Effects of varying acidic levels on dissolution, strength, organic content and surface texture of Pacific oysters (*Crassostrea gigas*) shells

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Abstract

Marine coastal organisms are exposed to periodic fluctuations in seawater pH driven by biological carbon dioxide (CO_2) production which may in the future be further exacerbated by the ocean acidification associated with the global rise in CO_2. There is widespread concern that these changes have direct impact on coastal organisms and alter the habitats severely. However, little or no attention has been given to the effects of the anticipated decrease in coastal pH on farmed oysters within the Namibian coastal waters. In this investigation, shells of the Pacific oysters, *Crassostrea gigas* were exposed to varying acidic levels under laboratory conditions; pH level 6.5 represented extreme hypercarpnia condition, 7.0 and 7.5 representing future predicted coastal pH levels. Shell dissolution rate, strength, organic content and surface texture were assessed after a two-week exposure period. Significant loss ($p < 0.05$) in weight and diameter were observed in shells exposed to 6.5, 7.0 and 7.5 pH levels compared to shells in the control groups (pH 8.1-8.2). With regard to organic content of the shell, significant reduction ($p < 0.05$) was only observed in shells exposed to 6.5 and 7.0 pH levels. Microscopic examination of the shell surface revealed reduced nacreous layer while the organic layer of the shells was sheared in acidic conditions. Visual inspection of the nacre region of shells exposed to 6.5, 7.0 and 7.5 pH showed straight edged tablets, with the nacre

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regions characterised by sparse with irregular shaped tablets within a reduced organic matrix. Ocean acidification can impact potential changes in morphometry and shell structure of pacific oysters during culture.

Keywords: Climate change, *Crassostrea gigas*, hypercapnic, Ocean acidification, pH, shell integrity

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1 Introduction

Ocean acidification is the lowering of oceanic pH and carbonate saturation that result from increasing atmospheric CO_2 concentrations. Observations indicate that oceanic pH declined by 0.1 unit in 2005 compared with pre-industrial values and it was projected to decrease further by the end of the century (Gazeau et al., 2010). The change in oceanic pH level will have indirect and potentially adverse biological and ecological consequences (Barker and Ridgwell, 2012), as most marine organisms are sensitive to pH reduction (Fabry et al., (2008). Calcifying organisms will be especially affected through decreased Calcium carbonate ($CaCO_3$) saturation, which affects calcification rates via disturbance to acid-base (metabolic) physiology (Sciandra et al. 2003).

The Pacific oyster, *Crassostrea gigas* which is native to temperate regions within the Pacific, have been introduced to many countries where it is being farmed as one of the promising species for the shellfish mariculture industry. Previous studies have found that early developmental stages of oysters are more susceptible to ocean acidification stresses, leading to various abnormal phenomena such as retard growth and development, and shell morphological abnormalities (Wei et al., 2015).

The shell of calcifying organisms act as a skeleton for the attachment of muscles, protects these organisms against predators and in burrowing species, it keep mud and sand out of the mantle cavity (Waldbusser et al. 2011). Its main component is Calcium carbonate and is formed by the deposition of crystals of this salt, an organic matrix of the protein, conchiolin. Calcium for shell growth is obtained from the diet or taken up from seawater (Gosling, 2003). The calcification of these shells depends on saturation of the carbonate mineral aragonite and the projected 30% decrease in the aragonite saturation as a result of increasing atmospheric CO_2 (Field et al., 2002) will have a major influence of the calcification rates of these organisms. Most calcifying marine organism produces some type of external organic layer that separates their shell from ambient seawater (Welladsen et al., 2010). However, the structure and composition of these protective organic layers vary widely amongst organisms, with oyster shells being mainly composed of three layers (the inner nacreous layer, the middle

prismatic layer and the outer organic layer (Gutierrez et al. 2003).

Shells are very important component structure of an organism. Exposure to adverse pH conditions and the destruction of the organic matrix of the shell surface as reported in the Pearl oyster by Fougerouse et al. (2008) could potentially be an assessment tool for survival of the oysters in increased acidic seawater.

