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Effects of clam size, food type, sediment characteristic, and seawater carbonate chemistry on grazing capacity of Venus clam *Cyclina sinensis* (Gmelin, 1791)*

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Abstract Aquaculture in saline-alkaline water has a major problem: microalgal blooming causes the pH of water to increase dramatically, thereby causing damage to the reared organisms. To solve this problem, we set out to find a candidate filter-feeding bivalve species suitable for saline-alkaline water to graze on microalgae and to control the pH. In the current study, we investigated the effect of carbonate alkalinity (CA, 2.5, 10.0, and 20.0 meq/L) and pH (8.0, 8.5, and 9.0) on the grazing capacity (GC) of the clam *Cyclina sinensis*. Additionally, the effect of clam size (small, medium, and large) and microalgae species (*Nannochloropsis oculata*, *Chaetoceros müelleri*, and *Isochrysis galbana*), and the effect of bottom sediment characteristic (mud, sandy mud, and muddy sand) and thickness (3 and 6 cm) were analyzed as well. The results show that the GC on *I. galbana* was the highest and small size had the maximum GC/*W* (*W*: wet weight including body and shells). No significant differences were observed between sediment type and thickness. Regarding CA and pH, a significant decrease in GC by the pH or by their interaction was found. The GC of *C. sinensis* was not greatly reduced in the treatments of pH≤8.5 and CA≤20.0, and also not affected by bottom sediment type, indicating that this clam is capable to manage microalgal concentrations and might be a candidate species for pH reduction in saline-alkaline water ponds.

Keyword: Venus clam *Cyclina sinensis*; carbonate alkalinity; pH; grazing capacity; saline-alkaline water

1 INTRODUCTION

Saline-alkaline water is distributed naturally throughout the world, particularly in Central and East Asia, South America, the Middle East, the former USSR and Australia and surrounding regions (Sharma and Minhas, 2005; Dendooven et al., 2010). In China, saline-alkaline lakes cover approximately 4.32 million hectares, which account for 54.4% of the total lake area (Yao et al., 2010). Of concern, the area of global saline-alkaline water has been rising due to natural factors such as increased evaporation and rising sea-levels caused by global warming (Rozema and Flowers, 2008) in addition to human activity such as the use of freshwater for agricultural irrigation and leaching in saline-alkaline regions (Rengasamy, 2006; Quantin et al., 2008). Saline-alkaline water has a high electrolytic conductivity, pH and carbonate alkalinity (CA), a poor buffering capacity, and an unstable proportion of major ions (Sharma and Minhas, 2005). Because of these characteristics, most saline-alkaline water remains neglected.

With the decreasing availability of freshwater resources, countries around the world have begun to exploit saline-alkaline water (Gómez-Bellot et al., 2013). Until now, two major approaches for saline-

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alkaline water utilization have been recognized. The first approach includes the practice of irrigation with desalination and dealkalization (Minhas, 1996; Birnhack et al., 2010) or breeding saline-alkaline tolerant crops irrigated directly with saline-alkaline water (Rozema and Schat, 2013); the other approach includes the use of aquaculture where saline-alkaline tolerant aquatic animals are transplanted from freshwater or seawater into saline-alkaline water (Allan et al., 2009). Generally, compared with the first approach, which requires vast amounts of labour, materials and financial resources, the second approach is more economical and manoeuvrable.

For more than 20 years, our team has engaged in the study of the use of saline-alkaline water for aquaculture. In 1994, the Chinese shrimp *Penaeus chinensis* was first farmed successfully in saline-alkaline waters in Northwest China (Wang et al., 1997). Soon after that advance, several species including freshwater prawn *Macrobrachium rosenbergii* (Wang, 1996), naked carp *Gymnocypris przewalskii* (Cao et al., 2009) and Siberian sturgeon *Acipenser baerii* (Huang et al., 2010) have been cultured commercially in saline-alkaline water throughout inland China, saving extensive freshwater resources and providing economic benefits to local farmers. Besides, we also found that aquaculture in saline-alkaline water has a positive effect on limiting the surrounding soil from further salinization/alkalization, even for recultivation. However, in recent years, as saline-alkaline water aquaculture has been developing, a major problem has emerged: it is prone to outbreaks of microalgal blooming in the middle and late rearing period. The microalgal blooming is mainly attributed to excessive feeding and long-term unchanged water (i.e., saline-alkaline water is not allowed to drain because it will quicken the surrounding soil secondary salinization/alkalization) (Lindholm et al., 1999; Barkoh et al., 2011). The damages of microalgal blooming to the reared organisms are serious and multifactorial. One of the most concerned is that blooming microalgae consume a large amount of CO_2 and result in the dramatic pH increase in rearing water, thereby causing direct damage to the reared organisms.

