to pH 7.4 (2.75 mg/clam/d) seawater in the second experiment were higher than the control (0.40 mg/clam/d), and the pH 7.7 (0.54 mg/clam/d) seawater (df=2, F=2.215, P=0.117). The results for the treatment exposed to the sediment (control, pH 7.4 and pH7.7) were 0.71, 0.54, and 1.28 mg/clam/d (df=2, F=0.339, P=0.714).

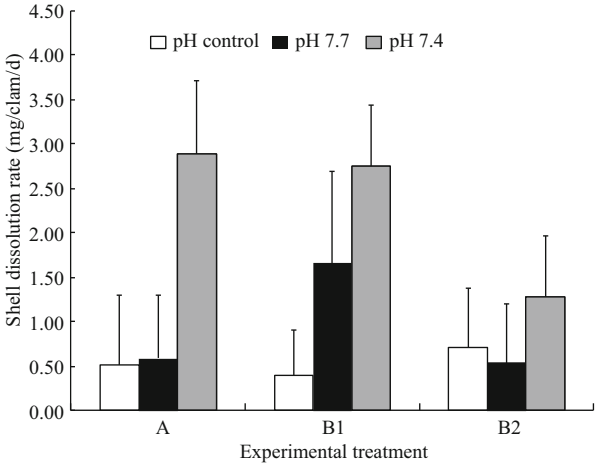

Fig.7 The shell dissolution rate of *Ruditapes philippinarum* at the end of both experiments

A: treated specimens were exposed to seawater in the acidification system without sediment (140 d); B1: treated specimens were exposed to seawater in the acidification system (170 d); B2: treated specimens were burrowed in sediment in the acidification system (170 d). Data were presented in mean±SD (n=30).

3.4 External surface structure of the shells

The structure of the external surface of *R. philippinarum* shells was examined by SEM at the end of both experiments (Fig.8). In the first experiment, we observed that the structure of the external surface exposed to acidic conditions showed notable damage compared with the control (Fig.8a, b, c). The structure of the shell's external surface from the control group had prominent and complete ribs (Fig.8a). Partial damage was observed in the pH 7.7 treatment (Fig.8b), whereas parallel ridges instead of ribs were observed in the pH 7.4 treatment (Fig.8c, arrows), accompanied by bumps and hollows (Fig.8c, ovals). In the second experiment, damage to the structure of the external surface of treated specimens from the seawater gradient depended on the seawater pH (Fig.8d, e, f), however specimens from the sediment treatment showed no clear difference between the control and the treatments (Fig.8g, h, i).

