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1 **ESTABLISHED AND EMERGING TECHNIQUES FOR CHARACTERISING THE FORMATION, STRUCTURE**  
2 **AND PERFORMANCE OF CALCIFIED STRUCTURES UNDER OCEAN ACIDIFICATION**

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41 Ocean acidification (OA) is the decline in seawater pH and saturation levels of calcium carbonate  
42 minerals that has led to concerns for calcifying organisms such as corals, oysters and mussels because  
43 of the adverse effects of OA on their biomineralisation, shells and skeletons. A range of cellular biology,  
44 geochemistry and materials science approaches have been used to explore biomineralisation. These  
45 techniques have revealed that responses to seawater acidification can be highly variable among species,  
46 yet the underlying mechanisms remain largely unresolved. To assess the large-scale impacts of global  
47 OA, researchers will need to apply a range of tools developed across disciplines, many of which are

48 emerging and have not yet been used in this context. This review outlines techniques that could be  
49 applied to study OA-induced alterations in the mechanisms of biomineralisation and their ultimate  
50 effects on shells and skeletons. We illustrate how to characterise, quantify and monitor the process of  
51 biomineralisation in the context of global climate change and OA. We highlight the basic principles as  
52 well as the advantages and disadvantages of established, emerging, and future techniques for OA  
53 researchers. A combination of these techniques will enable a holistic approach and better understanding  
54 of the potential impact of OA on biomineralisation and consequences for marine calcifiers and  
55 associated ecosystems.

## 56 **Introduction**

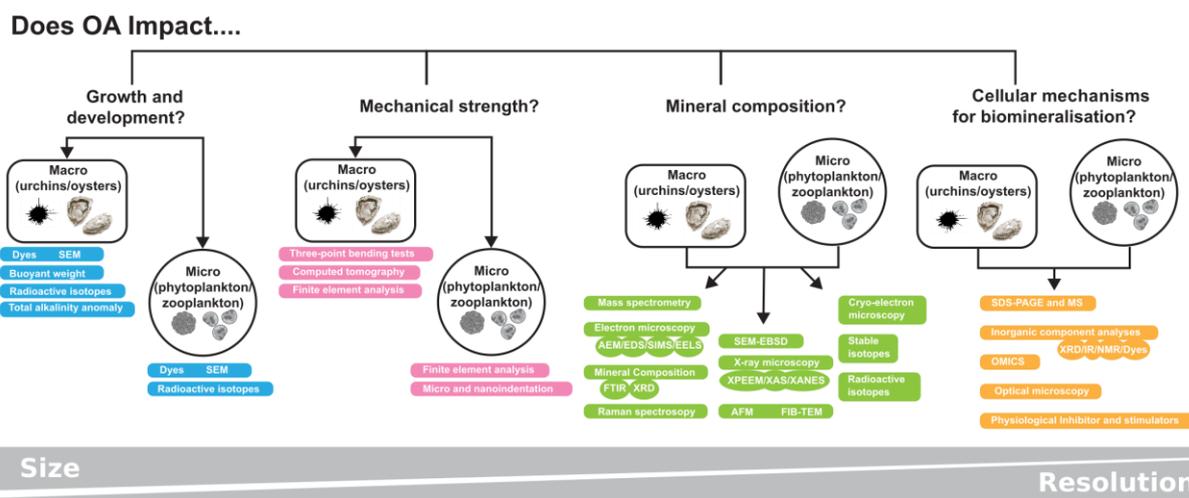
57 The precipitation of minerals such as calcium carbonate ( $\text{CaCO}_3$ ) for shells and skeletons using  
58 dissolved carbonate and calcium ions is commonly called ‘biomineralisation’ (Doney et al. 2009).  
59 Marine organisms have been producing calcium carbonate biominerals since the Precambrian and the  
60 resultant rich fossil record provides insight into the evolution of intricate, orderly and often beautiful  
61 structures (Wilkinson 1979). The composition of biominerals varies among taxa and, as the production  
62 of shell materials is dependent on the availability of mineral ion resources and on physiological  
63 conditions at the site of calcification (Wilbur 1964), mineral composition can reveal how organisms  
64 have interacted with environmental conditions over geological time to the present day.

65 Atmospheric  $\text{CO}_2$  levels have increased at a faster rate during the Anthropocene than in any previous  
66 time in Earth’s history causing a rapid decline in seawater pH and lowering the amount of calcium  
67 carbonate minerals (Orr et al. 2005).

68 Since concerns about OA were first highlighted it has become apparent that  $\text{CO}_2$ -driven acidification  
69 can lead to skeletal abnormalities and slower growth in many marine calcifiers (Hofmann et al. 2008,  
70 Vézina and Hoegh-Guldberg 2008, Wittmann and Pörtner 2013). Aragonite, calcite, vaterite  
71 (Wehrmeister et al. 2011), and amorphous calcium carbonate which is an important precursor of  
72 crystalline carbonate minerals (Addadi et al. 2003), are phases of  $\text{CaCO}_3$  whose production may be  
73 modified by OA. Organisms can be affected by OA as they need to maintain conditions that are

74 chemically suitable for the process of calcification (supersaturated with calcium [ $\text{Ca}^{2+}$ ] and carbonate  
75 [ $\text{CO}_3^{2-}$ ]) or for preventing dissolution (saturation state  $\Omega > 1$ ). Calcite is less susceptible to dissolution  
76 at lower pH values than aragonite, unless it contains high levels of magnesium (Ries et al. 2009, Chan  
77 et al. 2012). Production of any form of  $\text{CaCO}_3$  can be energetically expensive (Comeau et al. 2017a)  
78 and so the impact of OA on the production and maintenance of  $\text{CaCO}_3$  structures are modulated by  
79 energy acquisition (Melzner et al. 2011) and may be due to by  $\text{CO}_2$ -driven organism hypercapnia (Byrne  
80 et al. 2013).

81 To capture fully the impact of OA on biomineralisation, several key questions should be addressed.  
82 These include questions regarding the direct impact of OA on the process of biomineralisation itself  
83 and also about the functional consequences of these changes on shells and skeletons. Resolving these  
84 issues requires multidisciplinary research ranging from ‘-omics’ to cell culture, from physiological  
85 mechanisms to ecology, and from materials science to crystallography. The complexity of the task is  
86 reflected in the plethora of techniques that have been used to investigate biomineralization under OA  
87 conditions, including buoyant weight, total alkalinity anomaly, total calcium content, annual extension,  
88 calcein labelling and the use of radio isotopes (Table 1). This diversity of approaches allows  
89 investigators to tackle different questions related to the impact of OA on the process of  
90 biomineralisation, although there is a need to understand how different techniques compare when  
91 measuring similar processes (Schoepf et al. 2017). The selection and refinement of a technique is  
92 dependent upon scientific question and practical aspects related to the study question, experimental  
93 design and biological models (Figure 1).



94

95 **Figure 1. A schematic representation of analytical methods studying whole animal growth and**  
 96 **development (in blue), mechanical strength (in pink), mineral composition (in green), and**  
 97 **mechanisms of biomineralisation (in orange) that enables the answering of various level of**  
 98 **questions. These techniques can be strategically applied to macrobiota (e.g. urchin or oysters) or**  
 99 **microbiota (e.g. phytoplankton or zooplankton). From left to right, the figure shows the techniques**  
 100 **applicable to smaller scale samples with increasing resolution. At a lower resolution, macrobiota**  
 101 **can be measured in terms of various shell growth and development parameters (in blue) and**  
 102 **mechanical properties, while some approach has more limitations with microbiota. At a high**  
 103 **resolution, microbiota and macrobiota can be studied for their shell structure (in green) and**  
 104 **cellular mechanisms for biomineralisation (in orange).**

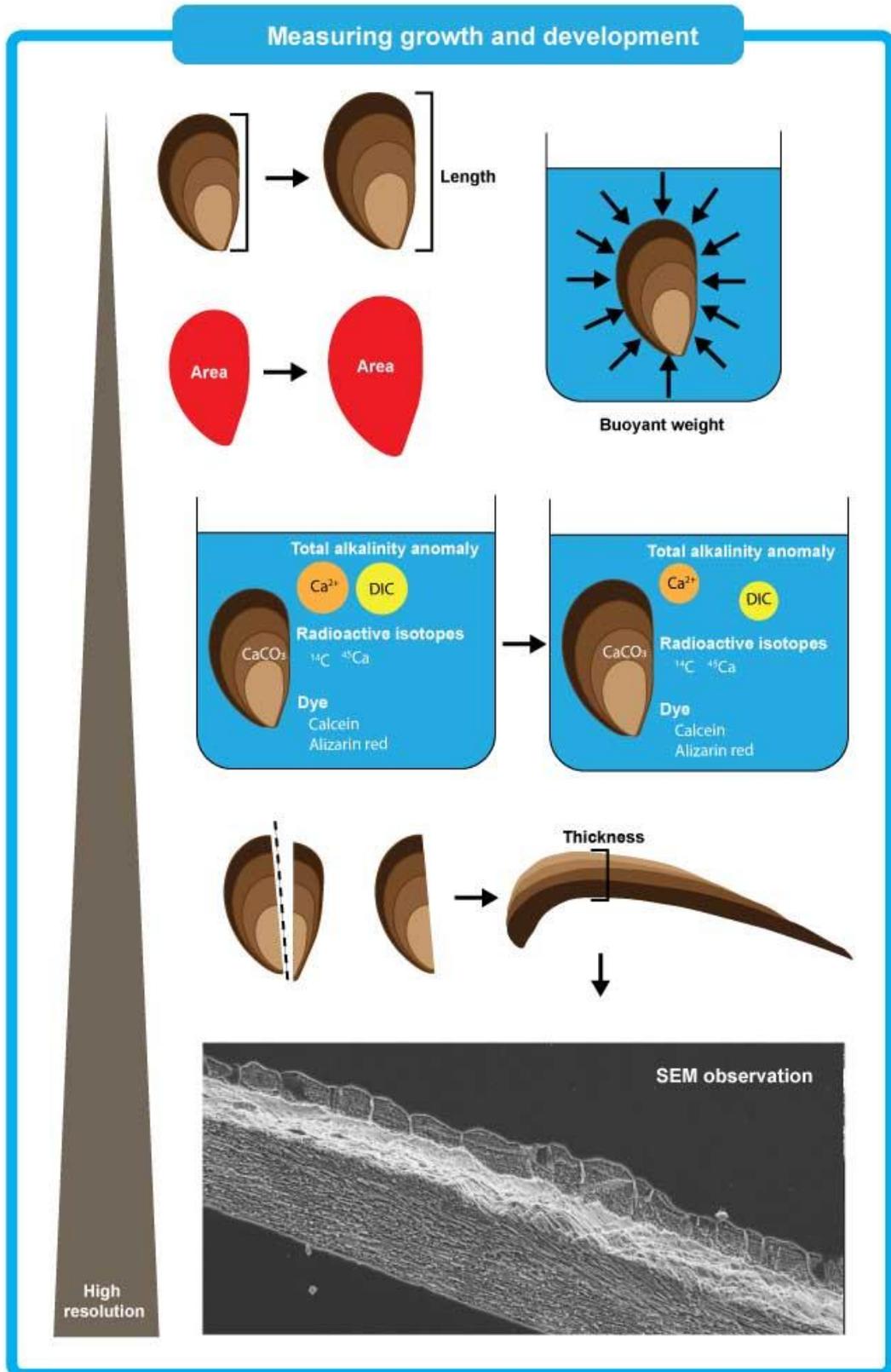
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106 Here we review an array of techniques used to explore the consequences of rising global CO<sub>2</sub> levels on  
 107 biomineralisation in marine organisms. We organize the techniques by categorizing the biological or  
 108 mineralogical parameters of interest. We evaluate their advantages and disadvantages so that future  
 109 work can more effectively measure the effects of OA on biomineralisation . We also highlight recent  
 110 advances in the study of the effects of ocean acidification on biomineralisation and how  
 111 interdisciplinary collaboration can advance the field.

112

*Growth and development*

113 When evaluating the impact of OA on biomineralisation, it is important to discriminate between  
114 methods measuring gross and net calcareous shell growth as the product of biomineralisation (Figure  
115 2). The term ‘gross calcification’ refers to the biologically controlled process of  $\text{CaCO}_3$  production  
116 through the formation of  $\text{CaCO}_3$  minerals from a super saturated solution ( $\text{CaCO}_3$  precipitation). In  
117 contrast, the term ‘net calcification’ is the net effect of gross calcification and dissolution (Cyronak et  
118 al. 2016).  $\text{CaCO}_3$  dissolution or decalcification is the dissolution of  $\text{CaCO}_3$  minerals in an under  
119 saturated solution. These processes combine to influence net calcification; for example, the upregulation  
120 in gross calcification rates of the limpet *Patella caerulea* helps to counteract higher shell dissolution  
121 rates (Rodolfo-Metalpa et al. 2011).



123 **Figure 2. Schematic representation of techniques for measuring growth and development in**  
 124 **biomineralising organisms. The tapering bar on the left indicates the changing level of resolution for**  
 125 **each technique. From top to bottom, growth and development can be measured on whole shells**  
 126 **and skeletons using techniques such as length and buoyant weight measurements. More sensitive**  
 127 **techniques are represented in the middle for the use of dyes, radioactive isotopes and total**  
 128 **alkalinity anomaly technique. At the bottom of the figure, sectional surface of a shell at higher**  
 129 **resolution can be visualized and thickness can be measured using techniques such as SEM**  
 130 **microscopy.**

131

### 132 *Dyes*

133 A range of chemical dyes (e.g. alizarin red, calcein) are used to mark shells or exoskeletons to assess  
 134 growth over time and have been used in OA studies to determine the impact on calcification in corals,  
 135 coralline algae and bivalves (Rodolfo-Metalpa et al. 2011, Dickinson et al. 2012a, Tambutté et al. 2012,  
 136 Bradassi et al. 2013, Venn et al. 2013, Fitzer et al. 2014b, Fitzer et al. 2015b). Calcein labelling is often  
 137 preferable because calcein was found better incorporated into foraminiferan calcite and emitted  
 138 fluorescence more strongly than the other markers such as Alizarin complexone, oxytetracycline, and  
 139 xylenol orange (Bernhard et al. 2004). Calcein has been applied in OA research to assess coralline algal,  
 140 coral and mollusc growth during experiments (Dickinson et al. 2012b, Bradassi et al. 2013, Venn et al.  
 141 2013, Fitzer et al. 2014b, Fitzer et al. 2015b). The techniques are low cost, less invasive as compared  
 142 to sacrificial shell sampling and the results are readily comparable.