This study aimed at determining the effects of predicted future pH levels of the ocean on the shell integrity of the Pacific oyster under laboratory conditions. Specifically, the effect on the shell dissolution rate, strength, organic content and surface texture were investigated. It is hoped that the findings of this study will be used as a proxies for possible impacts of ocean acidification on Pacific oysters.

2 Materials and Methods

2.1 Experimental set-up

This study was conducted at the Sam Nujoma Marine and Coastal Resources Research Centre (SANUMARC) of the University of Namibia located at Henties bay, Namibia. Prior to use, municipal tap water was stored in 10,000 L cylindrical fibreglass tanks to allow for de-chlorination before transferring into experimental 50 L glass aquaria

Twelve labelled experimental glass aquaria were set up in the laboratory with 25 L water with dechlorinated municipal tap water. For the hypercapnic treatments, CO_2 was vigorously bubbled from a gas proportionator/CO_2 gas cylinder to reach the desired pH level for each treatment and thereafter maintained through the shell exposure period of two weeks. The experimental set-up consisted of four aquaria with three replicates each. pH level for each treatment was maintained at 6.5, 7.0 and 7.5 designated as T1, T2 and T3 respectively with the pH 6.5 treatment (T1) representing extreme hypercapnia. One set of aquaria serving as the normocapnic treatment control and maintained at ambient pH (pH 8.2-8.4). The pH in the experimental tanks were constantly monitored and adjusted with gaseous CO_2 from the proportionator/CO_2 gas cylinder. All treatment aquaria were covered to avoid influx of atmospheric CO_2. The treatment and replicate aquaria were maintained at ambient temperature of 26.0 $\pm$ 1.0 OC. During the two week exposure period, the pH levels of the experimental tanks were within their desired levels, with fluctuations less than 0.12 for each treatment. The conductivity, salinity and temperature were consistent and within the desired range throughout the duration of the experiment (Table 1).

A total of 48 shells (12 for each Treatment) were used for the experiment, with three

Table 1: pH range and mean† values of conductivity, salinity and temperature recorded in the treatment and control aquaria during Pacific oyster shell exposure to varying ranges of hypercapnic pH levels.

Treatment†,‡	pH range	Conductivity (mS/cm)	Salinity (g/L)	Temperature (OC)
T1	6.50-6.60	53.0 (0.05)	34.8 (0.10)	19.31 (0.35)
T2	7.00-7.10	53.0 (0.05)	34.8 (0.03)	19.26 (0.35)
T3	7.50-7.66	53.0 (0.00)	35.2 (0.02)	19.25 (0.35)
Control	8.20-8.40	53.3 (0.28)	35.3 (0.06)	19.23 (0.37)

†$n = 6$

‡T1 to T3 representing extreme to mild hypercapnia in that order.
Values in parentheses are standard error of mean (SE).

shells per each pH treatment level. Fresh shells were obtained from a local oyster farm in Walvis Bay, Namibia. All meat was removed after shucking, and shells were placed into experimental conditions within 24 h of shucking.

2.2 Shell dissolution rate

Decrease on individual shell mass during and after the exposure period was used to estimate the shell dissolution rates at each pH level according to methods describe by Waldbusser et al. (2011). For the experimental mass loss over time, shells were weighted every 2 days by removing shells from treatment aquaria, blotting them dry, and allowing them to air dry for 15 minutes before measuring the mass. Final dry weights of shell were obtained by drying for 24h at 60°C, as described by Waldbusser et al. (2011). Within the two week exposure period in which the mass loss of shells was measured, the overall effects of shells and a positive effect of decreasing pH on the dissolution of shell were determined.

2.3 Determination of shell diameter and strength

The shell diameter was estimated by using Veiner® callipers in addition to photographs which were obtained by digital camera. A stereo-microscope (Zeiss) was used to examine the shell surfaces showing the differences in surface texture of the shells exposed at different pH treatment levels (Waldbusser et al., 2011). At the termination of the experiment, the strength of the shells in each treatment and control were determined by gentle application of force on the shell surface as described by Welladsen et al. (2010).