Bivalves are a group of filter feeders that graze mainly on planktonic microalgae (Nakamura and Kerciku, 2000). Hence, bivalves might be potential organisms to control microalgal blooming in saline-alkaline water ponds and to maintain the water pH below the lethal level. To verify this hypothesis, in our previous study, Venus clam *Cyclina sinensis*, a seawater bivalve species with a high microalgae-filtering capability and natural distribution along the sandy mud beaches, has been employed to investigate its CA and pH adaptation ranges (pH≤9.0 and CA≤20.0 meq/L) according to the clam survival and growth performances (Lin et al., 2013). The main objective of the current study is to get insight into the effects of CA and pH, clam size and microalgae species, and bottom sediment characteristic and thickness on the grazing capacity in the clam *C. sinensis*.

2 MATERIAL AND METHOD

2.1 Experimental microalgae

Three microalgae (*Nannochloropsis oculata*, *Chaetoceros müelleri* and *Isochrysis galbana*) were provided by FACHB-collection (Freshwater Algae Culture Collection at the Institute of Hydrobiology, Wuhan, China) and were scaled up and cultured in the laboratory. The microalgae were grown in Erlenmeyer flasks containing 5 L of sterilized seawater (salinity 28) mixed with F/2 nutrients (Guillard and Ryther, 1962) with aeration. The cultures were maintained in illuminating incubators (MLR-351, SANYO, Osaka, Japan) with a temperature of 26°C, relative humidity of 75% and illuminance of 6 000 lx (12 h:12 h light/dark). The cultured microalgal solution was concentrated by a centrifugation at 400×*g* for 5 min, then was resuspended with clam rearing seawater (see Section 2.2, temperature of 20±0.5°C and salinity of 15±1) or with CA & pH experimental water (see Section 2.4 and 2.5.3), and adjusted to a final concentration of (1.0–1.2)×10^7 cells/mL 1 h before being used in the experiment.

2.2 Experimental clams

The Venus clam *Cyclina sinensis* was sampled from a bivalve hatchery at Qingjiang Town, Yueqing City, Zhejiang Province, China. More than 1 000 clams of 1.2 to 4.0 cm in shell height (maximum linear dimension from apex to ventral edge) were sampled. Upon arrival at the laboratory, the clams were reared in five fibreglass tanks (length×width×height: 140 cm×70 cm×30 cm) with each tank containing approximately 200 clams. Each tank contained 200 L of sterilized and aerated seawater with a salinity of 15±1 (diluted with tap water), temperature of 20±0.5°C, pH of 8.0±0.1 and CA of 2.5±0.2 meq/L. Rearing seawater was changed daily.

The clams were fed daily with a mixture of microalgae. The clams were acclimated for two weeks and were not fed two days before the grazing experiments. Each clam in the experiment was used only once.

2.3 Experimental mud and sand

The sandy mud used in the bottom sediment experiment was collected from the intertidal zone in Qingjiang Town. To separate the mud and sand, the mixture was immersed in tap water and stirred. After settling for a few minutes, the sand sediment on the bottom was collected. The supernatant was siphoned out to another tank for overnight settling and the mud settlement was collected the following day. The mud and sand were then sterilised by boiling for 30 min and then sun-dried for at least one day. The particle diameters of the mud and sand were 11.6±1.5 and 121.8±3.3 μm, respectively.