### 143 *SEM*

144 Abnormalities and morphology can be assessed by optical microscopy. However, scanning electron  
 145 microscopy (SEM) is required for high resolution characterisation of biomaterial microstructures and  
 146 has been used to show modified skeletal phenotypes in a range of species grown under OA conditions  
 147 (Riebesell et al. 2000, Orr et al. 2005, Iglesias-Rodriguez et al. 2008, Lombardi et al. 2015). The

148 advantage of this approach include the ability to assess shape and malformation in net growth, while  
149 the disadvantages include higher costs and an extended preparation time.

150 When evaluating net shell growth as the overall product of biomineralisation, one of the simplest and  
151 most widely used methods to approximate net calcification is shell and exoskeleton length, as it is both  
152 non-invasive low-cost. However, this approach can fail to reveal impacts on gross and net calcification  
153 not expressed in the overall structure of the skeleton. For example, OA can cause skeletal malformations  
154 which can only be identified by microscopy (Langdon et al. 2000, Reynaud et al. 2003, Langdon and  
155 Atkinson 2005, Gazeau et al. 2007, Cooper et al. 2008, Jokiel et al. 2008, Ries et al. 2009). This can be  
156 partly addressed by including morphometric parameters to resolve shapes, and thickness, for example  
157 using 3-dimensional measurements generated from computed tomography (Rühl et al. 2017). It is  
158 therefore important to consider the net growth of the whole shell or skeleton.

159 Skeletal growth assessed as annual extension rate ( $\text{cm}^2 \text{yr}^{-1}$ ) is commonly used to determine growth  
160 rates of calcareous red algae and corals (Marsh 1970), with recent research applying photogrammetric  
161 methods based on digital photography and advanced image processing techniques for non-destructive  
162 measurements of area and volume (Mackenzie et al. 2014, Norzagaray-López et al. 2017). Densitometry  
163 using X-rays (Table 2) assesses the density of calcified structures (Carricart-Ganivet and Barnes 2007)  
164 and has been used to identify growth bands and to calculate growth rates of individuals or colonies  
165 (Cooper et al. 2008).

#### 166 *Buoyant weight*

167 To monitor changes in mineral content, buoyant weight determined by immersion is frequently used  
168 (Davies 1989, Herler and Dirnwöber 2011). Correction for seawater salinity and temperature variation  
169 between measurements is necessary (Fang et al. 2013). The buoyant weight technique is non-invasive  
170 (Molina et al. 2005) and remains one of the most common techniques to determine net calcification rate  
171 in OA studies, especially in corals (Herler and Dirnwöber 2011). Such an approach has shown that an  
172 array of temperate corals *Oculina arbuscula*, pencil urchins *Eucidaris tribuloides*, hard clams  
173 *Mercenaria mercenaria*, conchs *Strombus alatus*, serpulid worms *Hydroides crucigera*, periwinkles

174 *Littorina littorea*, bay scallops *Argopecten irradians*, oysters *Crassostrea virginica*, whelks *Urosalpinx*  
 175 *cinerea*, and soft clams *Mya arenaria* show mixed responses to CO<sub>2</sub>-induced acidification, thereby  
 176 highlighting the complexity of biomineralisation responses (Ries et al. 2009).

177 *Radioactive isotopes*

178 Naturally occurring radioactive isotopes can be used to measure growth by spiking organisms with a  
 179 radiotracer (Sabatier et al. 2012). Liquid scintillation counting is used to amplify the signal and  
 180 quantify the amount or rate of <sup>45</sup>Ca being incorporated into the biomineral structure (Rodolfo-Metalpa  
 181 et al. 2011, Rodolfo-Metalpa et al. 2015). <sup>45</sup>C is a non-natural radioactive isotope, therefore any changes  
 182 in <sup>45</sup>Ca quantity represents shell material accretion or loss by the calcification process that occurs during  
 183 the experiment and prior calcification is not taken in account (Furla et al. 2000). Similarly, synthetic  
 184 radioactive <sup>14</sup>C isotopes enable the measurement of carbon flux related to photosynthesis and  
 185 calcification (Guo et al. 2009, Li et al. 2015). The <sup>45</sup>Ca technique has been used in OA research to  
 186 determine the impact of increasing pCO<sub>2</sub> levels on cold-water corals, suggesting that calcification is not  
 187 disrupted under OA (Rodolfo-Metalpa et al. 2015). Gross calcification rates have been quantified using  
 188 <sup>45</sup>Ca in corals, limpets, mussels, foraminifera, coccolithophores and oyster larvae (McEnery and Lee  
 189 1970, Erez 1978, Satoh et al. 2009, Rodolfo-Metalpa et al. 2011, Rodolfo-Metalpa et al. 2015, Frieder  
 190 et al. 2016). In contrast, <sup>14</sup>C has mainly been applied in unicellular organisms such as coccolithophores  
 191 (Paasche 1963, Nimer and Merrett 1993, Gao et al. 2009), foraminifera (ter Kuile et al. 1989), and  
 192 diatoms (Li et al. 2015). Advantages include the improved spatial resolution, taking into account  
 193 material accretion during the incubation period. However, a major disadvantage of this technique is the  
 194 destructive nature of sampling unlike other techniques such as buoyant weight to determine calcification  
 195 rates.

196 *Total alkalinity anomaly technique*

197 Net calcification rate can be measured by determining the amount of CaCO<sub>3</sub> taken up by an organism  
 198 (Gazeau et al. 2007). When an organism precipitates a mole of CaCO<sub>3</sub> it takes up two moles of HCO<sub>3</sub><sup>-</sup>,  
 199 thereby reducing the alkalinity of the surrounding seawater over the incubation period (Langdon et al.

200 2000, Langdon and Atkinson 2005, Gazeau et al. 2007). The total alkalinity anomaly technique has  
201 been used as an alternative to the buoyant weight method to determine net calcification rates in a range  
202 of calcifying organisms including corals, mussels and oysters (Langdon et al. 2000, Langdon and  
203 Atkinson 2005, Gazeau et al. 2007). A recent study recommends that the technique is more suitable for  
204 shorter term (e.g. day/night) incubations whereas the buoyant weight method is suitable for longer term  
205 studies when resources are limited (Schoepf et al. 2017). Less frequently, calcium content has been  
206 determined directly using mass spectrometry as a proxy for calcification (Wood et al. 2008). Both the  
207 total alkalinity anomaly and the buoyant weight techniques are low cost and take into account skeleton  
208 malformations, however there is variability between incubation methods. Promisingly, there is  
209 agreement in the results obtained from the different methods, with the major trend of a reduction in net  
210 biomineralisation under OA shown by both techniques (Langdon et al. 2000, Langdon and Atkinson  
211 2005, Gazeau et al. 2007).

#### 212 **Mechanical tests - Protective function or ability to survive**

213 OA impacts the gross and net calcification in many marine calcifiers, and therefore it would be expected  
214 that OA would similarly impact the function of the shell or skeleton. Mechanical properties of  
215 shells/skeletons can be quantified by two parameters: a) hardness (resistance to irreversible  
216 deformation) and b) compressive strength (force needed to induce cracking). These parameters can be  
217 used to evaluate the functional impacts of changes in biomineralisation under OA. For example, changes  
218 in these parameters have implications on the vulnerability of reef-forming species and associated  
219 ecosystems, as well as consequences for predator-prey interactions (Fu et al. 2016).

#### 220 *Three-point bending tests*

221 As a classical, simplistic and low cost approach to examine mechanical features of brittle biomineral  
222 structures, three-point bending tests measure the flexural strength and modulus and commonly used to  
223 define material properties in its ability to resist bending. Three-point bending tests have been applied to  
224 measure the stiffness of the ambital plates in sea urchins grown in OA and found that there can be no  
225 significant impact on the protective function of the exoskeleton (Collard et al. 2016). Purpose-made

226 devices consisting of two supportive beams with appropriate span length and a loading beam can be  
227 built according to the specific morphology of the biomineral sample (Guidetti and Mori 2005, Asnaghi  
228 et al. 2013). These tests provide relevant information to the protective function of the shell or  
229 exoskeleton. The flexural response to a three-point bending test device mimics the deformation response  
230 to predatory attack by fish (Guidetti and Mori 2005). This exoskeleton robustness test was applied in  
231 the OA study of a sea urchin, and revealed increased  $p\text{CO}_2$  reduced the defense of a sea urchin to the  
232 predator (Asnaghi et al. 2013). If samples are to be directly compared, it is essential to first standardize  
233 the thickness and sectional area of the test material, which requires additional preparation time to ensure  
234 the biomineral samples are cut into a standard size. The three-point bending tests have the advantage of  
235 being able to measure the whole structure mechanical response; disadvantages are that it can be time  
236 consuming as purpose-made devices may be required for unusually shaped shell structures.

237

238

#### *Computed tomography*

239 Computed tomography (CT) and micro-computed tomography (Micro-CT) are powerful, non-  
240 destructive techniques to evaluate biomineralised structures. Micro-CT allows 3D visualisation of X-  
241 ray image series generated by scanning with axial rotation in small steps. This method enables  
242 examination of internal structural features at fine spatial resolution (Li et al. 2016). A micro-CT dataset  
243 allows a variety of quantifiable measurements, including thickness in terms of pixel distance, volume  
244 in terms of voxel counts and density in terms of brightness of each pixel at higher resolution compared  
245 to CT (Fantazzini et al. 2015, Tambutté et al. 2015, Chatzinikolaou et al. 2017).

246 With these 3D geometric morphometrics and measurements, the growth rate, density and morphological  
247 changes due to OA can be investigated. Micro-CT has been applied in OA studies on gastropods  
248 (Chatzinikolaou et al. 2017), tubeworms (Li et al. 2014, Li et al. 2016), and shrimp (deVries et al. 2016)  
249 to infer changes in the protective function of the exoskeletons. 3D model visualisation also enables the  
250 analysis of density distribution to understand the engineering of calcareous structures. Consequently,  
251 the presence of structurally vulnerable regions can be identified. Micro-CT analysis has been used in  
252 OA to determine the survival of coral through protective exoskeleton function (Tambutté et al. 2015).

253 Exoskeleton porosity often represents shell protective function. In particular, intertidal gastropods  
254 *Nassarius nitidus* and *Columbella rustica* exhibited density reduction in acidified conditions  
255 (Chatzinikolaou et al. 2017) while coral skeletons also showed an increased porosity at lower pH  
256 through micro-CT (Fantazzini et al. 2015, Tambutté et al. 2015).

257 The spatial resolution of most medical micro-CT is sufficient to provide a good measurement for large  
258 calcifiers, and typically have a resolution of 15 - 1000  $\mu\text{m}$  per pixel. As a consequence, however,  
259 observation of marine plankton and larvae remains as a challenge. Another limitation of micro-CT is  
260 the detection sensitivity which can generate false negatives through thin minerals where regions may  
261 appear as empty space in the 3D reconstruction. Therefore, it is important to verify the representative  
262 morphology by combining micro-CT with an SEM approach. All density measurements should be  
263 calibrated with a standard material which has a known bone mineral density (BMD,  $\text{g}\cdot\text{cm}^{-3}$ ) in terms of  
264 calcium hydroxyapatite and its corresponding pixel intensity for each scan (Li et al. 2014). Since there  
265 are no commercially available standards for calcium carbonate calibration, density measurements are  
266 relative and has limited comparability with other studies.

#### 267 *Finite element analysis*

268 The field of engineering and computational simulation can be applied creatively to understand structural  
269 impacts to biominerals caused by OA. In a simulation, any weakness in the architecture is highlighted  
270 and the loading capacity can be calculated from the shape and empirical data (Li et al. 2016). Therefore,  
271 the application of structural analysis can be performed when shapes and mechanical properties of the  
272 biological mineral are both known, providing a holistic picture of how well each calcified material  
273 functions as the protective or supportive structure.