2.4 Determination of shell organic content

At the end of the experiment, the organic content of the shells were estimated as ash-free dry weight (AFDW). The shells were dried and crushed into fine powder, followed by heating at 500°C overnight (12 hours). Thus the AFDW was determined by subtracting ash weight from previously determined dry weight and the percentage of the organic content was obtained by dividing ash weight with dry weight then multiplied by a hundred using the formula below:

$$\text{Shell organic content } (\%) = \frac{(W_1 - W_2)}{W_1} \times 100$$

where:
W_1 = dried shell weight before ash content determination
W_2 = dried shell weight after ash content determination

2.5 Shell strength and surface texture

This was determined using methods described by Welladsen et al. (2010) by placing the shells in a crushing plate and gently pressing with a handheld pestle. Total strength was obtained by dividing the number of weak shells with the total number of shells used multiplied by a hundred, to obtain the percentage using the formula below:

$$\text{Shell strength } (\%) = \frac{S_w}{S_T} \times 100$$

where:
S_W = Number of weak shells
S_T = Total number of shells exposed.

A stereo microscope at ×100 magnification was used to examine the shell surfaces to identify changes in surface texture after exposure at the different pH treatments.

2.6 Data analysis

Results obtained for each treatment were expressed as mean±SE and all levels of significance were determined at 95%. To test for significant difference in the shell dissolution, diameter and calcium content obtained between all treatments and control, a two-way ANOVA was

used. Values of parameters indicating significant differences were partitioned using LSD. All analyses were aided with the use of SPSS® computer software.

Table 2: Mean† weight of Pacific oyster shells before and after exposure to varying ranges of hypercapnic pH levels for two weeks under laboratory conditions.

Treatment†,‡	Weight (g)		Weight loss
	Before	After	(%)
T1	15.0	14.6	2.74
	(0.06)	(0.28)	(0.51)
T2	16.7	16.5	1.21
	(0.18)	(0.20)	(0.80)
T3	14.1	13.9	1.44
	(0.10)	(0.15)	(0.29)
Control	16.1	16.1	0.00
	(0.25)	(0.13)	(0.10)

†$n = 3$
‡T1 to T3 representing extreme to mild hypercapnia in that order.
Values in parentheses are standard error of mean (SE).

3 Results

3.1 Shell dissolution rate

The mean weights of shell before and after the exposure are shown in Table 2, while percentage weight losses after exposure period are illustrated in Figure 1. Statistical analysis indicated significant loss ($p < 0.05$) in weight of shells exposure to T1 compared to T2 and T3. Shells exposed to the normocapnic condition (Control) did not show any significant loss in weight ($p > 0.05$) before and after the exposure period.

3.2 Diameter of the shells

The mean changes in the diameter of shells before and after the exposure are shown in Table 3, while the percentage change in diameter after exposure period are illustrated in Figure 2. Statistical analysis indicated significant reduction ($p < 0.05$) in diameter of shells exposure to T1 compared to T2 and T3. Shells exposed to the normocapnic condition (Control) did not show any significant change in diameter ($p > 0.05$) before and after the exposure period.