2.4 Experimental water

The water used in the CA and pH experiment was prepared by addition of the required volume of 0.1 mol/L NaOH, or the required proportions of Na_2CO_3 and $NaHCO_3$ to the rearing seawater. CA was determined by the method of a strong acid titration to pH 4.5 using a changing-colour indicator. Specifically, 50 mL of water sample mixed with three drops of changing-colour indicator (a mixture (1:1, v/v) of 0.1% of methyl orange and 0.1% of aniline blue) was added into a 250-mL triangular flask, then HCl, a titrating solution whose concentration was pre-determined by the titrating method with 0.05 mol/L Na_2CO_3, was added into slowly until the solution colour in the flask change into light burgundy and keep for at least 3 min, then stopped titration and recorded the volume of HCl consumption. The unit of CA was expressed as the meq of HCl consumed by one litre of water sample. pH was measured with a pH-meter (FE20K, Mettler-Toledo, Zürich, Switzerland). The experimental water was maintained in plastic tanks for 24 h before use.

2.5 Experimental design

2.5.1 Experiment 1. The effect of clam size and microalgae species on the grazing capacity

Acclimated clams were pre-divided into three groups according to body sizes: small, medium and large (Table 1). The experimental set up consisted of a 3×3 factorial design with three clam sizes and three microalgae species (*N. oculata*, *C. müelleri* and *I. galbana*), resulting in a total of 9 treatments. Each treatment consisted of five replicates.

Table 1 Body and age parameters of the three size groups

	Small	Medium	Large
Shell height (cm)	1.52±0.10	2.44±0.14	3.68±0.14
Wet weight (g) (including body and shells)	0.97±0.15	4.12±0.47	14.84±1.22
Age (month) (beginning with a fertilized egg)	6–8	12–14	24–30

In each replicate, five clams were transferred from the acclimated tanks into a 5-L glass beaker containing 4 L of rearing seawater and recovered for a few moments until all of the clams stuck the ends of their siphons out between their shells. Then, 1 L of microalgal solution resuspended in rearing seawater was applied carefully (to avoid disturbing the clams) to the beaker, and the microalgal concentration (C_0) was measured using a haemocytometer under a microscope (IX71, Olympus, Tokyo). The microalgal concentration (C_{tf}) was measured again two hours later. Afterwards, the clams were taken out for weight (W: wet weight including body and shells) measurement. Beakers containing 4 L of rearing seawater and 1 L of microalgal solution, but no clams, were employed as a blank control. The grazing capacity was calculated using the following formula (Widdows and Navarro, 2007):

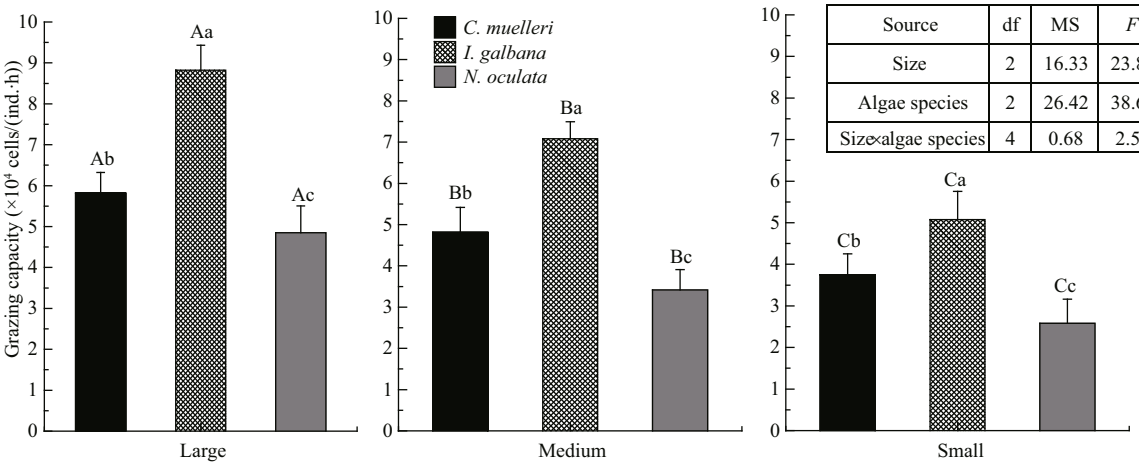
$$\mathrm{GC} = \frac{V}{N} \times \frac{\ln C_t - \ln C_{tf}}{t} \times \frac{C_{tf} - C_0}{\ln C_{tf} - \ln C_0},$$