274 The most widely applied numerical tool for computational simulation is finite element analysis (FEA)  
275 (Li et al. 2014; Li et al. 2016). To solve a problem using FEA, the problem is divided into smaller and  
276 simpler parts which are called finite elements. By assembling the solution of all finite elements  
277 mathematically, a total approximate solution of the large problem can be obtained. FEA enables the  
278 mechanical behaviours of complex biomineralised structures to be investigated accurately. The FEA

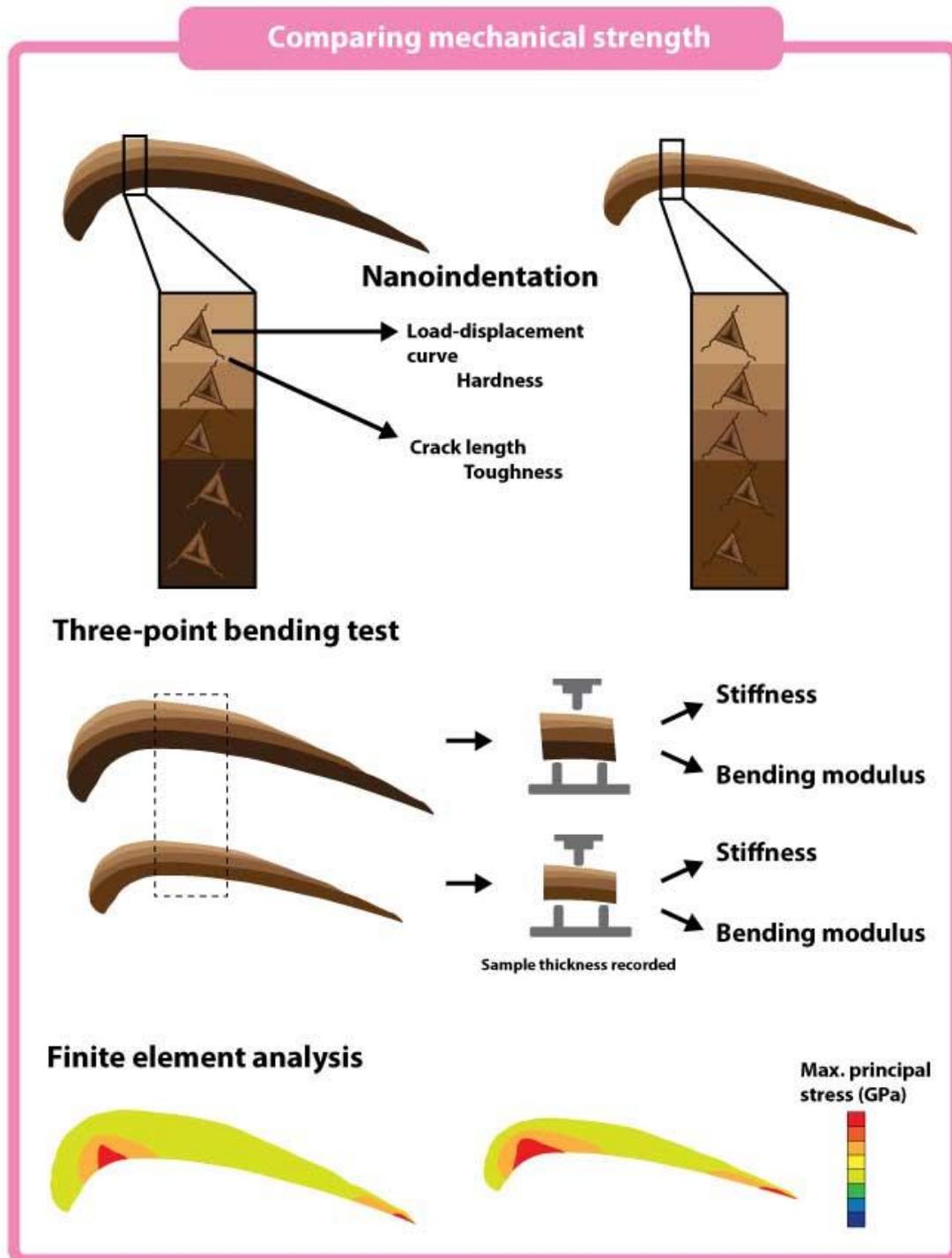
279 can include the different experimental mechanical properties, such as elasticities of different  
280 components of the shell structure, as well as simulate the effects of loading, for example the crushing  
281 forces associated with a predator attack.

282 With the diverse calcareous structures being produced by marine organisms, FEA can be applied to  
283 assess changes in mechanical performances due to morphological changes. For example, FEA has been  
284 applied to understand the mechanical response of a tubeworm under OA combined with low salinity  
285 and warming treatments. This enabled the identification of the most vulnerable region of the tube and  
286 the highest risk of fracture failure under predatory attack (Li et al. 2014, Li et al. 2016). This  
287 demonstrates that FEA can be developed as biologically accurate model to determine the impact of OA  
288 on the protective function of calcareous shells and exoskeletons. Beside its advantage, FE models  
289 especially when it is 2-D simplified (Ragazzola et al. 2012), often fail to account for heterogeneity,  
290 malformation and shape changes. In addition, 3-D models are complex and requires advanced  
291 computational efforts (Melbourne et al. 2015).

#### 292 *Micro- and nanoindentation*

293 Biominerals are composed of mineral crystals and an organic matrix framework. As a result, biogenic  
294 calcite has been reported to be 50-70% harder than geological calcite (Kunitake et al. 2012, Kunitake  
295 et al. 2013). Due to the high heterogeneity in morphology, structure and composition of mineralized  
296 shells and exoskeletons, hardness has been widely used as a comparable evaluation of mechanical  
297 properties (Beniash et al. 2010, Dickinson et al. 2012a, Fitzer et al. 2015b).

298 The strength of biomineralised structures can be characterised by a crushing or compressive test where  
299 machine applies and reads compressive force- versus displacement. The entire structure of specimens  
300 can be used to mimic a predatory attack (Byrne et al. 2014). However, biomineralised structures are  
301 typically not homogenous and using a single point allows a better mechanical understanding of the shell  
302 property.



303

304 Figure 3. Schematic representation of techniques to compare mechanical properties in  
 305 biomineralizing organisms. Nanoindentation and 3-point bending are two highlighted techniques  
 306 for assessing mechanical properties including hardness, elasticity, fracture toughness and stiffness

307 **of biominerals. Micro and nanoindentation in combination of computed tomography and finite**  
308 **element analysis project the impact of OA on mechanical properties of shells and skeletons during**  
309 **predatory attacks.**

310

311 Two main methods can be used to understand the impact of OA on shell mechanical properties (Figure  
312 3): 1) microhardness tests can measure Vickers hardness numbers (HV) determined by the ratio of the  
313 force applied by the indenter and the surface area of the final indent (Beniash et al. 2010, Dickinson et  
314 al. 2012a); and 2) nanoindentation which can measure the hardness and elasticity in a single indent.  
315 With the development of a depth-sensing indenter, the hardness and elastic modulus (a measure of  
316 stiffness) in units of pascals (Pa) from each indentation can be obtained from the loading-unloading  
317 curve by using the Oliver-Pharr model (Oliver and Pharr 1992). This method enables the measurement  
318 of shell hardness and has been used to address how the protective function of several mollusc species  
319 grown under experimental OA might be affected (Beniash et al. 2010, Dickinson et al. 2012a, Fitzer et  
320 al. 2015b). The results indicate no significant impact on microhardness in clams (Beniash et al. 2010)  
321 and oysters (Dickinson et al. 2012a) but an increase in microhardness in mussels (Fitzer et al. 2015c).

322 The advantage of nanoindentation is its precision at the nanometre scale. Nanoindentation enables  
323 spatial refinement where the mechanical properties profile can be examined (Li et al. 2014). At finer  
324 spatial resolution, mechanical features can be associated with the different textures of the mineral  
325 (Goffredo et al. 2014). The influence of OA can be reflected in different mineralised layers or structures  
326 of the marine invertebrate shell. Nanoindentation enables substantial refinement, for example, the  
327 hardness and modulus of the tubeworm from the exterior to the interior were mapped by  
328 nanoindentation in order to address questions about the protective function of the structure (Li et al.  
329 2014, Fitzer et al. 2015b). The mechanical properties of the exterior to the interior portion of the tube  
330 were shown to decrease under OA compared to the middle portion of a tubeworm (Li et al. 2014).  
331 Researchers can analyse the same polished sample using SEM, allowing correlation between structural  
332 alteration and mechanical performance. This has been done for mussels (Fitzer et al. 2015b) and

333 tubeworms (Chan et al. 2012, Li et al. 2014) grown in OA for extended periods of time showing that  
334 OA-induced structural alteration may lead to deteriorations in mechanical performance. Both hardness  
335 tests have the disadvantage of requiring a highly polished sample surface (Perez-Huerta and Cusack  
336 2009) and sample preparation can be both time-consuming and challenging (Milano et al. 2016).

337 In addition to hardness, the dimensions of the cracks generated around the indent can be used to  
338 determine the fracture toughness combined with the elasticity of the material as shown for bivalves  
339 grown under OA conditions (Beniash et al. 2010, Dickinson et al. 2012a, Fitzer et al. 2015b). By  
340 measuring the lengths of cracks, the plane-strain fracture toughness (K<sub>IC</sub>) can be calculated (Lawn et  
341 al. 1980, Anstis et al. 1981). However, it is difficult to define the local and bulk fracture behaviours by  
342 this technique, which makes it problematical to determine the accuracy of the fracture toughness values  
343 given by indentation (Kruzic et al. 2009). This technique has enabled the determination of the impact  
344 of OA on shell protective function: the fracture toughness of oyster shells and mussel shells was reduced  
345 (as measured by microhardness tests), which confirmed that the calcite shell became more brittle in OA  
346 conditions (Beniash et al. 2010, Dickinson et al. 2012a, Fitzer et al. 2015b).

#### 347 **Mineral composition - Biomineralisation mechanisms to enable shell growth**

348 Trace elements present in seawater are incorporated within the shell structure of calcifying organisms  
349 and several empirical relationships have been observed between the trace element to calcium ratio and  
350 environmental parameters of the surrounding water. For example, the Mg/Ca ratio of a shell is positively  
351 correlated with the temperature of the surrounding water (Nürnberg et al. 1996, Dwyer et al. 2002,  
352 Pérez-Huerta et al. 2008, Kamenos et al. 2013). Based on empirical and experimental calibration,  
353 several element to calcium ratios have been observed to reflect the environmental condition. Trace  
354 element to calcium ratios and particularly Mg/Ca and Sr/Ca have been widely used to understand the  
355 biomineralisation process (but noted that biological activity can influence the elemental composition in  
356 the mineral rather than recording the environmental conditions, Weiner and Dove 2003). For example,  
357 OA has been shown to affect trace element to calcium ratios in corals (Sinclair 2005), foraminifera  
358 (Elderfield et al. 1996, Keul et al. 2013, Not et al. 2018), ostracods (Dwyer et al. 2013), and tubeworms  
359 (Chan et al. 2015b), but not sea urchins (Byrne et al 2014). For sea urchins grown under OA and

360 warming from the juvenile to the adult stage, the Mg/Ca was not affected by OA but, as expected, was  
361 altered by temperature (Byrne et al. 2014). When exposed to OA, Mg/Ca increased in vermetid shells,  
362 suggesting a dissolution of aragonite and increase in calcite (Chan et al. 2012, Milazzo et al. 2014).  
363 Milazzo et al. (2014) applied inductively coupled plasma optical emission spectrometry (ICP-OES)  
364 techniques to understand impacts of OA on calcification as growth and suggested that under OA shell  
365 dissolution will occur with the potential to impact survival through weakened shell protection.

366 *Mass spectrometry*

367 A variety of methods are available to measure the elemental ratios of a biological mineral. Basically,  
368 calcifying organisms can be measured in a solid phase using techniques such as X-ray fluorescence  
369 (XRF) or laser ablation (LA) or in dissolved phase after dilution using a range of inductively coupled  
370 plasma spectrometry. Since trace elements are measured within the mineral, several cleaning steps are  
371 required to remove organic matter and potential lithogenic contamination (Martin and Lea 2002).  
372 Typically, analysis of a dissolved sample by inductively coupled plasma (ICP) spectrometry requires  
373 the preparation of the shell or skeleton sample by acid digestion, fusion, or ash drying. Techniques for  
374 solution analyses include, ICP- optical emission spectrometry (OES), ICP- atomic emission  
375 spectroscopy (AES), ICP-mass spectrometry (MS), and multiple collector (MC) ICP-MS. The  
376 differences between these ICP spectrometry techniques are the increase of precision of the analyses, the  
377 higher resolution and therefore lower detection limits of elements up to isotopic measurement with MC-  
378 ICP-MS, whereas the disadvantages are the cost and maintenance of the equipment.

379 *Electron microscopy AEM/ EDS/ SIMS/ EELS*

380 Spatial information of elemental distribution in the mineral provides valuable information to predict  
381 mechanical properties. Solid-sampling methods have been developed for ICP analysis for this purpose.  
382 Electro-thermal-vaporisation (ETV) and laser ablation (LA) are applied to generate vapour for  
383 characterisation. In combination with ICP-OES or ICP-MS, these techniques are suitable for analysis  
384 of a solid sample (Limbeck et al. 2017). However, LA-ICP-MS only provides resolution of  $> 5 \mu\text{m}$ ,  
385 while secondary ion mass spectrometry (SIMS) distinguishes sub-micrometre resolution (Becker et al.  
386 2010). Although SIMS has a sensitive detection level of 1 ppm, the technique is not directly quantitative

387 due to its dependence on a solid-state chemical standard, and the nonlinear and highly variable nature  
388 of the ionisation process of different elements in SIMS (Williams 1985). In addition, SIMS can be used  
389 to obtain depth profiles of mineral composition of shells (Jeffree et al. 1995). All of these methods have  
390 an advantage of giving spatial information on the elemental distribution, the differences lie in the  
391 resolution between ICP-MS, ICP-OES and SIMS. ICP-OES is already applied using acid digestion of  
392 collected individuals for determination of elemental ratios (Milazzo et al. 2014). The application of  
393 SIMS to OA research would enable the analysis of much smaller samples and to examine the response  
394 of individual calcifying organisms in terms of growth and survival (Eichner et al. 2017).