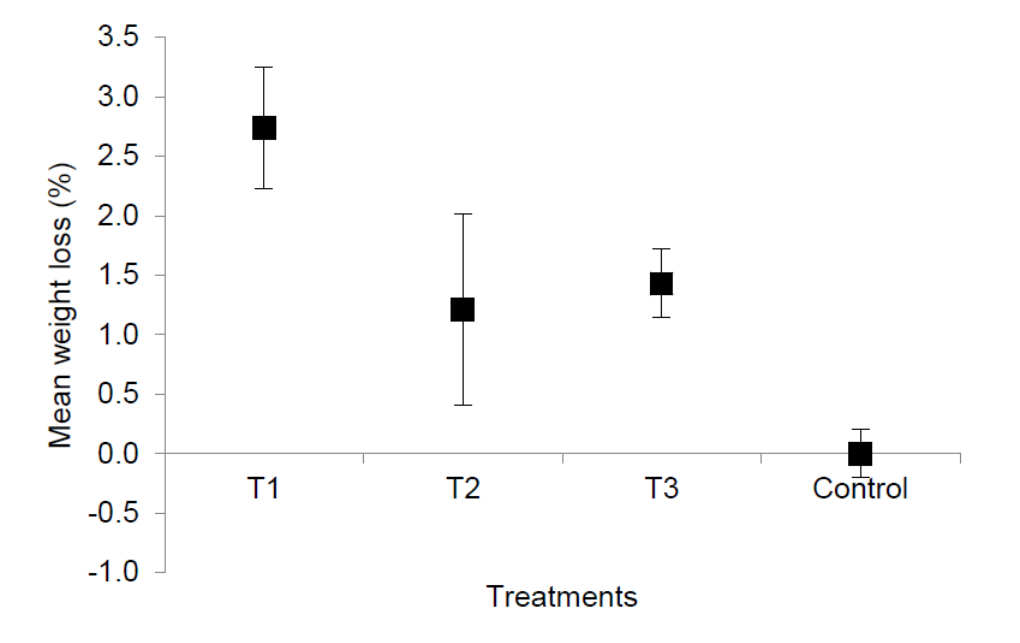

Figure 1: Changes in weight of Pacific oyster shells before and after exposure to varying ranges of hypercapnic pH levels for two weeks under laboratory conditions.

Table 3: Mean[†] diameter of Pacific oyster shells before and after exposure to varying ranges of hypercapnic pH levels for two weeks under laboratory conditions.

Treatment[†,‡]	Diameter (mm)		Difference
	Before	After	(%)
T1	49.26	48.66	1.23
	(0.12)	(0.18)	(0.36)
T2	48.49	48.13	0.75
	(0.05)	(0.22)	(0.10)
T3	49.62	49.55	0.14
	(0.11)	(0.08)	(0.01)
Control	49.04	49.09	-0.10
	(0.05)	(0.07)	(0.05)

[†]$n = 3$
[‡]T1 to T3 representing extreme to mild hypercapnia in that order.
Values in parentheses are standard error of mean (SE).

3.3 Total organic content of the shells

The mean changes in the shell organic content before and after the exposure is illustrated as Figure 3. There was a significant reduction ($p < 0.05$) in the organic content of shells

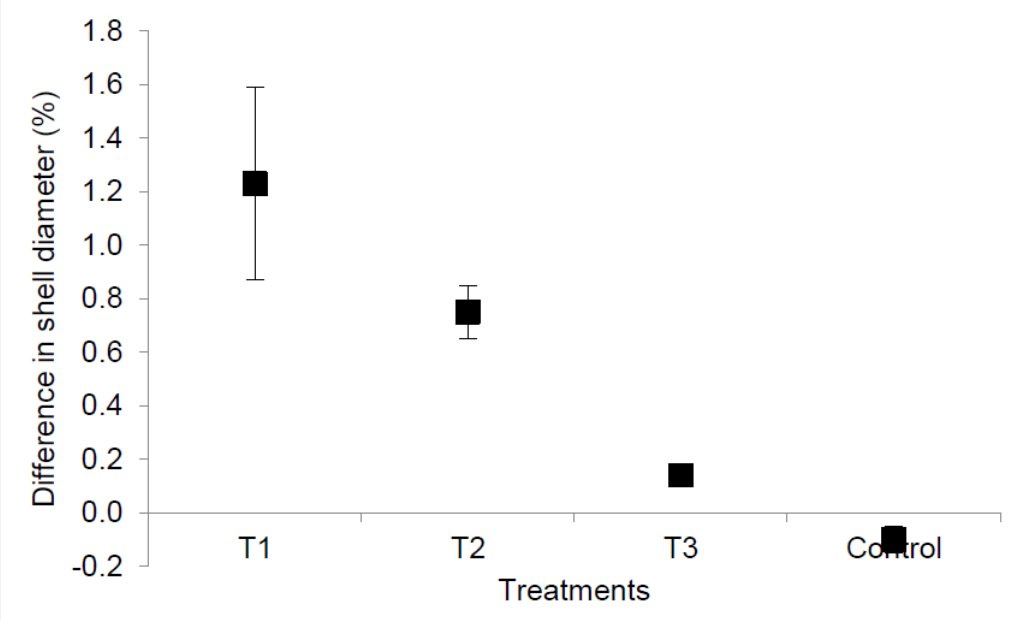

Figure 2: Changes in mean shell diameter of Pacific oyster shells before and after exposure to varying ranges of hypercapnic pH levels for two weeks under laboratory.