where GC: grazing capacity (cells/ind./h); t: total time of the experiment (h); V: volume of the microalgal solution in the experimental container (L); N: number of clams in the experimental container (ind); C_0 and C_{tf}: microalgal concentration at times 0 and t in the experimental group (cells/mL); C_t: microalgal concentration at time t in the blank control (cells/mL).

According to the result of experiment 1, the treatment with the highest value of GC/W was selected for the bottom sediment characteristic and thickness, CA and pH experiments.

2.5.2 Experiment 2. The effect of bottom sediment type and thickness on the grazing capacity

The experimental design consisted of a 3×2 factorial trial with three types of bottom sediment (mud; sandy mud, with a sand to mud ratio of 1:3; and muddy sand, with a sand to mud ratio of 3:1) and two levels of thickness of 3 cm and 6 cm (i.e., in a natural state, to stick out their siphons from the sediment, the



Source	df	MS	*F*	*P*
Size	2	16.33	23.88	0.006
Algae species	2	26.42	38.64	0.002
Size×algae species	4	0.68	2.52	0.078

Fig.1 The effect of body size (small, medium and large) and microalgae species (*N. oculata*, *C. müelleri* and *I. galbana*) on the grazing capacity of *C. sinensis*

Values with different capital letters indicate a significant difference in the grazing capacity among the clam sizes compared within the same microalgae species. Values with different lower-case letters indicate a significant difference in the grazing capacity among the microalgae species compared within the same clam size.

diving depth of clams *C. sinensis* into sediment is not deep, usually of 3–6 cm); additionally, a no sediment treatment was as control to simulate rocky sediment, resulting in a total of 7 treatments. Each treatment contained five replicates.

In each replicate, the dried sediment was placed on the bottom of a 5-L glass beaker. Four litres of rearing seawater were poured slowly along the beaker wall into the beaker and let rest until the sediment and water were completely layered. Five clams were then placed into the beaker and once they all burrowing into the sediment (for no sediment, once all of the clams stuck the ends of their siphons out between the shells), 1 L of microalgal solution resuspended in rearing seawater was added carefully into beaker. The microalgal concentrations were measured at times 0 and 2 h for calculation of the grazing capacity.

2.5.3 Experiment 3. The effect of CA and pH on the grazing capacity

According to the CA and pH tolerant ranges for *C. sinensis* survival and growth reported by Lin et al. (2013), the current experiment consisted of a 3×3 factorial design with three levels of CA (2.5, 10.0 and 20.0 meq/L) and pH (8.0, 8.5 and 9.0), resulting in a total of 9 treatments. The preparation of experimental water and the required levels of the CA and pH in each treatment were followed the methods in Lin et al. (2013). Each treatment contained five replicates.

In each replicate, five clams were placed into a 5-L glass beaker containing 4 L of experimental water and were acclimated until all of them stuck the ends of their siphons out between the shells. After being placed in the water, the time when the clam sticking its siphon out was recorded as an indicator of stress. Subsequently, 1 L of microalgal solution resuspended in experimental water was added carefully into the beaker, and the microalgal concentrations at 0 and 2 h were measured for the grazing capacity calculations.

2.6 Statistical analyses

All the data from the grazing experiments were expressed as the mean±standard deviation. Prior to the analyses, normality of the data was evaluated using Shapiro-Wilk W-test and the homogeneity of variances was assessed using Levene's test with the SPSS statistical software (version 16.0, Chicago, Illinois, USA). The effects of clam size & microalgae species, bottom sediment type & thickness and CA & pH were analysed using two-way analysis of variance (ANOVA) followed by a post hoc test with Bonferroni correction if there is a significant interaction. Otherwise, a Tukey's multiple comparison test was applied within one factor fixed if interaction is not significant. The level of significance was defined as $P<0.05$.