395 The spatial detection of trace elements on a bulk material surface can be achieved through electron  
396 microscopy (Müller et al. 2011). Analytical electron microscopy (AEM) with energy dispersive X-ray  
397 spectrometry (EDS) and wavelength-dispersive X-ray spectroscopy (WDS) provides data at the  
398 nanometre scale (Newbury 1998). EDS offers an advantage of greater specimen area than the high  
399 resolution method of electron energy loss spectrometry (EELS), which also requires a 10 nm thick  
400 specimen to be prepared. Therefore, EDS is a more efficient and accessible AEM approach for OA  
401 research. Notably, detection levels of AEM-EDS are around 1000 ppm, EELS 10 ppm and structured  
402 illumination microscopy (SIMS) 1 ppm. EDS and WDS both enable the microanalysis of biominerals  
403 and provide additional spatial information of the elemental profile. The cost of EDS is considerably  
404 lower than WDS and has a high acquisition speed. In comparison, the spectral resolution of WDS is  
405 superior to that of EDS. These techniques have been applied to address the question of whether OA  
406 would have an impact on the calcification of shrimp and tubeworm skeletons, in order to understand  
407 the impact on exoskeleton critical function including protective defense against predators (Chan et al.  
408 2012, Taylor et al. 2015). EDS was employed to determine magnesium content in the exoskeleton of  
409 shrimps grown under OA with the finding that increased calcium content with lowered pH resulted in  
410 a greater Mg/Ca ratio (Taylor et al. 2015). Mg/Ca as an environmental indicator of calcite has been  
411 suggested to increase as aragonite saturation decreases (Chan et al. 2012).

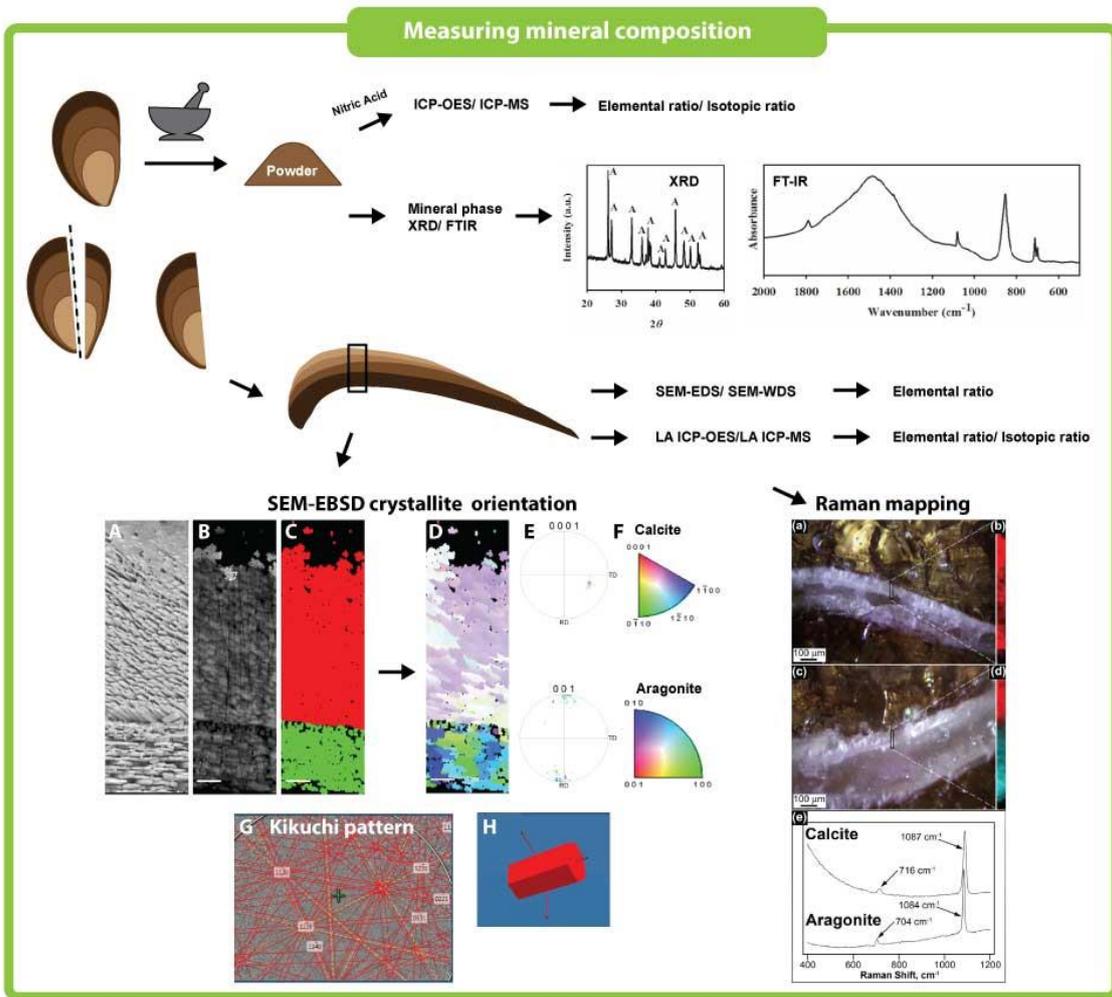
412 Taken together, there has been significant growth in a number of techniques available for quantifying  
413 elements present in liquid and solid materials. Some of the surface techniques such as LA-ICP-MS, and  
414 other analytical electron microscopy (AEM) techniques, such as SIMS, AEM-EDS and AEM-EELS,

415 have different resolutions and sample preparation requirements which should be considered in the  
416 context of experimental objectives. The recent development of MS analytic methods for bioimaging  
417 opens opportunities to investigate mineralising tissues at the biomolecule level (Becker et al. 2010).  
418 Complementing optical and electron microscopy techniques as discussed in the following section, these  
419 tools will enable a better understanding of the mechanism of OA impacts on the processes involved in  
420 the production of biominerals. However, these techniques at present have the disadvantages of requiring  
421 sample specific standards for calibration, time consuming sample preparation, observation confined to  
422 a tiny area of interest and the destructive nature of sample analysis.

423

424 *Mineral composition analyses by FTIR and XRD*

425 Mineral composition characterisation techniques target the comparison of mineral phases, elemental  
426 ratios and amorphous calcium carbonate to clarify the intricate process of biomineralisation (Figure 4).  
427 In the context of OA, it is important to understand the process of biomineralisation mechanisms to  
428 appreciate how continued growth will be possible under future environmental change.



429

430 **Figure 4. Schematic representation of highlighted techniques for characterizing mineral composition**  
 431 **in biomineralising organisms. Shell power samples are digested prior to mass spectrometry**  
 432 **characterisation, e.g. ICR-OES/ICP-MS, for determining elemental ratio or isotopic ratio. Sectional**  
 433 **surface of the shell are inspected for elemental ratio mapping using electron microscopy techniques**  
 434 **such as SEM-EDS or SEM-WDS; or using mass spectrometry approaches by LA-ICP-MS or LA-ICP-OES.**  
 435 **Additionally, a sample of shell powder provides mineral phase indentification using FTIR, XRD,**  
 436 **figures modified from Chan et al., (2012). SEM-EBSD, SEM-WDS and Raman spectroscopy are**  
 437 **highlighted as techniques to determine the mineral composition and crystallographic orientation in**  
 438 **a mussel shell to determine the impacts on shell growth under OA as an alternative to techniques**  
 439 **such as ICP-OES or ICP-MS to determine elemental or isotope ratios in biominerals. SEM-EBSD figure**

440 data taken from Fitzer et al., (2014a). Secondary electron images of the crystal structure from an  
441 etched (a) and polished (b) sample, the mineral composition can be seen in the phase map where  
442 calcite is shown in red and aragonite in green (c). The crystallographic orientation map (d),  
443 corresponding pole figures (e) and colour keys (f) are indicated for calcite and aragonite. EBSD uses  
444 Kikuchi patterns to identify the mineral phase (g) and the crystallographic orientation (h). Raman  
445 mapping figure modified from Chan et al., (2015b). Raman microscopy has been used to provide  
446 photomicrographs of younger (a) and older regions (c), of a juvenile tubeworm, phase maps  
447 indicate regions of aragonite only (b) and aragonite and calcite (d) in the tube. A Raman spectra  
448 (e) of aragonite and calcite has been measured in the same specimen (c).

449

450 Fourier-transform infrared (FTIR) spectra can be used to determine the relative quantity of amorphous  
451 calcium carbonate from an intensity ratio ( $I_{\max V_2}/I_{\max V_4}$ ) between the absorption bands. The major  
452 disadvantage of the FTIR approach is its semi-quantitative nature; results can only be compared within  
453 the same experimental dataset. This method has been adopted in OA research on a marine tube worm  
454 where amorphous calcium carbonate content was found to be higher at low pH (Chan et al. 2012, Leung  
455 et al. 2017). Chan et al. (2012) suggest that this result indicates the presence of an active shell repair  
456 mechanism when animals are counteracting shell weakening by OA. The advantage of FTIR is that it  
457 takes as little as 1 mg of mineral sample and so may be applied to larval specimens.

458 The ratio of calcite and aragonite content, that has implications for the vulnerability of shells and  
459 skeletons to OA, can be quantified by X-ray diffraction (XRD). This is an advantage over FTIR, but  
460 XRD has its own drawback, i.e. the loss of spatial resolution due to the need for powdered samples.  
461 XRD approach has been used to assess shell or exoskeleton growth under OA. Unless containing large  
462 amounts of Mg, calcite is considered to be less susceptible to dissolution at lower pH values than  
463 aragonite (Ries et al. 2009, Chan et al. 2012). OA relevant changes in thickness of the calcite and  
464 aragonite layers was first noted in mussels transplanted into low-pH environments (Hahn et al. 2012).  
465 Calcite: aragonite ratios have been shown to change under OA, leading to a thinner and more vulnerable

466 aragonite layer in mussel shells (Fitzer et al. 2015a). The shell thickness index in comparison to the  
467 measured thickness of the aragonite or calcite layers uses the thickness, the length, height and dry mass  
468 of the shell and is considered to produce a lower measurement error compared to direct measurement  
469 (Freeman and Byers 2006, Naddafi and Rudstam 2014, Fitzer et al. 2015a). The shell thickness index,  
470 in comparison to XRD, has an advantage of being non-destructive to the sample, but a disadvantage  
471 with a loss in spatial resolution.

#### 472 *Raman spectroscopy*

473 Raman spectroscopy is a non-destructive technique that enables molecular bonds and mineralogical  
474 information to be precisely characterised at a submicron resolution. By illuminating a sample with a  
475 monochromatic laser beam, a Raman spectrum is generated that contains unique peaks that are  
476 diagnostic of mineral polymorphs due to their characteristic Raman shifts (Eisenstein et al. 2016).  
477 Structural components, such as calcite, aragonite and collagen, have been identified and mapped with  
478 resolution as fine as 1  $\mu\text{m}$  (Eisenstein et al. 2016, Taylor et al. 2016). Advantages of Raman  
479 spectroscopy over other spectroscopic methods, such as FTIR, include the improved spatial resolution  
480 and the direct relevance of this method for biomineralisation, as well as the potential to examine samples  
481 in their native state (Eisenstein et al. 2016, Von Euw et al. 2017). This technique is considered a  
482 complementary method to FTIR, and is perhaps more suitable for OA studies. Raman microscope  
483 imaging has been applied to OA research to identify the mineral composition and polymorphic forms  
484 to assess rates of calcification under increasing  $p\text{CO}_2$  in coralline algae and limpets (Kamenos et al.  
485 2013, Langer et al. 2014). For instance, Raman mapping of the shells of limpets from a  $\text{CO}_2$  vent  
486 demonstrated that the polymorph distribution pattern is maintained despite living at low pH (Langer et  
487 al. 2014). In coralline algae, although calcification continues under OA, Raman has identified disorder  
488 in the molecular position of the carbonate ions which suggests a weakened skeletal structure (Kamenos  
489 et al. 2013). These applications were used to assess exoskeleton structural weaknesses which can impact  
490 the protective function of the calcified structures under OA.

#### 491 *SEM-EBSD*

492 Electron backscatter diffraction (EBSD) is widely used to determine the crystallographic orientation of  
493 biogenic minerals. EBSD provides additional information to precursory SEM imaging of shell  
494 dissolution or exoskeletal microstructure as it allows for the examination of microstructure at the  
495 individual crystal level. The technique identifies Kikuchi patterns (Kikuchi 1928, Nishikawa and  
496 Kikuchi 1928) as scattered electrons are reflected as per Bragg's law from the crystal lattice onto a  
497 phosphorus screen. It was first used to observe the impact of OA on the shell ultrastructure of the mussel  
498 *Mytilus galloprovincialis* (Hahn et al. 2012) and was further applied across a broad range of species,  
499 including an argonaut (Wolfe et al. 2013) and corals (Fitzer et al., 2014b; Hennige et al., 2015). The  
500 effects on crystalline structure identified using this technique in OA research have been used to address  
501 the question of how changes in seawater environment can impact the orderly arrangement of shell or  
502 exoskeleton structures which has an implication to the animal's ability to survive.

503

504

*X-ray microscopy XPEEM – XAS and XANES*

505 An alternative emerging technique for determining the mineral composition of marine skeletons is high-  
506 spatial resolution synchrotron X-ray photo emission electron microscopy (XPEEM) combined with X-  
507 ray absorption spectroscopy (XAS) (Fitzer et al. 2016). The benefits of the XPEEM and XAS over SEM  
508 is the high level of spatial resolution, and the fact that it can detect without the need for an energy filter  
509 by measuring the secondary electrons yield as a function of photon energy. This technique can be  
510 applied alongside electron backscatter diffraction (EBSD) to identify mineral phases throughout the  
511 shell structure (Fitzer et al. 2016). XANES and XPEEM techniques use the principles of X-ray  
512 absorption fine structure (XAFS) which interpret the scattering of photo-electrons emitted from an  
513 absorbing atom in a structure when excited by high energy photons (Politi et al. 2008, Fitzer et al. 2016).  
514 XANES has been used as a tool to examine the phase transformation mechanisms of amorphous calcium  
515 carbonate into calcite particularly in sea urchin larval spicules (Politi et al. 2006, Politi et al. 2008, Gong  
516 et al. 2012). Recently, XPEEM combined with XAS has been used as a tool to examine amorphous  
517 calcium carbonate in mussels reared under OA conditions (Fitzer et al. 2016) showing more induced  
518 amorphous calcium carbonate with less crystallographic control over shell formation. This technique

519 was applied to address the question of OA impact on biomineralisation and shell repair to determine  
520 the protective function of the shell under changing environments (Fitzer et al. 2016). The technique  
521 requires the embedding and fine-polishing of samples, similar to SEM-EBSD preparation (Politi et al.  
522 2008, Perez-Huerta and Cusack 2009, Fitzer et al. 2016). XANES and XPEEM have the advantage of  
523 providing high spatial resolution to locate amorphous calcium carbonate embedded within the shell  
524 structure when applied in combination with SEM techniques (Politi et al. 2008). Disadvantages include  
525 the high instrumentation cost and lengthy sample preparation time.