exposure to T1 and T2. Shells exposed to T3 and the normocapnic condition (Control) did not show any significant reduction in organic content ($p > 0.05$) after the exposure period.

3.4 Strength of the shell

This study found a significant decline in shell strength as treatment pH levels decreased. The maximum force that shells in T1 could withstand was significantly lower ($p < 0.05$) than that for shells held in other treatments and the control. In terms of percentages, shells held in T1 were 100% weaker than those in other treatments. Also, shells in T2 was 33.3% weaker, while T3 and control groups had full strength

3.5 Surface texture and microscopic analysis

The texture of the shells exposed in T1 and T2 appeared smoother when compared to the shells in control. Fading of the shells were observed in T1, T2 and T3 when compared to the control (Figure 4). Shells held in T1 were mostly discoloured and a chalklike appearance

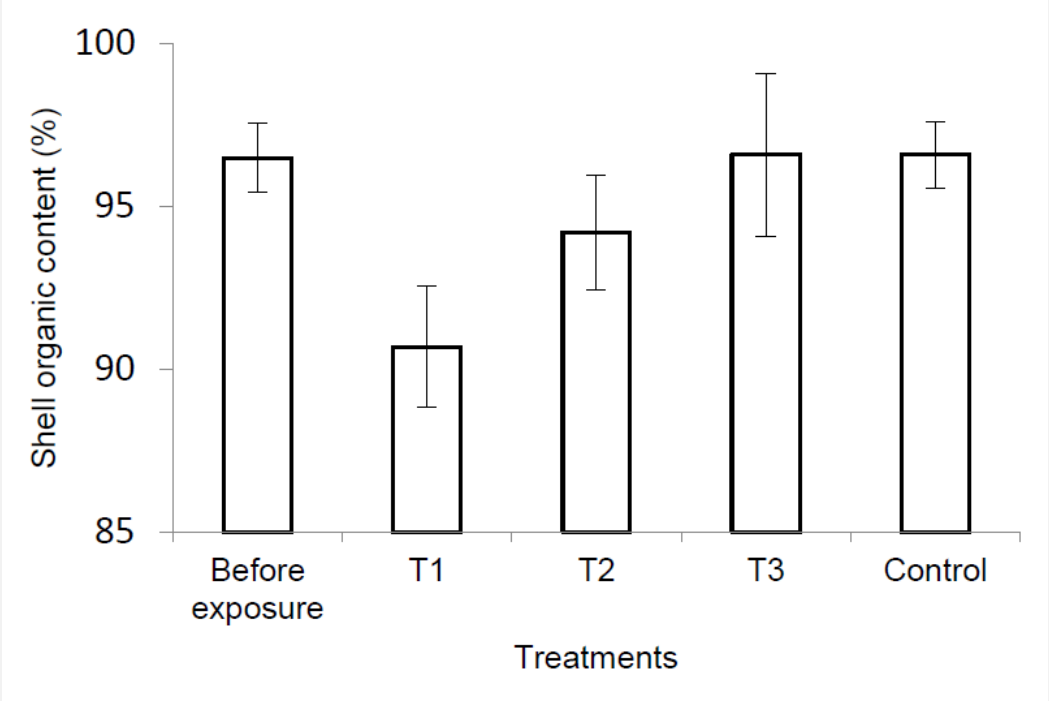

Figure 3: Changes in organic content of the Pacific oyster shells before and after exposure to varying ranges of hypercapnic pH levels for two weeks under laboratory conditions.

was observed on their surfaces. Under a microscopic examination (Figure 5), all the layers of the shells that were exposed to the hypercapnic conditions were affected. The shell edges of the nacreous layer were reduced while the organic layer of the shells was sheared in acidic conditions. Closer inspection of the nacre region of shells in the normocapnic conditions revealed straight edged tablets, while the nacre regions of shells held in the hypercapnic conditions were characterised by sparse, irregular shaped tablets within a reduced organic matrix (Figure 5).