3 RESULT

3.1 The effect of clam size and microalgae species on the grazing capacity

There was a significant difference in grazing capacity among the three sizes of clams when grazing on the same microalgae species ($P=0.006$), with the large size exhibiting the highest grazing capacity and the small size exhibiting the lowest (Fig.1). However,

Source	df	MS	*F*	*P*
Size	2	31.10	120.3	<0.001
Algae species	2	3.54	13.68	<0.001
Size×algae species	4	1.06	4.09	0.015

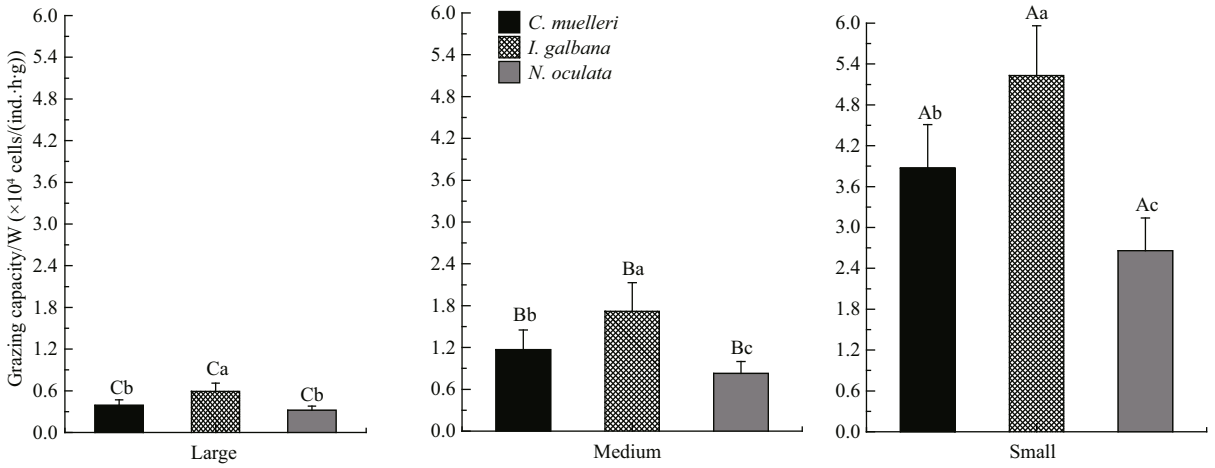


Fig.2 The effect of body size (small, medium and large) and microalgae species (*N. oculata*, *C. müelleri* and *I. galbana*) on the grazing capacity per unit body weight of *C. sinensis*

Values with different capital letters indicate a significant difference in the grazing capacity per unit weight among the clam sizes compared within the same microalgae species. Values with different lower-case letters indicate a significant difference in the grazing capacity per unit weight among the microalgae species compared within the same clam size.

when the grazing capacity was expressed in GC/*W* (cells/(ind.·h·g)), the results showed the opposite trend; i.e., the GC/*W* of the small clam was the highest and the large was the lowest (Fig.2). Additionally, among the three microalgae species, a clear difference was observed when grazed by the same size clam (*P*=0.002), where *I. galbana* incurred the maximum and *N. oculata* the minimum. The combined effect between clam size and microalgae species was not significant (*P*=0.078) (Fig.1).

3.2 The effect of bottom sediment type and thickness on the grazing capacity

The results from six treatments displayed no significant effects of bottom sediment type (*P*=0.67), thickness (*P*=0.30), as well as their interaction (*P*=0.24) (Fig.3). Besides, the results of Tukey's multiple comparison test (7 levels) did not showed significant differences among no sediment and the other six treatments.

