



# Ocean acidification reduces the crystallographic control in juvenile mussel shells



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## ABSTRACT

Global climate change threatens the oceans as anthropogenic carbon dioxide causes ocean acidification and reduced carbonate saturation. Future projections indicate under saturation of aragonite, and potentially calcite, in the oceans by 2100. Calcifying organisms are those most at risk from such ocean acidification, as carbonate is vital in the biomineralisation of their calcium carbonate protective shells. This study highlights the importance of multi-generational studies to investigate how marine organisms can potentially adapt to future projected global climate change. *Mytilus edulis* is an economically important marine calcifier vulnerable to decreasing carbonate saturation as their shells comprise two calcium carbonate polymorphs: aragonite and calcite. *M. edulis* specimens were cultured under current and projected  $p\text{CO}_2$  (380, 550, 750 and 1000  $\mu\text{atm}$ ), following 6 months of experimental culture, adults produced second generation juvenile mussels. Juvenile mussel shells were examined for structural and crystallographic orientation of aragonite and calcite. At 1000  $\mu\text{atm}$   $p\text{CO}_2$ , juvenile mussels spawned and grown under this high  $p\text{CO}_2$  do not produce aragonite which is more vulnerable to carbonate under-saturation than calcite. Calcite and aragonite were produced at 380, 550 and 750  $\mu\text{atm}$   $p\text{CO}_2$ . Electron back scatter diffraction analyses reveal less constraint in crystallographic orientation with increased  $p\text{CO}_2$ . Shell formation is maintained, although the nacre crystals appear corroded and crystals are not so closely layered together. The differences in ultrastructure and crystallography in shells formed by juveniles spawned from adults in high  $p\text{CO}_2$  conditions may prove instrumental in their ability to survive ocean acidification.

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## 1. Introduction

Global climate change is having a dramatic impact on the oceans which absorb atmospheric CO<sub>2</sub> increasing the partial pressure ( $p\text{CO}_2$ ) and causing ocean acidification. Projections suggest that an increase from 384  $\mu\text{atm}$  to 1000  $\mu\text{atm}$  will result in a reduction of 0.4 pH by the year 2100 (IPCC, 2007; Doney et al., 2009). This ocean acidification poses a threat to those marine calcifying organisms that produce calcium carbonate exoskeletons and shells; due to projected future chemistry changes leading to reduced ocean carbonate saturation state ( $\Omega$ ) (Doney et al., 2009). It is suggested that early life history stages of organisms are most vulnerable to environmental changes (Doney et al., 2009; Byrne, 2012). Impacts of increased  $p\text{CO}_2$  on juvenile molluscs include increased mortality, decreased shell deposition and reduced microhardness of oyster *Crassostrea virginica* shells (Beniash et al., 2010; Talmage and Gobler, 2010). Generally,

mollusc larvae seem to have reduced survival and shell size when exposed to increased  $p\text{CO}_2$  (Byrne, 2012). However, for the newly settled post larvae of the genus *Mytilus edulis*, there was a high tolerance for increased  $p\text{CO}_2$  with no impact on survival, but markedly reduced growth and calcification (Thomsen et al., 2013).

Multi-generational studies are a powerful way of assessing the ability of marine organisms to adapt. However such studies remain difficult to address within laboratory conditions. Most studies examine juveniles or adults which have been grown from adults under ambient conditions and transferred to experimental exposure to  $p\text{CO}_2$  (Thomsen et al., 2013). Very few multi-generational studies have attempted to study the possibility of adaptation by transferring resistance to  $p\text{CO}_2$  from the parent to the gametes (Fitzer et al., 2012; Kurihara and Ishimatsu, 2008), and multi-generational studies have not been undertaken in larger marine invertebrates such as *M. edulis* before. Further long-term and multi-generational study is vital to predict the possibility of acclimation and potential adaptation by marine organisms to future ocean acidification.