526 *AFM*

527 Atomic force microscopy (AFM) provides atomic resolution analysis of material properties. As a type  
528 of scanning probe microscopy, AFM scans and interacts with a sample directly using a tip that is  
529 connected to a cantilever spring. The vertical deflection and the force-distance curve are recorded by a  
530 piezoelectric translator (Butt et al. 2005). In tapping mode, AFM generates fine topographical images  
531 with nanometre resolution. The time-dependent relationship between applied pressure (stress) and  
532 deformation (strain) represents viscoelastic properties (Butt et al. 1995) enabling the measurement of  
533 local mechanical properties.

534 Easy sample preparation and high resolution are the major advantages of using AFM over conventional  
535 microscopy methods (Butt et al. 1995). The typically small interacting surface for AFM must be smooth  
536 and homogeneous requiring polished and etched surfaces similar to EBSD sample preparation (Dalbeck  
537 et al. 2011). Therefore, AFM complements the observations of SEM-EBSD analysis which can provide  
538 high resolution textural data to OA studies (Dalbeck et al. 2011). AFM performed in the presence of an  
539 electrolyte solution is possible (Butt et al. 1995), therefore, enabling better biological relevance. In order  
540 to obtain comparable regions of interest, correlative SEM or light microscopy data are often helpful to  
541 effectively navigate at AFM resolution (Sikes et al. 2000). AFM has yet to be applied to determine the  
542 impact of OA on biomineralisation. Once applied, this technique will provide a correlation with EBSD  
543 data to address the question of biomineralisation mechanisms in shell growth and hence survival.

544 *FIB-TEM*

545 The finest biological observation have been via transmission electron microscopy (TEM), providing  
546 resolutions of nanometres down to below an ångström (Nellist et al. 2004) exceeding ‘super-resolution’  
547 microscopy. In addition, TEM is an important characterisation tool that collects X-ray diffraction with  
548 a micrograph enabling subcellular features and location of crystals to be analysed together. A  
549 nanofabrication technique using the focused ion beam (FIB) system has emerged as a powerful tool for  
550 precise TEM specimen preparation, where milling and cutting of a sample is performed inside an SEM  
551 or scanning ion microscopy (SIM) (Titze and Genoud 2016). This preparation approach overcomes  
552 technical challenges of manual preparation of ultrathin TEM sections, with the localisation of the region  
553 of interest, and reduces the risks of sample loss (Chan et al. 2017).

554 Suzuki et al. (2011) revealed the details of five microstructures in the limpet shell using FIB-TEM. The  
555 FIB technique was used to separate each microstructure in cross section to determine crystal  
556 morphology and orientation. The FIB technique is powerful in handling tiny larval or juvenile shells  
557 (Yokoo et al. 2011, Chan et al. 2015a, Chan et al. 2017), and reduces costs of analysis time for TEM  
558 on samples with poor orientation or an unfocussed area of interest. FIB-TEM ensures a consistent  
559 cutting angle at the nanoscale, so providing comparable observation of a larger number of experimental  
560 samples.

561 Depending on institutional resources, fine spatial resolution, long-hour procedures performed using  
562 FIB-TEM can be costly in a centralised facility. In summary, FIB-TEM is currently a qualitative  
563 observational method, but it has potential to be applied in a more quantitative setting.

#### 564 *Cryo-electron microscopy*

565 In cryo-electron microscopy, biological samples can be visualised by a freeze fracture process which is  
566 achieved by rapid freezing of fixed tissue samples by vitrification (Alfredsson 2005). A hydrated sample  
567 that is close to the native state can be observed in high resolution without the requirement of destructive  
568 conventional preparation procedures for SEM and TEM (Levi-Kalisman et al. 2001, Khalifa et al. 2016,  
569 Thompson et al. 2016). Electron microscopy also enables X-ray diffraction characterisation essential  
570 for identification of minerals. Ice from humidity in the environment can contaminate the sample,

571 therefore, samples must be prepared after vitrification (Thompson et al. 2016). Technical disadvantages  
 572 associated with cryo- methods include the need for stabilising detergents for structure (Singh and  
 573 Sigworth 2015) which, in addition to its high cost, will make the application of this technique  
 574 challenging for large scale OA experiments.

575 *Stable isotopes*

576 Biogenic stable isotopes have been used extensively to reconstruct the paleoclimates, in terms of  
 577 temperature, pH and salinity (Lear et al. 2000, Parkinson et al. 2005, Ghosh et al. 2006, McConnaughey  
 578 and Gillikin 2008, Martin et al. 2016, Stewart et al. 2016). They can also be used to understand  
 579 biomineralisation mechanisms of ion transport at the site of calcification (Furla et al. 2000, Rae et al.  
 580 2011, Allen et al. 2012, Allison et al. 2014). The detection of Mg, Sr, and Ca, the detection of the  
 581 isotopes,  $\delta^{13}\text{C}$ ,  $\delta^{18}\text{O}$ ,  $^{10}\text{B}$  and  $\delta^{11}\text{B}$  requires acid digestion prior to mass spectrometry analyses (Krief et  
 582 al. 2010). Here, we list some of the target isotopes that have promise for OA research.

583 Quantification of isotopic elements can be applied to detect the consequence of stress on calcification  
 584 pathways (Rae et al. 2011, Allison et al. 2014, Stewart et al. 2016). Brachiopods, in particular, form  
 585 their exoskeletons in good isotopic equilibrium with the seawater (Parkinson et al. 2005). Since pH is  
 586 dependent on two boron species: boric acid ( $\text{B}(\text{OH})_3$ ) and the borate ion ( $\text{B}(\text{OH})_4^-$ ) (Hemming and  
 587 Hanson 1992, Stewart et al. 2016, Zhang et al. 2017), the species of boron isotopes found in shells  
 588 represents the dissolved inorganic carbon (DIC) chemistry of the calcification fluid (Allison et al. 2014).  
 589 Stable isotope techniques have been applied in OA research to understand the mechanisms of  
 590 biomineralisation, specifically, whether material is laid down under control by the organism irrespective  
 591 of the seawater isotopes (Krief et al. 2010). For example, seawater pH impacts the skeletal  $\delta^{13}\text{C}$  and  $\delta$   
 592  $^{18}\text{O}$  in corals, but there is an offset in the  $\delta^{11}\text{B}$  between the calcified material and that of seawater  
 593 suggesting control of biomineralisation by ion-transport enzymes (Krief et al. 2010). This technique  
 594 addresses the question of calcification mechanism change under OA and whether there is a reduced  
 595 metabolic incorporation of isotopes through enzyme control, hence reduced growth and survival under  
 596 OA. The influence of carbonate ion concentration on  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  is still being explored, particularly  
 597 in foraminifera (Spero et al. 1997).

598 Isotopic approaches have the disadvantage of requiring a relatively large amount (~2 mg) of biogenic  
599 calcium carbonate powder. Sample preparation with micro-milling is time-consuming and can be  
600 technically challenging, especially when investigating different polymorphs and seasonal growth bands  
601 in smaller specimens (Stewart et al. 2016). Advances in stable isotope techniques will consist of  
602 improved ways of separating organics from biominerals, micro milling samples for biomineral powder,  
603 and laser ablation methods that increase spatial resolution of measurements (Fietzke et al. 2010, Wall  
604 et al. 2016).

#### 605 *Radioactive isotopes*

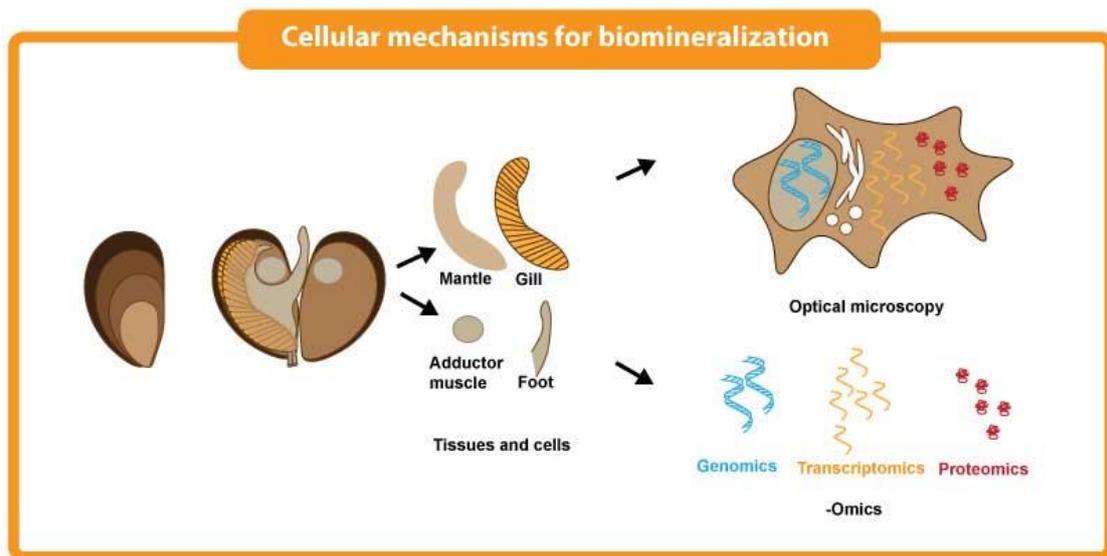
606 In addition to using  $^{45}\text{Ca}$  for estimation of calcification, radioactive isotopes can also contribute to a  
607 mechanistic understanding of the calcification process. Furla et al. (2000) used a double radioactive  
608 isotope experimental design ( $\text{H}^{14}\text{CO}_3$  and  $^{45}\text{Ca}$ ) to show inorganic carbon transport. Comprehensive  
609 measurement of both the DIC species and Ca for calcification was accomplished in terms of net  
610 radioactivity by  $^{14}\text{C}$  and  $^{45}\text{Ca}$  (Furla et al. 2000). These data also unraveled the carbonate concentrating  
611 mechanisms within coral cells (Furla et al. 2000).

612 The use of radioactive isotopes ( $^{45}\text{Ca}$ ,  $^{14}\text{C}$ ) is specific and sensitive to experimental conditions.  
613 Moreover, the maintenance of radioactive substances can be challenging. Before adopting the use of  
614 radioactive isotopes within an experimental culture, a protocol must be optimised to adequately label  
615 specimens and avoid contamination. Therefore, even though the approach is quite well-established in  
616 environmental geochemistry (Parkinson et al. 2005, Ghosh et al. 2006, McConnaughey and Gillikin  
617 2008), disadvantages of requiring continual radioactive tracer application and the destructive nature of  
618 the sample digestion by acid means that it has yet to be applied more widely in OA studies (Furla et al.  
619 2000).

#### 620 **Cellular biomineralisation mechanisms**

621 The processes by which organisms control the substrate for calcification are complex and can occur  
622 internally within tissues, or outside the organism, but both need to modify the seawater chemistry for  
623 calcification to take place (Roleda et al. 2012). It is thought that  $\text{HCO}_3^-$  is the choice of substrate for

624 biomineralisation, which can be taken directly from seawater or metabolised from CO<sub>2</sub> (Roleda et al.  
 625 2012). The mechanisms revolve around producing an abundance of ions and the right conditions to  
 626 favour the precipitation of CaCO<sub>3</sub> from available Ca<sup>2+</sup> and CO<sub>3</sub><sup>2-</sup> (Roleda et al. 2012). An understanding  
 627 of biomineralisation mechanisms under forecasted OA conditions will require the use of a wide range  
 628 of techniques (Figure 5) but also working at different levels, from genes to organisms and ecosystems.  
 629 This can be achieved by taking advantage of established techniques from other disciplines. For example,  
 630 methods to culture larval sea urchin primary mesenchyme cells facilitates the study of the calcification  
 631 process *in vitro* (Basse et al. 2015). Identifying and evaluating the roles of organic molecules in shells  
 632 is a major topic in biomineralisation as the mechanical properties of shells are highly influenced by  
 633 their 1% content of organic components. In the context of OA impacts on marine calcifiers, the response  
 634 of organic molecules provides an insight into cellular mechanisms for the ability to reproduce and  
 635 develop and can be applied to all marine organisms irrespective of size (Figure 1, schematic of question  
 636 vs scale). Mechanisms of biomineralisation are largely unresolved and vary from species to species.