4 Discussion

The study aimed at investigating the shell dissolution, strength, organic content and surface texture under varying acidic level. It was observed that the shells of pacific oyster held in hypercapnic conditions showed weaker strength and declined organic contents that those held at normocapnic unit after 14 days exposure period. Results in this study are consis-

Figure 4: Visual examination of Pacific oyster shells after exposure to varying ranges of hypercapnic and normocapnic pH levels for two weeks under laboratory conditions.(A) shell held in T1 (pH 6.5), (B) shell held in T2 (pH 7.0), (C) shell held in T3 (pH 7.5) and (D) shell in the control (pH 8.2).

tent with those of prior studies of calcifying organisms in the ocean, such as the Antarctic bivalves reported by McClintock et al. (2009) and on the Akoya pearl oyster by Welladsen et al. (2010) when exposed to elevated acidity levels. Our results indicate that the ocean acidification will have a direct impact on pacific oyster through shell dissolution and reduced calcification rates.

This study demonstrated that hypercapnic conditions resulted in significant mass weight and diameter loss in Pacific oyster shells ranging from 1.44 to 2.74% and 0.14 to 1.23% respectively in all media. Similar loss in shell weight was reported by Waldbusser et al. (2011) when they exposed shells of the Eastern oyster, *Crassostrea virginica* to hypercapnic conditions. In addition, Waldbusser et al. (2011) reported that fresh shells had more weight loss than dredged and weathered shells. However, in this study only fresh shells were used. The significant level of decrease in shell diameter observed when the shells were held in pH of 6.5 and 7.0 is in agreement with the observation of Talmage and Gobler (2009) when the Eastern oyster shells were exposed to hypercapnic conditions. At pH 7.5, the study by