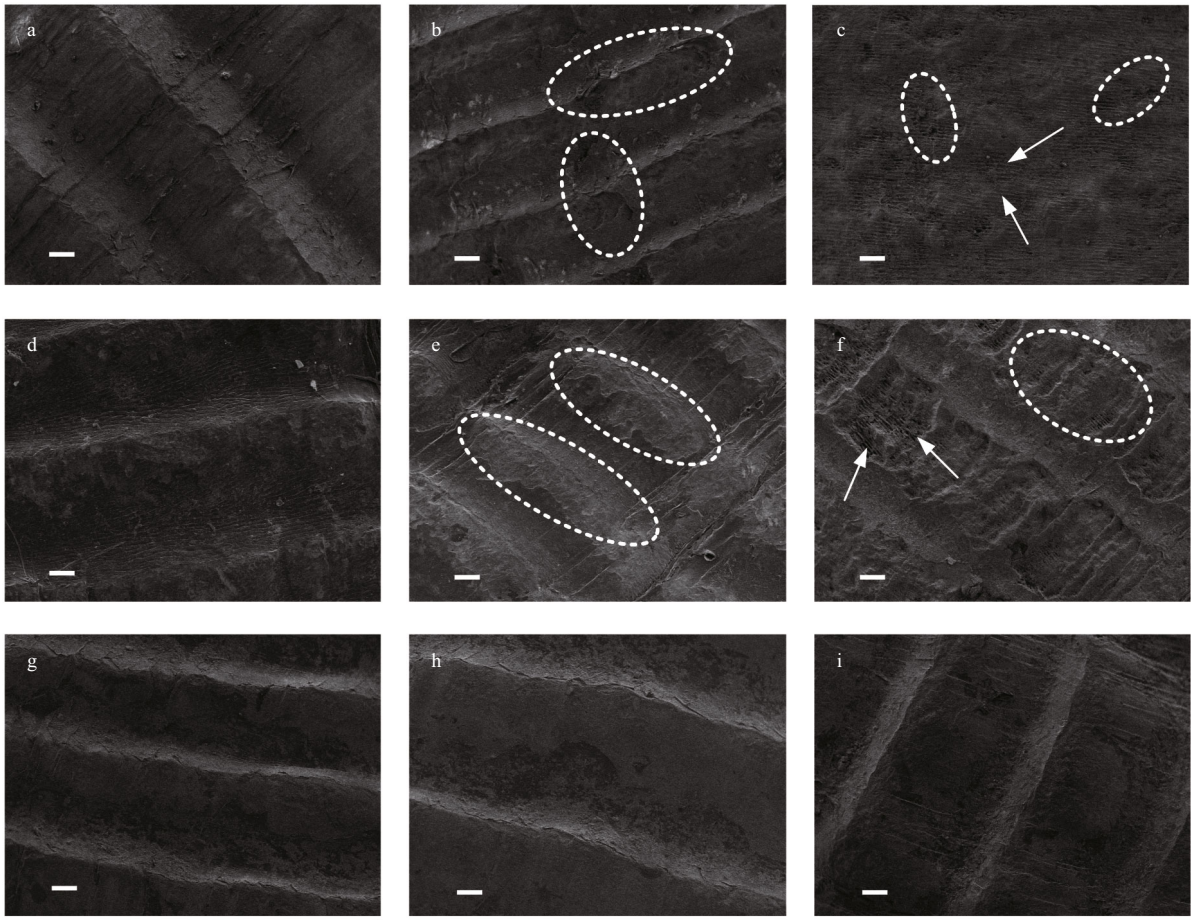

Fig.8 SEM micrographs of the shell external surface of *Ruditapes philippinarum* at the end of both experiments

The treated specimens in the first row (a, b, and c) were exposed to seawater in the acidification system without sediment (140 d). a: control; b: pH 7.7; c: pH 7.4. The treated specimens in the second row (d, e, and f) were exposed to seawater in the acidification system with sediment (170 d); d: control; e: pH 7.7; f: pH 7.4. The treated specimens in the third row (g, h, and i) were exposed to sediment in the acidification system (170 d); g: control; h: pH 7.7; i: pH 7.4. Ovals show the damaged structure and arrows point to the ridge (prismatic layer). Bar=100 μm.

In contrast to specimens from the sediment, shells of treated specimens from acidic seawater showed notable damage under the same conditions (Fig.8e, f, h, i). In addition, although the duration of the second experiment (170 d) was longer than the first experiment (140 d), damage to the external surface of treated specimens with pH 7.4 seawater was more serious in the first experiment than in the second experiment (Fig.8c, f).

4 DISCUSSION

In the first experiment, a decrease in shell weight corresponding to an increase in the dissolution rate were observed at pH 7.4, at which shell color disappeared and presented as white. In the second experiment (170 d), the obvious effects were recorded in shell dissolution rate and shell color in the shell specimens exposed to the overlying seawater, but not found in the shell specimens exposed to sediment. We found that the shell of *R. philippinarum* would be damaged under direct exposed to acidified seawater conditions, and the sediment can protect the shell of adult clams from the negative effects of ocean acidification.

Shallow-water sediment, representing a large reservoir of $CaCO_3$ that can react to the decreasing saturation state of seawater, thereby releasing alkalinity to the overlying water column, can play an important role in the global carbonate cycle (Navarro et al., 2013). Gazeau et al. (2014) investigating the impact of ocean acidification on sediment processes in shallow water in the Arctic Ocean, showed that higher T_A levels were observed for the 3 000 μatm treatment due to a significant release of T_A during 24 h incubations. In our study, higher T_A levels were observed in the pH 7.4 treatment in the second experiment, whilst Ω_{ca} and Ω_{ar} values were higher in the second experiment than the first. It is widely believed that a part of the negative impacts of ocean acidification derives from declining $CaCO_3$ saturation, thus sediments play an important role in reducing these negative impacts.