637  
 638 **Figure 5. Schematic representation of techniques to examine the cellular mechanism of**  
 639 **biomineralisation. Techniques with OMICS, optical microscopy are established techniques that**  
 640 **could be applied on various tissues and cells to determine the impact of OA on molecular**  
 641 **biomineralisation.**

642

643

*SDS-PAGE and MS*

644 Shell protein extraction is achieved by recovering the protein from decalcified shell. Sodium dodecyl  
645 sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), or 2D gel electrophoresis enables a  
646 comparison of protein profiles. In addition, mass spectrometry (MS) also enables the identification of  
647 proteins from available databases. As marine organisms studied in OA research are usually not ‘model-  
648 organisms’, protein identification is challenging. Protein purification, protein sequencing, and protein  
649 digestion, may be required in the identification process (Marxen et al. 2003, Suzuki et al. 2004). When  
650 a large protein (more than 30 kDa) needs to be identified, cloning methods can be used to recover the  
651 nucleotide sequence for deduction of the protein sequence (Miyamoto et al. 1996, Samata et al. 1999,  
652 Suzuki et al. 2009, Suzuki et al. 2011). Many genome and transcriptome databases of calcifying  
653 invertebrates generated by next generation sequencing are also available as open resources (Takeuchi  
654 et al. 2012, Zhang et al. 2012).

655 MS is also a promising technique to understand shell proteins. The MS/MS spectra of peptides allow  
656 determination of amino acid sequence without the need for a protein sequencer and has become a low  
657 cost and high throughput technique. The combination of MS/MS and databases from next generation  
658 sequencing is dramatically increasing the efficiency of protein identification (Joubert et al. 2010,  
659 Marie et al. 2010, Marie et al. 2011). The potential disadvantage of generating big data from the high  
660 throughput process is covered in more detail in the next **Section**. These techniques have been applied  
661 to show the proteome responses of invertebrates grown in OA conditions. For example, the shell  
662 matrix proteins of the larval pacific oyster (*Magallana gigas*) were observed to decrease under OA  
663 (Harney et al. 2016).

664 SDS-PAGE and MS offer advantages of low cost and in the case of MS high throughput protein  
665 identification to understand why physiological responses may be influenced by ocean acidification.  
666 The disadvantages of these techniques include time-consuming sample preparation to extract and  
667 purify proteins for analysis and production of big data which can be difficult to interpret.

668 *Insoluble organic component analyses using XRD, IR and pyrolysis NMR, and dyes*

669 Insoluble organic components in the shell, such as chitin, are an essential polysaccharide that connects  
670 organic matrices and the initial deposition of the mineral (Nakayama et al. 2013); it serves as a scaffold  
671 for organic materials during the deposition of minerals. For example, the molluscan periostracum is  
672 made of chitin and it serves as a waterproof layer of a calcification compartment. Chitin is commonly  
673 found in the forms of  $\alpha$  and  $\beta$ -chitin. Crustaceans use  $\alpha$ -chitin in their exoskeletons and molluscs use  $\beta$ -  
674 chitin in their shells, as such, chitin is an essential additive to greatly improve the mechanical properties  
675 of biomaterials. Due to the insoluble and organic nature of chitin, the impact of OA on chitin content is  
676 currently unknown. There are many techniques to identify chitin, including X-ray diffraction (Weiner  
677 and Traub 1980, Levi-Kalishman et al. 2001, Falini et al. 2003), infra-red (IR) spectroscopy in the finger  
678 printing region ( $700\text{-}1800\text{ cm}^{-1}$ ) (Pearson et al. 1960), and nuclear magnetic resonance (NMR) or MS  
679 detection of the glucosamine hydrochloride after hydrolysis (Nakayama et al. 2013). The analysis of  
680 pyrolysis (thermal decomposition of materials in a vacuum) GC-MS is able to identify characteristic  
681 chitin decomposition markers (Furuhashi et al. 2009). However, many other contaminants (such as  
682 proteins) produce a complex of unknown peaks that make the identification of chitin in biominerals  
683 difficult and, in the case of insufficient crystallinity, a clear diffraction pattern may be hard to obtain.  
684 Recently, a colorimetric assay of chitin has been developed to quantify chitin (Katano et al. 2016). The  
685 workers found that upon complete hydrolysis of chitin in strong acid (5M HCl), characterization of  
686 depolymerized glucosamine is possible using colorimetry at the absorbance maximum at 750 nm. The  
687 method requires small amount of sample (10 mg), it is low cost, simple and quantitative. However, the  
688 detection is robust and cannot distinguish polymorphs and spatial distribution of chitin.

689 Chitin can be visualised microscopically by calcofluor white which binds strongly to cellulose and  
690 chitin, and wheat-germ agglutinin (WGA) which binds to N-acetyl-D-glucosamine and sialic acid  
691 (Suzuki et al. 2007). Due to the non-specific nature of calcofluor-white and WGA, more specific  
692 detection is accomplished by using chitin-binding domain fused with green fluorescent protein (CBD-  
693 GFP) as shown in the larval shell of *Mytilus galloprovincialis* (Weiss and Schönitzer 2006) and the

694 prismatic layer of *Atrina rigida* (Nudelman et al. 2007). Quantifying the expression of the chitin  
695 synthase gene is an alternative approach to measure chitin production (Cummings et al. 2011).

696 While the role of chitin may be essential in providing a waterproof cover and biomineralisation  
697 framework to the shell formation process, the plasticity of chitin synthesis under OA environmental  
698 stress has not yet been investigated. Chitin has many important biological roles in a diverse taxonomic  
699 group of animal models (Lee et al. 2011), therefore, the detection of chitin could provide valuable  
700 information on both the structural and functional responses to OA. The disadvantages of XRD, IR, and  
701 pyrolysis NMR or GC-MS techniques for chitin analysis such as the low abundance of organic materials  
702 in calcareous structures and therefore time-consuming sample preparation may limit the application of  
703 this approach to future OA studies. On the other hand, visualizing chitin with dyes such as calcofluor  
704 white and WGA are prone to non-specific reactivity to other molecules than chitin. The more specific  
705 CBD-GFP labeling requires bioengineering protocol to express and purify the chitin probe.

#### 706 *OMICS*

707 Omics studies are generating “big data”. However, these data cannot be simply used as proxies of fitness  
708 (Feder and Walser 2005). As a consequence, little information can be extracted from purely exploratory  
709 studies (e.g. comparisons between two OA scenarios). A more powerful approach involves the analysis  
710 of the data to test a hypothesis based on physiological or ecological experiments. For example, De Wit  
711 et al. (2016) filtered a large-scale transcriptomic database to select genes following the same pattern  
712 identified at the physiological level in copepods exposed to OA (Thor and Dupont 2015, De Wit et al.  
713 2016). The future use of omics to investigate the effects of OA on biomineralisation is promising,  
714 especially using the integration of omics technique with other physiological endpoints.

715 The three main omics approaches to consider are transcriptomics, proteomics and metabolomics.  
716 Transcriptomics is a study of mRNA and actively expressed genes, while proteomics investigates the  
717 total protein profile, and metabolomics capture the biochemical status of an organism.

718 Transcriptomic changes can provide insights into genetic pathways involved in calcification by  
719 comparison of gene expression, for example at different stages of calcification, or under different

720 environmental conditions. That is, when applied in a time series, changes in transcriptomes of  
721 developing larvae provide useful information relevant to the onset of biomineralisation (Zhang et al.  
722 2012). De Wit et al. (2018) used OA as a tool to delay calcification in early stage of oyster development  
723 and using a time series were able to identify genes involved in larval shell calcification (De Wit et al.  
724 2018). Under OA, transcriptomic studies are used to assess the physiological capacity of organisms by  
725 studying not just the biomineralisation molecular pathways but also the other related pathways giving  
726 a complete picture in understanding the consequences of living in a high CO<sub>2</sub> oceans (Todgham and  
727 Hofmann 2009).

728 Proteomics investigates the total protein profile. Since proteins are the active functional units of an  
729 expressed gene, these data are closer to the organism at the functional level and phenotype. Proteomes  
730 can be altered by OA, as shown by different calcification protein expression in oysters (Dineshram et  
731 al. 2015). The organic matrix proteins that are associated to shell deposition have been profiled in corals  
732 (Drake et al. 2013), oysters (Suzuki and Nagasawa 2013), and blue mussels (Suzuki et al. 2011),  
733 although this information has not been profiled in the OA context.

734 In common with the transcriptome, some precautions should be taken during the interpretation of a  
735 proteome. The quantity of a protein commonly cannot be directly linked to the fitness of an organism  
736 as regulatory post-translational modifications play a key role (Mann and Jensen 2003). This is an  
737 essential consideration for the study of shell proteins which are often heavily glycosylated,  
738 phosphorylated or tyrosine sulfated as required for calcium binding (Zhang and Zhang 2006).

739 A major disadvantage of applying OMICS to the study of biomineralisation is the low abundance of  
740 about 5% organic content in calcareous structures (Zhang and Zhang 2006). In order to isolate sufficient  
741 protein or polysaccharide for analysis, a large amount of shell must be used. In addition, the extraction  
742 and purification of the organic content is greatly influenced by decalcification, and the shell can often  
743 contain organic impurities (Watabe 1965). Researchers should be aware of technical limitations using  
744 different characterisation techniques, for example 2D gels have a lower sensitivity than iTRAQ analysis  
745 (Wiese et al. 2007). In addition, the interpretation of OMICS data is highly dependent on genomic  
746 information, therefore, it remains as a challenging method for non-model organisms.

747 *Cellular pH imaging*

748 When live imaging is conducted, inverted microscopy enables living marine organisms to be fully  
749 submersed in seawater during observation (Venn et al. 2011, Stumpp et al. 2012, Tambutté et al. 2015,  
750 Chan et al. 2017). In some imaging methods, synthetic ratiometric images are generated by sequential  
751 images of the same region of interest. Mobile organisms can be immobilized (e.g. 2-4% agarose  
752 seawater or using micropipettes) to enable image acquisition of the region of interest. For longer periods  
753 of observation, a perfusion chamber is necessary to remove metabolic waste and allow exchange of  
754 aerated experimental seawater with enriched CO<sub>2</sub>.

755 Fluorescent microscopy has been applied to visualise the calcification compartment during  
756 mineralisation at a low seawater pH using markers including calcein, alizarin and calcofluor white for  
757 *in situ* analysis of calcification and tracking of calcification as shown for coralline algae (Lewis and  
758 Diaz-Pulido 2017). Newly deposited minerals can be quantified from their fluorescent appearance at  
759 their respective excitation (Exλ) and emission wavelengths (Emλ), e.g. alizarin: Exλ = 530-560 nm,  
760 Emλ = 580 nm; calcein: Exλ = 494 nm, Emλ = 517 nm; calcofluor white: Exλ = 365 nm, Emλ = 435  
761 nm. Calcein is a preferable marker because of its high efficiency, non-invasiveness (Lewis and Diaz-  
762 Pulido 2017) and it is relatively low in cost. In addition, the fluorescent region can be isolated and  
763 characterised using the techniques described for measuring growth and development in the earlier  
764 sections .

765 The heterogenous distribution of carbon sources, e.g. CO<sub>3</sub><sup>2-</sup> ions at the calcification site, can be  
766 monitored by measuring intracellular pH (de Nooijer et al. 2008, Venn et al. 2011, Venn et al. 2013,  
767 Tambutté et al. 2015). Similar to the carbonate dynamics in the ocean, a shift in pH influences the DIC  
768 abundance in the biomineralisation compartment, in terms of CO<sub>3</sub><sup>2-</sup> and HCO<sub>3</sub><sup>-</sup>. As shown in  
769 foraminifera, pH of the site of calcification increases during calcification, while the surrounding  
770 ambient pH decreases probably through active proton pumping (Toyofuku et al. 2017). A higher pH  
771 value facilitates the conversion of CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> to CO<sub>3</sub><sup>2-</sup> (Toyofuku et al. 2017), and both the CO<sub>3</sub><sup>2-</sup>  
772 concentration and calcium carbonate saturation state can then be calculated (Venn et al. 2011, Venn et  
773 al. 2013, Tambutté et al. 2015). Furthermore, it is possible to estimate the amount of emitted proton by

774 image processing of pH sensitive ratiometric microscopy. Ratiometric fluorescent dyes enable the  
775 monitoring of intracellular and extracellular pH (Chan et al. 2015a, Comeau et al. 2017b, Toyofuku et  
776 al. 2017). Several pH sensitive dyes are available depending on the tested pH range. When the cell  
777 permeable dye SNARF-1 acetoxymethyl ester is excited at a wavelength of 543 nm, the ratio of  
778 fluorescence captured at emission wavelengths of  $585 \pm 10$  nm and  $640 \pm 10$  nm shows a linear  
779 relationship to intracellular pH (Venn et al. 2013). Similarly, cell impermeable SNARF-1 can be used  
780 to measure pH in the calcifying fluid in corals; 2'-7'-bis(carboxyethyl)-5(6)-carboxyfluorescein  
781 (BCECF) for intracellular pH of echinoderm larvae (Stumpp et al. 2012) and pyranine for foraminifera  
782 (Toyofuku et al. 2017). This technique has been applied to OA to investigate how growth and  
783 calcification rates are impacted by increasing  $p\text{CO}_2$  (Stumpp et al. 2012), where extracellular pH was  
784 actively compensated.

785 More recently, measurement of intracellular pH employs the use of microelectrodes between 10 and 15  
786  $\mu\text{m}$  tip diameter for direct in-tissue measurement (Cai et al. 2016), using pH polymeric membrane  
787 microelectrodes (Zhao and Cai 1999), and  $\text{CO}_3^{2-}$  electrodes (Cai et al. 2016). Using this approach, pH  
788 and  $\text{CO}_3^{2-}$  were observed to sharply increase in the calcifying fluid of various coral species, confirming  
789 the presence of  $\text{H}^+$  pump (Cai et al. 2016). In addition to intracellular pH determination, microelectrodes  
790 can be designed to monitor dissolved oxygen and calcium concentration to enable analysis of a wider  
791 range of parameters (Glas et al. 2012a, Glas et al. 2012b). The cellular pH imaging techniques have an  
792 advantage of high spatial resolution for direct in-tissue measurement, however, the disadvantages  
793 include time consuming sample preparation. This includes the challenge that organism immobilization  
794 strategies varies and the protocol requires optimization to ensure the organism is capable of generating  
795 biominerals. Ratiometric pH probe also requires calibration before the ratios can be converted to pH  
796 values.