Source	df	MS	*F*	*P*
Sediment	2	0.08	0.48	0.67
Thickness	1	0.31	1.93	0.30
Sediment×thickness	2	0.16	1.60	0.24

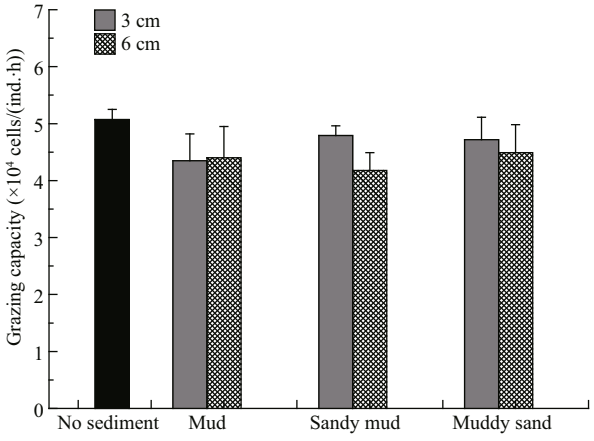


Fig.3 The effect of the bottom sediment type (mud, sandy mud and muddy sand) and thickness (3 cm and 6 cm) on the grazing capacity of *C. sinensis*

3.3 The effect of CA and pH on the grazing capacity

After the clams were transferred from the seawater into saline-alkaline water, they required more recovery time before sticking out their incurrent and excurrent siphons as the pH, CA or both increased (Fig.4). It took approximately 7–13 min for clam to recover in the treatments of CA 2.5 & pH 8.0, CA 2.5 & pH 8.5 and CA 10 & pH 8.0. In the high pH (9.0) or high CA (20.0) environment, on the other hand, the

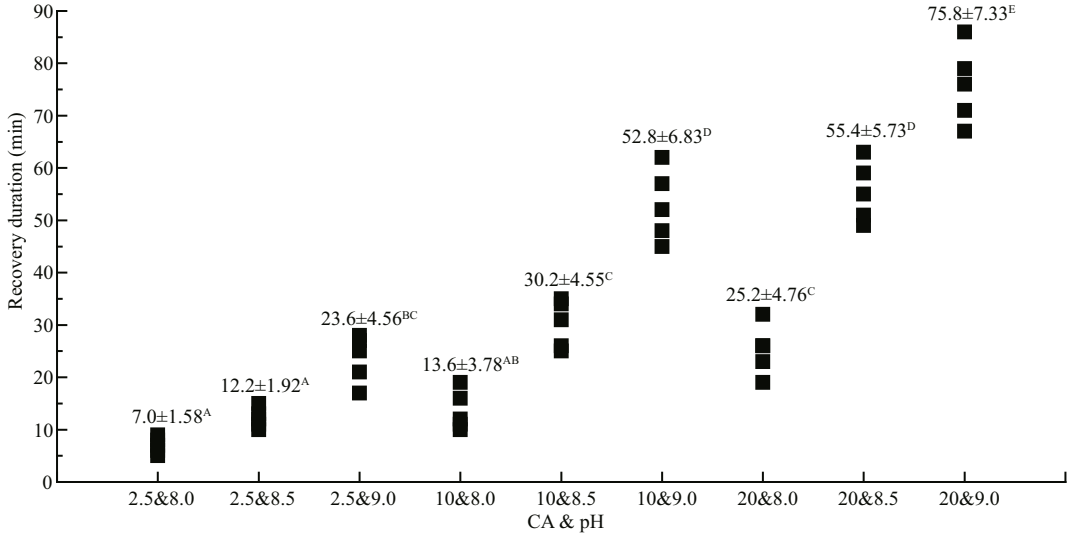


Fig.4 The time needed by *C. sinensis* to stick the ends of their siphons out when transferred from seawater into different CA (2.5, 10 and 20 meq/L) and pH (8.0, 8.5 and 9.0) level waters

Black square (■) represents an individual clam. The values with different superscript capital letters indicate a significant difference among the different treatments.

clams took a significantly longer period to recover; more than 50 min in the treatments of CA 10.0 & pH 9.0 and CA 20.0 & pH 8.5, and more than 75 min in the treatment of CA 20.0 & pH 9.0.

After being exposed to high CA and pH, the clams' grazing capacities were decreased (Fig.5). Specifically, highest grazing capacity occurred at low pH (8.0) together with low CA (2.5 and 10) environments. Increases in both the CA and pH resulted in a synergistical decrease in grazing capacity. In the treatments of CA 10.0 & pH 9.0 and CA 20.0 & pH 8.5, the grazing capability was reduced by approximately 31.0%, and it was reduced by 67.9% in the treatment of CA 20.0 & pH 9.0. The effect of CA was not significant (P=0.079), but the effect of pH was significant (P=0.027). Besides, their interaction (P<0.001) was also significant, which means the weakening effect of CA on grazing capacity is changed with pH.