Several studies showed that the low sediment pH is negative to the infaunal bivalve. Clements et al. (2018) found that shell mass loss was 1% and 7% for *E. heros*, and 0% and 4% for *L. littorea* in 800 and 4 000 μatm, respectively, compared to 0% in the control for both species. The infaunal bivalves can avoid the negative impact of sediment acidification by reducing burrowing and increasing dispersal in more acidic sediments, irrespective of species or life stage (Clements et al., 2017). But in our study, the sediment can protect the clam from the ocean acidification to some extent. However, none of the previous studies demonstrate the sort of protective effect of sediment. Widdicombe et al. (2009) indicated that lowering pH in overlying seawater can decrease the pH of sediment pore water. The sediment pH is generally lower than the water column pH due to microbial activity in the sediment (Clements et al., 2018). The infaunal bivalve in sediment will still be exposed to low pH sediment porewater. A study showed that the gonads development of the clams (*R. philippinarum*) exposed to the sediment were more quickly than the clams exposed to the overlying seawater at low pH (Tiankun Han, unpublished). We thought that the shell dissolution happened on the outer shell surface, the process should be governed by chemical processes, rather than biological. The sediment pore water is less diffusive, when the pore water react with shell, the alkalinity will increase, thus the saturation will decrease, preventing the shell dissolution. We also thought it can be explained by buffering within sediments.

According to previous studies, the effects of acidic seawater on clam mortality varies among species. For example, Jansson et al. (2013) found the survival of larval *M. balthica* was significantly lower in more acidified water-column conditions (pH 7.2 and 7.4) in comparison to control (pH 7.7). In contrast, Range et al. (2011) recorded mortality of *Ruditapes decussatus* juvenile clams reared for 75 d under low pH conditions (7.84 and 7.46) to be lower compared to mortality rates under control conditions. Our results for both experiments indicated that there was no clear increase in average mortality per day in *R. philippinarum* maintained under acidic conditions. In the second experiment, average mortalities of clams exposed to seawater were higher than clams exposed to sediment under the same pH conditions. This suggests that clams burrowing in sediment can decrease mortality. Furthermore, the second experiment showed that increasing the concentration of CO_2 had a negative impact on the live weight of *R. philippinarum* directly exposed to seawater. This result was in accordance with previous reports by Duarte et al. (2015), who observed that increased concentration of CO_2 negatively affected the growth rate (total weight) of *Mytilus chilensis*. Interestingly, specimens in the sediment treatment recorded no significant difference between the control treatment and the two low pH treatments. This result indicates that sediment can

have a protective effect against ocean acidification for clams.

Several studies on shelled molluscs have described shell color to whiten and shell veins to degrade under high seawater acidification levels, for example the *Littorina littorea* and *E. heros* (Clements et al., 2018), the clam *Chamelea gallina* (Bressan et al., 2014) and pearl oysters *Princtadamar garitifera* (Le Moullac et al., 2016). Similarly, at the end of the first experiment, we observed a gradient in the bleaching of shell color and degradation of shell veins depending as pH levels decreased, suggesting that shell external surface of the clams was damaged by seawater acidification.

As clams live for the majority of their life-span in seawater sediment, one of the aims of the second experiment was to investigate protective effects of sediment against acidic seawater on their shell color, an area we currently know little about. Our results indicated that the shell color of specimens living within the sediment showed no clear difference between the control samples and those under low pH treatments. This result demonstrates that sediment could protect shell color from the negative effects of ocean acidification. In addition, although the duration of our second experiment (170 d) was longer than the first experiment (140 d), the bleaching of shell color of treated specimens from pH 7.4 seawater was more serious in the first experiment than in the second experiment. Our previous result showed that the Ω_{ca} and Ω_{ar} values were higher in the second experiment than in the first experiment, thus we propose that this result could be related to $CaCO_3$ saturation in seawater.