The common blue mussel *M. edulis* is an ideal calcifying marine organism for the study of increasing  $p\text{CO}_2$ , with shell calcium

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carbonate production comprising both calcite (prismatic layer) and aragonite (nacreous layer, or mother of pearl). As increasing  $p\text{CO}_2$  reduces carbonate saturation states within the oceans, aragonite, the less stable polymorph of calcium carbonate, is likely to become more vulnerable to dissolution in comparison to calcite, the more stable polymorph (Doney et al., 2009). In addition, *M. edulis* is an important economical species cultured for food, globally comprising 23.6% (14.2 million tonnes) of an annual \$119.4 billion industry (FAO, 2012). Calcifying organisms in general provide a vital carbon sink; as such a shift in carbon usage as a result of increasing  $p\text{CO}_2$  could feed back into the climate, reducing the capacity of oceans to absorb atmospheric  $\text{CO}_2$ .

Physical characteristics such as shell dissolution and reduced growth are one way to examine ocean acidification impacts on marine calcifying organisms (Beniash et al., 2010; Byrne, 2012; Thomsen et al., 2013). Moreover, increased biological stress through increased environmental  $p\text{CO}_2$  could also affect shell formation, as well as shell dissolution from external seawater under saturated with respect to carbonate.

Here we report the impacts of ocean acidification on second generation juvenile mussels grown for 6 months in experimental culture from spawning of exposed adults. In this study the physical impact of ocean acidification was assessed by examining shell ultrastructure and crystallography in second generation juvenile *M. edulis* grown under current and future projected ocean acidification scenarios (380, 550, 750, 1000  $\mu\text{atm } p\text{CO}_2$ ).

## 2. Methods

### 2.1. Mussel collection and culture

Mussels (*M. edulis*) were obtained from the Loch Fyne, Argyll, Scotland (Loch Fyne Oysters Ltd.) during October 2012. Mussels were placed into experimental tanks (six L) supplied with natural filtered (1  $\mu\text{m}$  and UV) seawater at Loch Fyne temperatures (7 °C) and ambient  $p\text{CO}_2$  (~380  $\mu\text{atm}$ ). Mussels were fed 10 ml of cultured microalgae (five species of zooplankton, *Nannochloropsis* sp., *Tetraselmis* sp., *Isochrysis* sp., *Pavlova* sp., *Thalassiosira weissflogii* (stock from Reefphyto, UK)) per tank every other day. Feeding was conducted during a two week acclimation and throughout the experimental period. Over a six month experimental culture period adults mussels (~1 year old) spawned and juveniles (0.5–1 cm) settled onto the six L plastic tank surfaces.

### 2.2. Environmental conditions

Seasonal experimental temperatures and day length (light) mirrored those at the collection site. Experiments were conducted at 380, 550, 750 and 1000  $\mu\text{atm } p\text{CO}_2$ . Seawater  $p\text{CO}_2$  concentrations were increased up to experimental levels (380, 550, 750 and 1000  $\mu\text{atm } p\text{CO}_2$ ) over a one month period.  $\text{CO}_2$  was mixed into

air lines supplying all experimental tanks (Findlay et al., 2008). Gas concentrations were logged continuously using LI-COR®Li-820  $\text{CO}_2$  gas analysers (Table 1). Seawater was topped up with a mixture of seawater and freshwater once a week to simulate fresh water pulses experienced by mussels in their natural environment. This is reflected in calcite ( $\Omega$  Ca) and aragonite ( $\Omega$  Ar) saturation states which are similar to other ocean acidification studies examining brackish water environments (Thomsen and Melzner, 2010) and the natural variability present at the collection site (Table 1 Loch Fyne samples). Loch Fyne sample site natural variability for sampling site measurements have been reported in Fitzner et al. (2014). Seawater salinity, temperature, and dissolved oxygen (DO) were checked daily and recorded once a week (YSI Pro2030). Seawater samples were collected (once per month) and spiked with 50  $\mu\text{l}$  of mercuric chloride for subsequent total alkalinity ( $A_T$ ) analysis via semi-automated titration (Metrohm 848 Titri-no plus) (Dickson et al., 2007) combined with spectrometric analysis using bromocresol indicator (Yao and Byrne, 1998) (Smart pH cuvettes, Ocean Optic Ltd.) (Hach DR 5000™ UV-Vis). Certified seawater reference materials for oceanic  $\text{CO}_2$  (Batch 123, Scripps Institution of Oceanography, University of California, San Diego) were used as standards to quantify the error of analysis (Dickson et al., 2007). Seawater  $A_T$ , salinity, temperature and  $p\text{CO}_2$  were used to calculate other seawater parameters using  $\text{CO}_2\text{SYS}$  (Riebesell et al., 2007).