#### 797 *Physiological inhibitors and stimulators*

798 The use of physiological inhibitors or stimulators is a useful approach to investigate the biochemical  
799 pathways and pumps involved with biomineralisation (Basse et al. 2015). How biological pathways  
800 may be influenced by specific inhibitors can explain the mechanism of shell formation under OA

801 conditions. For example, treatment with adenylyl cyclase inhibitors alleviate the negative effects of OA  
802 in Pacific oysters, suggesting the potential mechanism change under OA (Wang et al. 2017). This result  
803 confirmed the role of adenosine triphosphate (ATP) generation is essential to support shell production  
804 (Pan et al. 2015). Not requiring complete genetic information is greatest advantage of using inhibitors  
805 and stimulators to evaluate the mechanisms of biomineralization in a reductionist approach (Toyofuku  
806 et al. 2017). However, the choice of inhibitors may be non-specific to a single pathway and its action  
807 requies verification by a known physiological end-point.

### 808 **Combining techniques**

809 The described techniques can be employed individually to answer specific scientific questions to  
810 determine the impact of OA on marine biomineralisation. However, it is important to consider  
811 combining techniques to address complex scientific questions.

#### 812 *Combining x-ray microscopy techniques*

813 Due to the development of optimum sample preparation for the analysis of a flat surface (Perez-Huerta  
814 and Cusack 2009), the output of XPEEM with XAS (Fitzer et al. 2016) and SEM-EBSD (Hahn et al.  
815 2012, Wolfe et al. 2013, Fitzer et al. 2014a, Fitzer et al. 2014b) can be readily compared. This has  
816 allowed the influence of OA on both the biomineral structure and composition to be determined for  
817 corals (Rodolfo-Metalpa et al. 2011), sea urchins (Bray et al. 2014), and mussels (Melzner et al. 2011).  
818 Likewise, the simple mapping of calcite and aragonite is applicable across a wide variety of species  
819 including mussels and limpets (Hahn et al. 2012, Langer et al. 2014, Fitzer et al. 2015a). In contrast,  
820 comparison of mineral composition between high-resolution microscopy with spectral techniques such  
821 as XRD, FT-IR, and XPEEM with XAS is more complex. The use of SEM imaging, and calcite and  
822 aragonite thickness by EBSD, or species-specific visual inspection using compound microscopy (Fitzer  
823 et al. 2014b), have their merits to examine larger areas of shell erosion. However, to examine the  
824 intricate details of biomineralisation and potential changes under OA conditions these methods should  
825 be used in conjunction with XRD, FT-IR, XPEEM with XAS, and SEM-EBSD.

#### 826 *Multi-omics data integration*

827 Several -omics approaches can be integrated, i.e. multi-omics (Huang et al. 2017). For example, the  
828 mantle transcriptome and shell proteomes were integrated to study the shell formation of the pearl oyster  
829 (Joubert et al. 2010, Berland et al. 2011), enabling proteomics data to be analysed without a complete  
830 genome. Similarly, proteomics and metabolomics were studied together in oysters (Wei et al. 2015). A  
831 more complex multi-omics study examined the genome, transcriptome and proteome in the Pacific  
832 oyster (Zhang et al. 2012). Such a multi-omics approach also provided insights on the phosphate  
833 biomineralisation in brachiopods (Luo et al. 2015). This is a promising approach in the context of OA  
834 to understand the fitness or survival of organisms.

### 835 **Conclusions: What now for OA research on biomineralisation?**

836 A range of tools are shown in this article to help researchers to determine the impact of OA on  
837 biomineralisation mechanisms at physiological and molecular levels, and thus on shell or skeleton  
838 structural mechanics. The purpose of this review is to discuss commonly available biomineralisation  
839 tools for understanding this one physiological response to ocean acidification. However, organisms  
840 have a complex physiological profiles, and it is important to note that biomineralisation is not an isolated  
841 process, and not is it the only physiological process that needs to be considered in this context. Given  
842 the nature of biomineralisation mechanisms and their complex responses to OA, a variety of  
843 physiological, materials science, and crystallography tools are needed to thoroughly understand the  
844 biomineralisation process and its vulnerability to OA. This review outlines techniques that can be used  
845 to characterise, quantify and monitor the process of biomineralisation in a variety of calcifying marine  
846 organisms, especially when they are cultured under OA experimental conditions. It also highlights basic  
847 principles and the advantages and disadvantages of established, emerging and future techniques for OA  
848 researchers. The key to developing a strategy aimed at better understanding the potential consequences  
849 of OA is to define clear questions and hypotheses for testing. This would naturally lead to constrains  
850 (e.g. tested species, quantity of material available, size) that, when combined with practicalities (e.g.  
851 budget, equipment), will lead to selection of the appropriate experimental approach.

852 Recently, more attention has been paid to the underlying biological and physiological mechanisms of  
853 biomineralisation. For example, tissues and external organic layers can protect the shell from corrosion

854 in under-saturated waters (Rodolfo-Metalpa et al. 2011). To address the underlying mechanisms  
855 affected by the impacts by OA, various methods need to be combined. For instance, determination of  
856 amorphous calcium carbonate is important to characterise mineral choice and relate phase transitions at  
857 the earliest stage of biomineral formation. Mechanisms of cellular involvement and specific  
858 biomolecules for biomineralisation can be examined using fluorescent microscopy and omics. The  
859 interactions of proteins in the extrapallial fluid and shell interface can be understood by applying  
860 techniques in isotope labelling and microscopy. It is also crucial to consider the fitness consequences  
861 of observed changes. For example, in a prey species, shell strength should be considered in relation to  
862 predator behaviour. The combination of established, emerging and future techniques will enable a  
863 holistic approach and better understanding of the potential impact of OA on biomineralisation by marine  
864 species and consequences for marine calcifiers and associated ecosystems.

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1455 **Table 1.** Summary of established techniques on Growth and Development to measure calcification and morphology under OA conditions with details of  
1456 measurements, advantages and disadvantages of each application.

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1458 **Table 2.** Summary of emerging techniques on Mechanical tests to investigate mechanical properties under OA conditions with details of measurements,  
1459 advantages and disadvantages of each application.

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1461 **Table 3.** Summary of emerging techniques on Mineral composition to investigate mechanical properties under OA conditions with details of measurements,  
1462 advantages and disadvantages of each application.

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1464 **Table 4.** Summary of emerging techniques on Cellular biomineralization mechanisms to investigate mechanical properties under OA conditions with details  
1465 of measurements, advantages and disadvantages of each application.

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<i>1. Growth and development</i>			
<b>Technique</b>	<b>Measurement(s)</b>	<b>Advantages</b>	<b>Disadvantages</b>
<i>Dyes - alizarin red and calcein</i>	<ul style="list-style-type: none"> <li>• Alizarin red stains calcium rich structures a red/light purple colour</li> <li>• Calcein stains calcium rich structures a fluorescent green colour</li> </ul>	<ul style="list-style-type: none"> <li>• Low cost, non-invasive</li> <li>• Track shell growth of living organisms during exposure to experiment</li> <li>• Data comparable to published work</li> <li>• Dye location can be analysed with advanced characterisation methods</li> </ul>	<ul style="list-style-type: none"> <li>• Alizarin red also binds free Ca</li> <li>• Calcein also binds Ca<sup>2+</sup>, Mg<sup>2+</sup>, Zn<sup>2+</sup></li> <li>• Is not compatible with other fluorescence techniques such as internal pH measurement</li> <li>• No mineral phase information</li> </ul>
<i>SEM</i>	<ul style="list-style-type: none"> <li>• High resolution characterisation</li> <li>• Ultrastructures of minerals</li> </ul>	<ul style="list-style-type: none"> <li>• Low cost</li> <li>• Data are comparable</li> <li>• Provide structural information</li> </ul>	<ul style="list-style-type: none"> <li>• No mineral phase information</li> </ul>
<i>Buoyant weight</i>	<ul style="list-style-type: none"> <li>• Mineral content determined from submerged weight of organism</li> </ul>	<ul style="list-style-type: none"> <li>• Low cost</li> <li>• Non-invasive</li> <li>• More accurate than length or area measurements</li> <li>• Mineral density changes are reflected in measurement</li> </ul>	<ul style="list-style-type: none"> <li>• Seawater density varies when temperature and salinity change</li> <li>• Purpose-made setup needed</li> </ul>
<i>Radioactive isotopes</i>	<ul style="list-style-type: none"> <li>• <sup>45</sup>Ca incorporation rate</li> <li>• <sup>14</sup>C incorporation</li> <li>• Represents mineralisation process during an experiment</li> </ul>	<ul style="list-style-type: none"> <li>• More accurate than length or area measurements</li> <li>• Synthetic isotopes are specific to the study</li> </ul>	<ul style="list-style-type: none"> <li>• Invasive acid digestion of mineral samples are needed for scintillation measurement</li> <li>• Requires handling of radioactive substances</li> </ul>
<i>Total alkalinity anomaly technique</i>	<ul style="list-style-type: none"> <li>• Alkalinity reduction surrounding an organism</li> </ul>	<ul style="list-style-type: none"> <li>• Low cost</li> <li>• Accurate</li> </ul>	<ul style="list-style-type: none"> <li>• Incubation in individual organism required</li> </ul>

			<ul style="list-style-type: none"> <li>• <i>Not suitable for long-term studies</i></li> </ul>
<b>2. Mechanical tests - Protective function or ability to survive protection</b>			
<b>Technique</b>	<b>Measurement(s)</b>	<b>Advantages</b>	<b>Disadvantages</b>
<i>Three-point bending tests</i>	<ul style="list-style-type: none"> <li>• <i>Elastic modulus</i></li> <li>• <i>Fracture toughness</i></li> </ul>	<ul style="list-style-type: none"> <li>• <i>Mimic predatory attack</i></li> <li>• <i>Simple operation</i></li> <li>• <i>Low cost</i></li> </ul>	<ul style="list-style-type: none"> <li>• <i>Requires a tailor-made device</i></li> <li>• <i>Test samples are cut into a standard size for testing</i></li> </ul>
<i>Computed tomography</i>	<ul style="list-style-type: none"> <li>• <i>Shell thickness</i></li> <li>• <i>Shell volume</i></li> <li>• <i>Shell density</i></li> </ul>	<ul style="list-style-type: none"> <li>• <i>3D visualisation of shell shape for morphometric analysis</i></li> </ul>	<ul style="list-style-type: none"> <li>• <i>Hard to detect planktonic and larval samples (15 - 1000 <math>\mu\text{m}</math> per pixel)</i></li> <li>• <i>Standard density calibrated with bone mineral density (BMD, in <math>\text{g.cm}^{-3}</math>)</i></li> </ul>
<i>Finite element analysis</i>	<ul style="list-style-type: none"> <li>• <i>Visualize structural weakness of a material</i></li> <li>• <i>Provide a numerical model for material properties</i></li> </ul>	<ul style="list-style-type: none"> <li>• <i>Links nanoindentation data to whole sample measurements</i></li> <li>• <i>Takes shell shape changes into account</i></li> <li>• <i>Data can be verified by mechanical tests</i></li> </ul>	<ul style="list-style-type: none"> <li>• <i>Requires computational skills</i></li> <li>• <i>Shape information requires simplified experimental data</i></li> <li>• <i>FEA Models need experimental verification</i></li> </ul>
<i>Microindentation</i>	<ul style="list-style-type: none"> <li>• <i>Compressive force using 4- Vickers tip</i></li> <li>• <i>Hardness</i></li> <li>• <i>Elasticity Modulus</i></li> </ul>	<ul style="list-style-type: none"> <li>• <i>Broader Vickers tip is less localised than nanoindentation</i></li> <li>• <i>Lower cost than nanoindentation</i></li> <li>• <i>Provides microscale spatial resolution</i></li> </ul>	<ul style="list-style-type: none"> <li>• <i>Localised measurement</i></li> <li>• <i>Does not represent shape and mechanical behaviour of the whole structure</i></li> <li>• <i>Destructive to the sampling area of the specimen</i></li> </ul>
<i>Nanoindentation</i>	<ul style="list-style-type: none"> <li>• <i>Compressive force using Berkovich tip</i></li> <li>• <i>Hardness</i></li> <li>• <i>Elasticity Modulus</i></li> </ul>	<ul style="list-style-type: none"> <li>• <i>Sharper Berkovich tip enables higher spatial refinement of measurements</i></li> <li>• <i>Provides both hardness and elasticity data in one measurement</i></li> </ul>	<ul style="list-style-type: none"> <li>• <i>Localised measurement</i></li> <li>• <i>Does not represent shape and mechanical behaviour of the whole structure</i></li> <li>• <i>Destructive to the sampling area of the specimen</i></li> </ul>