In our first experiment, adult clams were exposed to seawater in the acidification system without sediment. After 140 days, adult clams from the pH 7.4 treatment recorded lower shell weights and higher shell dissolution rates than those in the control and the pH 7.7 treatment. No significant difference in shell density was found between the control and low pH treatments. This result is in agreement with the findings of Ries et al. (2009), where net shell dissolution was observed under the highest pCO_2 treatment applied (pH 7.45) in both hard clams (*M. mercenaria*) and soft clams (*Mya arenaria*). Conversely, Range et al. (2011) did not detect any shell damage in *R. decussatus* after exposure to pH 7.4 for 75 days. In contrast to the carbonate concentration in the two studies, Ries et al. (2009) observed that the experimental seawater was under-saturated with respect to aragonite and calcite; under-saturated conditions were not observed by Range et al. (2011). Bressan et al. (2014) found that shell thickness of treated (pH 7.4) animals was significantly thinner than control specimens after three months for clams (*Chamelea gallina*) and after six months in mussels (*Mytilus galloprovincialis*). Zhao et al. (2017) found that ocean acidification impairs the calcification process and inner shell surface integrity. There is no doubt that a decrease in shell thickness can seriously threaten survival. Although we did not evaluate actual shell thickness, we can conclude that shell thickness of adult clams could be negatively affected by seawater acidification since shell density and volume was not changed, but shell weight was decreased under pH 7.4 conditions.

Larvae and juveniles are certainly more sensitive to ocean acidification than adults. Clements and Hunt (2014) showed that sediment acidification can negatively impact the burrowing success and dispersal of juvenile soft-shell clams (*Mya arenaria* L.). They also found that there was a significant positive relationship between the percent of juvenile clams (*Mya arenari*a) burrowed and pore water acidification in the lab (Clements and Hunt, 2018). Green et al. (2009) considered the *M. mercenaria* (0.2, 0.4, and 0.6 mm) were reared in sediments at Ω_{ar}=0.4, losses of living individuals were 14.0%, 9.6% and 2.8%/d, respectively. These appeared to prove the smaller one was more sensitive to sediment acidification. The sediment acidification had more serious negative effects to the larvae and juveniles.

Recent studies have not considered the importance of sediment in the evaluation of the effects of ocean acidification. In our second experiment, adult clams were exposed to seawater in the acidification system with sediment. Interestingly, shell weight, shell density and shell dissolution rate of adult clams exposed to sediment were not changed by seawater acidification; as per the first experiment, shell weight and shell dissolution rate of adult clams exposed to seawater were affected under pH 7.4 conditions. This result showed that in contrast to adult clams exposed to acidified seawater, the shells of clams burrowing in sediment could have increased protection from the effect of seawater acidification. In this regard, we can predict that marine bivalves with burrowing behavior will have more adaptability than those without burrowing behavior for future predicted increased in seawater acidification conditions. To our knowledge, this effect of seawater acidification on bivalves is reported for the first time.

5 CONCLUSION

The findings of our study demonstrate that the shell of *R. philippinarum* could be damaged under direct exposed to acidified seawater conditions, and that sediment can protect the shell of adult clams from the negative effects of ocean acidification. Hence, from the standpoint of a defense function for the shell, we can predict that marine bivalves with burrowing behavior might have more adaptability than non-burrowing bivalves in regard to future acidification of ocean water. In addition, SEM results showed that the effect of seawater acidification on the shell of adult clams directly exposed to high pCO_2 seawater in the acidification system without sediment was higher than in the acidification system with sediment. However, studies simulating future ocean acidification conditions aimed at evaluating the effects of acidification on marine bivalves have neglected the effects sediment provides in the marine environment. Further investigations should consider sediment as an important factor in the ocean acidification problem, regardless of the burrowing behavior of the marine bivalves.

6 DATA AVAILABILITY STATEMENT

The data used to support the findings of this study are available from the corresponding author upon request.

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