Source	df	MS	F	P
CA	2	3.99	5.11	0.079
pH	2	7.97	10.20	0.027
CA×pH	4	0.78	37.24	<0.001

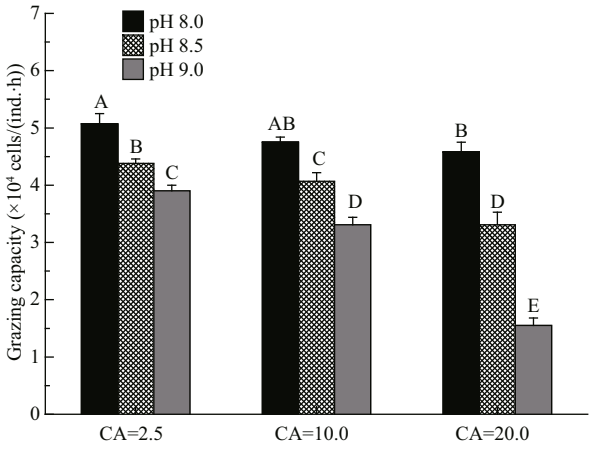


Fig.5 The effect of CA (2.5, 10 and 20 meq/L) and pH (8.0, 8.5 and 9.0) on the grazing capacity of *C. sinensis*

Values with different capital letters indicate a significant difference among the different treatments.

4 DISCUSSION

In China, saline-alkaline water is varied. It can be classified into inland and coast types, low- and high-salinity types, carbonate, chloride and sulphate types based on the criteria of location, salinity and dominant anion, respectively (Wang et al., 2010). Up to now, most of the saline-alkaline water types have been successfully developed for aquaculture, and have gained good economic and ecological benefits. However, in recent years, as saline-alkaline water aquaculture has been developing, a major problem has emerged: microalgal blooming causes a rapid increase in water pH in the middle and late rearing period, and results in reduced performances in reared organisms. The blooming microalgae vary with saline-alkaline water types. In the low-salinity & carbonate type, they are dominated by Cyanophyta (particularly *Microcystis*, *Oscillatoria* and *Arthrospira*), and Chlorophyta (particularly *Chlorella* and *Nannochloropsis*) (Wang et al., 2010). In the low-salinity & chloride type, they are

dominated by Bacillariophyta (particularly *Cyclotella* and *Chaetoceros*, and Cyanophyta) (Zhao et al., 2002). In the high-salinity & chloride type, they are dominated by Chrysophyta (particularly of *Prymnesiacee* and *Isochrysis*) (Wang et al., 2015). Based on the key blooming microalgae species in the saline-alkaline water in the published results, three microalgae species *N. oculata*, *C. müelleri* and *I. galbana* were selected in the current study, which respectively represent Chlorophyta, Bacillariophyta and Chrysophyta and have been demonstrated as the ideal grazing objects in a large number of bivalves (Leverone et al., 2007; Velasco, 2007; Jones et al., 2011).

The results have showed that *C. sinensis* preferred to graze *I. galbana*. The selectivity to *I. galbana* may be attributed to the chemical composition or algal metabolites in this algal species which could act as an activator of grazing, similar to the functions of those compounds extracted from certain microalgae that could greatly induce the clearance rates of scallops (Ward et al., 1992; Navarro et al., 2000; Velasco, 2007). Many of the physiological capacities of bivalves could vary with their body sizes (Cranford et al., 2011). For instance, small clams of the species *Meretrix meretrix* exhibited higher oxygen consumption and ammonia excretion rates than their large counterparts after a starvation stress (Fan et al., 2009), while large oysters of the species *Crassostrea gigas* consumed a greater proportion of sea lice larvae than their small and medium counterparts (Webb et al., 2013). Similarly, we found that the GC of *C. sinensis* is positively related to body size, but the GC/*W* value was the greatest in the small size group. The trend of GC/*W* value indicates that if no or limited predators exist, choosing small size clam might be a better practical option, not only because of its lower cost (i.e., small *C. sinensis* is much cheaper than their large size counterparts), but also its stronger algae-filtering capacity compared with the large size clams.