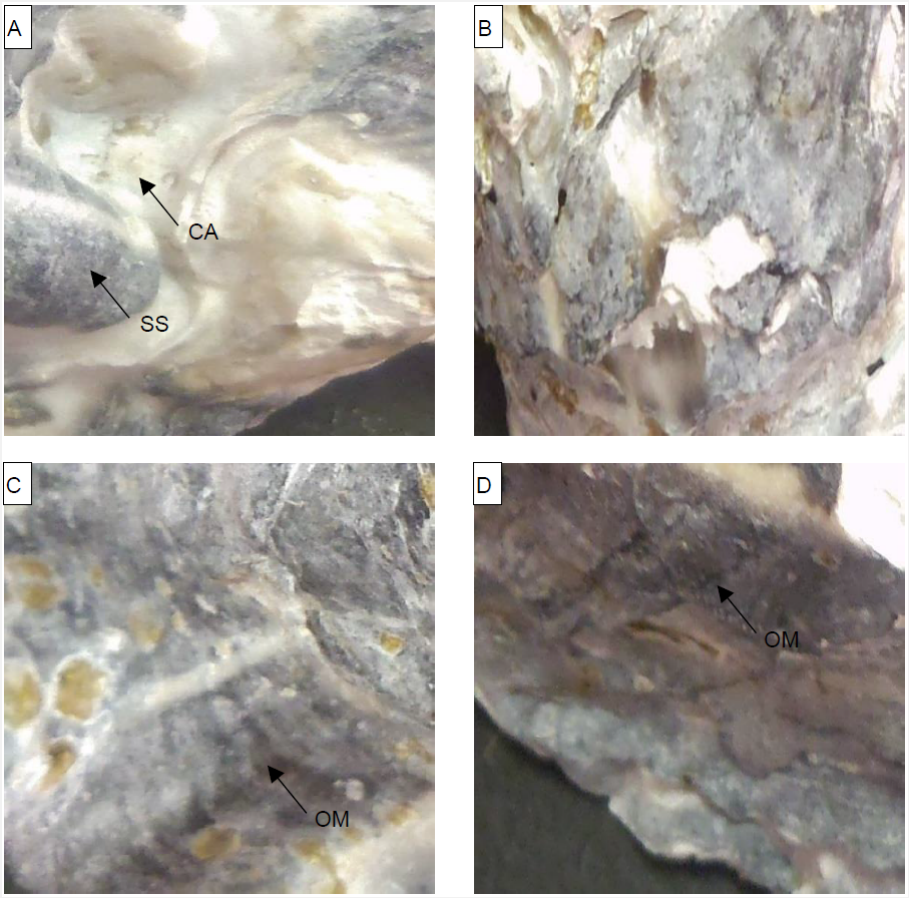

Figure 5: Stereo-microscopic images (×100) of surface texture and appearance of the Pacific oyster shells after exposure to varying ranges of hypercapnic and normocapnic pH levels for two weeks under laboratory conditions. (A) shell held in T1 (pH 6.5), (B) shell held in T2 (pH 7.0), (C) shell held in T3 (pH 7.5) and (D) shell in the control (pH 8.2). CA = chalklike appearance, SS = smooth surface, OM = organic matrix.

Talmage and Gobler (2009) indicated much decrease in shell diameter than in this present study. This difference could be attributed to the differences in the oyster species.

Loss in shell mass weight and diameter recorded in this investigation is an indication of dissolution of the shell as a result of the adverse effects of low pH levels. Similar dissolution as reported in this study was observed by Watson et al. (2009) when they held shells of the Sidney rock oyster, *Saccostrea glomerata* in same range of hypercapnic conditions. Michaelidis et al. (2005), Gazeau et al. (2007) and Ries et al. (2009) had earlier postulated that shell dissolution was attributed to reduced calcification rates when bivalve shells were

exposed to acidified seawater with similar pH levels as used in this study. This study is also in support of this postulation as evidence by the significantly reduced organic content after exposure to the hypercapnic pH levels used in this study. Since, the calcium and carbonate ions of the shells dissociate due to acidic conditions, the shells lose these most important components for calcification. Consequently, the organic content of the shells were reduced. Shells exposed to the highest acidic level used in this investigation recorded over 8% reduction in its organic content. Welladsen et al. (2010) observed similar reduction in shell organic content when the Pearl oysters were held in near-future pH levels under laboratory conditions.

Shell discolouration with chalklike appearances on their surface at hypercapnic pH levels were observed in this study. Microscopic examination of discoloured shells further revealed evidence of altered mechanical properties of the shells under hypercapnic conditions. The foliated layer of oyster are multi-layered nanomaterials, comprised of thin calcite laths bound together by the extracellular matrix molecules as reported by Checa et al. (2007), Lee et al. (2008). Lee et al. (2008) further reported that mechanical properties of the shells are determined by the morphology and organization of crystalline laths and by the shell matrix component. Lower hardness values of hypercapnic shells can also be due to the alterations in the organic matrix (Lee et al. 2008); however, currently we do not have the data to support this hypothesis.

This study has attested to the fact that acidification of the oceans has the potential to affect a wide variety of marine calcifying organisms such as the Pacific oyster. These organisms have received much attention in ocean acidification research, due to the potential for dissolution and reduced organic content of the calcified structures when exposed to reduced pH as earlier reported by Fabry et al. (2008) and Welladsen et al. (2010) respectively. In this study, the integrity of the Pacific oyster shells was significantly impaired after exposure to hypercapnic conditions.

The results from this study have broad implications for both the ecology and culture of this oyster species. For example, shells held for only 14 days at the least hypercapnia (pH 7.5) revealed reduced shell strength which, presumably, would render this valuable oyster species more susceptible to predation and environmental hazards thereby negatively affecting the oyster industry. The results of this study suggest that the physiological process of oyster shells may be affected by ocean acidification.

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