### 2.3. Shell preparation and analysis by Electron Backscatter Diffraction

Electron Back Scatter Diffraction (EBSD) was used to identify calcium carbonate polymorph and determine crystallographic orientation across the middle section of the length of mussel shell at the calcite/aragonite interface. Juvenile mussel shells were carefully prepared as to not damage internal shell layers. Shells were dissected and rinsed to remove tissues with milli-Q water before drying within an oven for 48 h at 60 °C before being embedded into epoxy resin. Resin blocks were polished by hand for 2–4 min using grit papers (P320, P800, P1200, P2500, and P4000), followed by further polishing for 4 min on cloths using 1  $\mu\text{m}$  and 0.3  $\mu\text{m}$  Alpha alumina, and 2 min using colloidal silica to ensure a smooth shell surface. A beam voltage of 20.0 kV was applied under low vacuum mode (~50 Pa) on an FEI Quanta 200F with the stage tilted to 70° to examine backscatter Kikuchi patterns (Perèz-Huerta and Cusack, 2009). Diffraction intensity, phase and crystallographic orientation maps were produced through OIM Analysis 6.2 software. Data points with a confidence index of less than 0.1 were removed. EBSD results are presented as diffraction intensity, phase and crystallographic orientation maps and pole figures with each colour representing a particular crystallographic orientation. Pole figures were examined for spread of crystallographic orientation using 5 degrees angle grid-lines (Supplementary Fig. 1).

**Table 1**

Experimental seawater chemistry parameters; salinity, dissolved oxygen (DO),  $p\text{CO}_2$ , total alkalinity ( $A_T$  ± standard deviation from the mean,  $n = 8$ ). Loch Fyne natural seawater was collected from three sites in duplicate for the chemistry parameters reported. Salinity, DO and temperature are averages collected manually throughout experiments, and  $p\text{CO}_2$  is the averaged values logged throughout the six months of experiments (logging every 5 min) using LI-COR®software. Bicarbonate ( $\text{HCO}_3^-$ ) and carbonate ( $\text{CO}_3^{2-}$ ), calcite saturation state ( $\Omega$  Ca), and aragonite saturation state ( $\Omega$  Ar) (reported on a log scale) were calculated from measured parameters using  $\text{CO}_2\text{SYS}$ .

Experimental condition	Salinity (ppt)	DO (%)	Temperature (°C)	$p\text{CO}_2$ ( $\mu\text{atm}$ )	$A_T$ ( $\mu\text{mol kg}^{-1}$ )	$\text{HCO}_3^-$ ( $\mu\text{mol kg}^{-1}$ )	$\text{CO}_3^{2-}$ ( $\mu\text{mol kg}^{-1}$ )	$\Omega$ Ca	$\Omega$ Ar
380 $\mu\text{atm}$ Ambient	32.78 ± 1.42	95.58 ± 1.84	9.40 ± 0.36	375.62 ± 9.69	635.24 ± 28.93	590.3	11.9	0.29	0.18
550 $\mu\text{atm}$ Ambient	32.74 ± 1.56	99.04 ± 2.19	10.01 ± 0.56	553.59 ± 62.65	970.76 ± 186.48	908.9	19.7	0.47	0.30
750 $\mu\text{atm}$ Ambient	28.42 ± 4.07	98.64 ± 4.78	10.28 ± 0.34	768.74 ± 41.63	753.64 ± 55.16	725.7	8.4	0.21	0.13
1000 $\mu\text{atm}$ Ambient	34.18 ± 4.58	98.66 ± 1.97	10.23 ± 0.40	1132.53 ± 31.74	698.32 ± 5.77	678.2	5.6	0.13	0.08
Loch Fyne (Average)	19.33 ± 7.46	99.36 ± 12.99	15.70 ± 4.15	341.17 ± 102.57	1261.95 ± 416.39	1170.56 ± 430.42	34.37 ± 18.99	0.88 ± 0.47	0.52 ± 0.29
Loch Fyne (Lowest total alkalinity)	12.80	17.80	11.6	7.95	876.10 ± 12.62	798.39 ± 11.75	29.19 ± 0.44	0.68 ± 0.01	0.39 ± 0.01

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