The clam *C. sinensis* is an infaunal bivalve and has a habit of diving into the coastal sandy mud (Liu et al., 2002), however, in saline-alkaline water areas, the bottom sediment types can vary from mud, sand, sandy mud to rock. The results from the current study showed that effects of bottom sediment types, thickness, and their interactions were not significant. Similar observations were also found in the clams *Macoma balthica* and *Soletellina alba* (Huxham and Richards, 2003) and in the cockle *Cerastoderma edule* (Matthews and Fairweather, 2006): the sediment choice of the small individuals in these three bivalves showed no correlation with sediment characteristics.

The CA and pH are two important factors that determine the adaptation of aquatic animals to saline-alkaline water. High CA leads to a low solubility of CO_2 in the water and to a reduced pCO_2 in the gill water during respiratory CO_2 exchanges (Truchot and Forgue, 1998; Velo et al., 2013). A number of studies have reported immune suppression, hypocapnia and respiratory alkalosis as a result of reduced pCO_2 (Truchot, 1984; Goss et al., 1994; Yao et al., 2010). High pH leads to acid-base imbalances, Ca^{2+} and Mg^{2+} deficiency, an increase in oxygen consumption rates, a decrease in ion transporter activity or inactivation, etc. (Wang et al., 2002; Pan et al., 2007; Li and Chen 2008). In the current study, prolonged recovery duration and weakened grazing capacity both demonstrated that high pH could exhibit an inhibitory effect upon the physiological state of *C. sinensis*. However, no inhibitory effect of CA was noted which may be due to *C. sinensis* with a strong tolerance to high CA (Lin et al., 2012), or pH more susceptible to pCO_2 fluctuation than CA (Gazeau et al., 2011).

In addition to the significant effect of pH, in this study, a significant interaction between CA and pH was also found, i.e., pH combined with CA could cause a stronger weakening effect than the single pH or CA. This relationship can be explained by the following reaction equations:

I $CO_2+H_2O \leftrightarrow HCO_3^- + H^+$ ($pK_{a1}=6.35$),

II $HCO_3^- \leftrightarrow CO_3^{2-} + H^+$ ($pK_{a2}=10.33$).

According to the above equations, CO_2 is the predominant component at pH≤6.35, and CO_3^{2-} is predominant at pH≥10.33. HCO_3^- dominates at 6.35<pH<10.33, where within this pH range, increasing the pH will lead to the transformation of HCO_3^- into CO_3^{2-}, as shown in equation II. The loss of HCO_3^- caused by this transformation is negligible, but CO_3^{2-} gains greatly (Truchot and Forgue, 1998; Yao et al., 2010). Many studies have noted that the toxic effect of CO_3^{2-} on aquatic animals is far greater than HCO_3^- (Lei et al., 1985; Thomas and Poupin, 1985). In summary, a higher pH promotes a higher proportion of CO_3^{2-}, resulting in a higher toxicity.

5 CONCLUSION AND FUTURE DIRECTION

In this study, no correlation between bottom sediment type and grazing capacity was found, indicating that *C. sinensis* could be potentially be reared in various saline-alkaline waters regardless of their bottom sediment characteristics. Additionally,

although the effect of pH, and pH combination effect of CA were significant, the decreasing magnitude was within 30% in most treatments (except for CA 20.0 & pH 9.0), indicating that *C. sinensis* could keep a high microalgae-filtering capability in saline-alkaline water. The capability in managing microalgal concentrations in saline-alkaline water would suggest that *C. sinensis* could be applied to manage pH fluctuations. To improve our understanding on pH control with this bivalve species, studies on more parameters would be needed, such as grazing on cyanobacteria, long-term effect of CA and pH, and their synergic effects with other non-biological (such as temperature and salinity) and biological (such as size and sex) factors.

6 ACKNOWLEDGEMENT

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