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<i>3. Biomineralisation mechanisms to enable growth</i>			
<i>Elemental analysis</i>			
<b>Technique</b>	<b>Measurement(s)</b>	<b>Advantages</b>	<b>Disadvantages</b>
<i>Inductively coupled plasma (ICP) spectrometry</i>	<ul style="list-style-type: none"> <li>• <i>Element to calcium ratios, e.g. Mg/Ca, Sr/Ca</i></li> <li>• <i>Analyse acid digested samples</i></li> </ul>	<ul style="list-style-type: none"> <li>• <i>Quantitative</i></li> <li>• <i>Data are comparable</i></li> <li>• <i>Coupling with OES, MS or AES provides different sensitivity at various costs</i></li> </ul>	<ul style="list-style-type: none"> <li>• <i>Destructive sample preparation</i></li> <li>• <i>Requires elemental standards</i></li> <li>• <i>More sensitive instruments are more costly</i></li> </ul>
<i>laser ablation (LA)</i>	<ul style="list-style-type: none"> <li>• <i>Element to calcium ratios, e.g. Mg/Ca, Sr/Ca</i></li> <li>• <i>Analyse solid samples</i></li> </ul>	<ul style="list-style-type: none"> <li>• <i>Spatial resolution</i></li> <li>• <i>Less destructive than ICP approach</i></li> <li>• <i>Data are comparable</i></li> </ul>	<ul style="list-style-type: none"> <li>• <i>Spatial resolution of &gt; 5 μm, less than SIMS</i></li> <li>• <i>Less sensitive than ICP approach</i></li> </ul>
<i>Analytical electron microscopy (AEM) with energy dispersive X-ray spectrometry (EDS)</i>	<ul style="list-style-type: none"> <li>• <i>Element to calcium ratios, e.g. Mg/Ca, Sr/Ca</i></li> <li>• <i>Microanalysis provides a compositional map with spatial resolution</i></li> </ul>	<ul style="list-style-type: none"> <li>• <i>Low cost and accessible</i></li> <li>• <i>Spatial resolution</i></li> <li>• <i>Non-destructive to specimen surface, therefore can be followed by EBSD, LA-ICP-MS or nanoindentation</i></li> </ul>	<ul style="list-style-type: none"> <li>• <i>Requires elemental standards</i></li> <li>• <i>Detection level at 1000 ppm</i></li> </ul>
<i>AEM with wavelength-dispersive X-ray spectroscopy (WDS)</i>		<ul style="list-style-type: none"> <li>• <i>Moderate cost</i></li> <li>• <i>Nanometer-scale resolution</i></li> </ul>	<ul style="list-style-type: none"> <li>• <i>More costly than AEM-EDS</i></li> </ul>
<i>Structured illumination microscopy (SIMS)</i>		<ul style="list-style-type: none"> <li>• <i>Spatial resolution of SEM</i></li> <li>• <i>Detection sensitivity of 1ppm</i></li> </ul>	<ul style="list-style-type: none"> <li>• <i>High cost</i></li> <li>• <i>Destructive to the sampling area of the specimen</i></li> </ul>

Electron energy loss spectrometry (EELS)		<ul style="list-style-type: none"> <li>• 10 ppm detection limit</li> <li>• High resolution compositional map</li> </ul>	<ul style="list-style-type: none"> <li>• High cost</li> <li>• Require 10 nm thick samples</li> <li>• Small region of interest</li> </ul>
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3. Biomineralisation mechanisms to enable growth (cont.)			
Mineral composition analyses			
Technique	Measurement(s)	Advantages	Disadvantages
Fourier transform infrared spectroscopy (FTIR)	<ul style="list-style-type: none"> <li>• Intensity ratio (<math>I_{\max\nu_2}/I_{\max\nu_4}</math>) between the absorption bands</li> <li>• Identifies the presence of aragonite or calcite</li> </ul>	<ul style="list-style-type: none"> <li>• Low cost</li> <li>• Provides a comparable measurement of ACC</li> <li>• Requires ~1 mg of mineral sample</li> </ul>	<ul style="list-style-type: none"> <li>• Semi-quantitative</li> <li>• No spatial resolution</li> <li>• Destructive</li> </ul>
X-ray diffraction (XRD)	<ul style="list-style-type: none"> <li>• Identifies the presence of aragonite or calcite</li> </ul>	<ul style="list-style-type: none"> <li>• Allows for quantification of calcite and aragonite ratios</li> <li>• Peak position suggests Mg content</li> <li>• Powdered sample can be acid-digested to provide elemental data</li> </ul>	<ul style="list-style-type: none"> <li>• Requires more powdered samples than FTIR</li> <li>• No spatial resolution</li> <li>• Destructive</li> <li>• Quantitative measurement requires the addition of <math>\text{CaF}_2</math> as an internal standard</li> </ul>
Raman spectroscopy	<ul style="list-style-type: none"> <li>• Identifies the distribution of aragonite or calcite</li> </ul>	<ul style="list-style-type: none"> <li>• Spatial resolution</li> <li>• Area of calcite and aragonite is quantifiable</li> <li>• Specimen surface can be analysed by SEM and AEM methods for ultrastructure and elemental contents</li> </ul>	<ul style="list-style-type: none"> <li>• Requires sectional surfaces</li> <li>• Bleaching is necessary to remove organic contaminants</li> <li>• Limited spatial resolution</li> </ul>
SEM-EBSD	<ul style="list-style-type: none"> <li>• Identifies the distribution of calcite and aragonite</li> </ul>	<ul style="list-style-type: none"> <li>• Allows visual comparison of mineral crystals</li> </ul>	<ul style="list-style-type: none"> <li>• Requires fine polishing</li> </ul>

	<ul style="list-style-type: none"> <li>• High resolution crystallographic orientation data</li> </ul>	<ul style="list-style-type: none"> <li>• Provides quantifiable data on thickness of polymorphs</li> <li>• Spatial resolution</li> </ul>	<ul style="list-style-type: none"> <li>• Sectional axis and plane of observation must be standardised</li> <li>• Moderate cost</li> </ul>
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<i>3. Biomineralisation mechanisms to enable growth (cont.)</i>			
<i>Mineral composition analyses (cont.)</i>			
<b>Technique</b>	<b>Measurement(s)</b>	<b>Advantages</b>	<b>Disadvantages</b>
<i>X-ray photo emission electron microscopy (XPEEM)</i>	<ul style="list-style-type: none"> <li>• High-spatial resolution composition map</li> <li>• Localises and characterises ACC</li> <li>• Identify mineral phases</li> </ul>	<ul style="list-style-type: none"> <li>• High spatial resolution</li> <li>• Sample preparation enables SEM observation and SEM-EBSD characterisation</li> </ul>	<ul style="list-style-type: none"> <li>• High cost</li> <li>• Requires fine polishing</li> <li>• Time-consuming</li> </ul>
<i>X-ray atomic spectroscopy (XAS)</i>			
<i>X-ray absorption near edge structure (XANES)</i>			
<i>Atomic force microscopy (AFM)</i>	<ul style="list-style-type: none"> <li>• Records force-distance curve</li> <li>• Visualises fine topographical features</li> </ul>	<ul style="list-style-type: none"> <li>• Nanometre resolution</li> <li>• Simple sample preparation</li> <li>• Possible to measure hydrated samples in electrolyte solution</li> </ul>	<ul style="list-style-type: none"> <li>• High cost</li> <li>• Small area of interest</li> <li>• Time-consuming</li> </ul>
<i>Focused ion beam Transmission electron microscopy FIB-TEM</i>	<ul style="list-style-type: none"> <li>• Fine spatial resolution</li> <li>• FIB prepares TEM sections</li> </ul>	<ul style="list-style-type: none"> <li>• Selective region of interest</li> <li>• High spatial resolution</li> <li>• Suitable for small samples</li> </ul>	<ul style="list-style-type: none"> <li>• High cost</li> <li>• Time-consuming</li> <li>• Small area of interest</li> </ul>

<i>Cryo-electron microscopy</i>	<ul style="list-style-type: none"> <li>• High resolution study of biological sample after rapid freezing</li> </ul>	<ul style="list-style-type: none"> <li>• High resolution</li> <li>• Provides information on composition and crystallography</li> </ul>	<ul style="list-style-type: none"> <li>• High cost</li> <li>• Requires stabilising detergents for structure</li> </ul>
<i>Stable isotopes</i>	<ul style="list-style-type: none"> <li>• Detection of stable isotopes, e.g. <math>\delta^{13}C</math>, <math>\delta^{18}O</math>, <math>\delta^{10}B</math> and <math>\delta^{11}B</math></li> </ul>	<ul style="list-style-type: none"> <li>• Data are comparable</li> <li>• Measures changes in metabolic activity in biomineralisation</li> </ul>	<ul style="list-style-type: none"> <li>• Destructive</li> <li>• Some proxies are not well established</li> </ul>
<i>Radioactive isotopes</i>	<ul style="list-style-type: none"> <li>• Calcification rate</li> <li>• Labelling with radioactive isotopes, e.g. <math>^{45}Ca</math> and <math>^{14}C</math></li> </ul>	<ul style="list-style-type: none"> <li>• Sensitive technique</li> <li>• Specific to experimental exposure</li> </ul>	<ul style="list-style-type: none"> <li>• Continual spiking of radiotracer required during incubation</li> <li>• Destructive</li> </ul>

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<b>4. Cellular biomineralization mechanisms</b>			
<b>Technique</b>	<b>Measurement(s)</b>	<b>Advantages</b>	<b>Disadvantages</b>
<i>SDS-PAGE and MS</i>	<ul style="list-style-type: none"> <li>• Characterises shell proteins</li> </ul>	<ul style="list-style-type: none"> <li>• Established protocol for protein identification</li> <li>• Low cost and high throughput of samples</li> </ul>	<ul style="list-style-type: none"> <li>• Sensitivity insufficient to detect shell proteins occurring at low quantity</li> <li>• Protein extraction and purification is time consuming</li> <li>• Difficult to interpret sequences from non-model marine organisms</li> </ul>
<i>Insoluble organic component analysis using X-ray diffraction (XRD)</i>	<ul style="list-style-type: none"> <li>• Characteristic spectroscopy correlated to chitin</li> </ul>	<ul style="list-style-type: none"> <li>• Provides evidence on the presence of chitin</li> <li>• Analyses insoluble solid samples</li> </ul>	<ul style="list-style-type: none"> <li>• Destructive</li> <li>• Sufficient sample is hard to obtain</li> <li>• Information has poor relevance to the cells and tissue associate with chitin</li> </ul>
<i>Insoluble organic component analysis using infra-red (IR) spectroscopy</i>			

<i>Pyrolysis NMR gas chromatography–mass spectrometry (GC-MS)</i>	<ul style="list-style-type: none"> <li>• <i>Detects decomposition product of chitin after chitinase action</i></li> </ul>	<ul style="list-style-type: none"> <li>• <i>Analyses the digested products</i></li> <li>• <i>Digested product peaks can be quantified and compared</i></li> </ul>	<ul style="list-style-type: none"> <li>• <i>Requires chemical standards of digested products</i></li> <li>• <i>Protocol optimisation can be time-consuming</i></li> </ul>
<i>Colorimetric Assay for acid hydrolysate of chitin</i>	<ul style="list-style-type: none"> <li>• <i>Detects decomposition product of chitin after acid hydrolysis</i></li> </ul>	<ul style="list-style-type: none"> <li>• <i>Low cost</i></li> <li>• <i>Quantitative</i></li> <li>• <i>Simple protocol</i></li> </ul>	<ul style="list-style-type: none"> <li>• <i>Destructive</i></li> <li>• <i>Cannot distinguish forms of chitin</i></li> <li>• <i>Spatial information is lost</i></li> </ul>
<i>Calcofluor-white</i>	<ul style="list-style-type: none"> <li>• <i>Visualises chitin in relation to cells and tissues</i></li> </ul>	<ul style="list-style-type: none"> <li>• <i>Non-destructive to tissues and cells</i></li> <li>• <i>Low cost</i></li> <li>• <i>Commercially available</i></li> </ul>	<ul style="list-style-type: none"> <li>• <i>Also binds to cellulose</i></li> </ul>
<i>Wheat-germ agglutinin (WGA)</i>			<ul style="list-style-type: none"> <li>• <i>Also binds to N-acetyl-D-glucosamine and sialic acid</i></li> </ul>
<i>Chitin-binding domain fused with green fluorescent protein (CBD-GFP)</i>			<ul style="list-style-type: none"> <li>• <i>Requires a time-consuming bioengineering approach to express and purify CBD-GFP</i></li> </ul>

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<i>4. Cellular biomineralization mechanisms (cont.)</i>			
<b>Technique</b>	<b>Measurement(s)</b>	<b>Advantages</b>	<b>Disadvantages</b>
<i>OMICS – transcriptomics</i>	<ul style="list-style-type: none"> <li>• <i>mRNA of expressed genes</i></li> </ul>	<ul style="list-style-type: none"> <li>• <i>Assesses the molecular pathways of the organism from expressed genes or proteins</i></li> <li>• <i>Provides a whole physiological picture</i></li> </ul>	<ul style="list-style-type: none"> <li>• <i>mRNA presence may not represent protein activities</i></li> <li>• <i>Tissue-specific response</i></li> <li>• <i>Costly</i></li> <li>• <i>Identification of transcripts in a non-model marine species is difficult</i></li> </ul>
<i>OMICS – proteomics</i>	<ul style="list-style-type: none"> <li>• <i>Total protein profile</i></li> </ul>		<ul style="list-style-type: none"> <li>• <i>Shell proteins occurring at low quantity of 5%</i></li> <li>• <i>High-sensitivity method like iTRAQ is very costly</i></li> </ul>

			<ul style="list-style-type: none"> <li>• <i>Identification of protein in a non-model marine species is difficult</i></li> </ul>
<i>Cellular pH imaging</i>	<ul style="list-style-type: none"> <li>• <i>Intracellular pH indicated by a pH sensitive ratiometric probe</i></li> <li>• <i>Visualises the sites of biomineralisation</i></li> </ul>	<ul style="list-style-type: none"> <li>• <i>Examines living organisms</i></li> <li>• <i>High spatial resolution</i></li> </ul>	<ul style="list-style-type: none"> <li>• <i>Not well established and optimisation</i></li> <li>• <i>Ratiometric pH probes require calibration</i></li> </ul>
<i>Physiological inhibitors and stimulators</i>	<ul style="list-style-type: none"> <li>• <i>Enables examination of the physiological response of a non-model organism</i></li> <li>• <i>The importance of a biological pathway is evaluated by a specific inhibitor</i></li> </ul>	<ul style="list-style-type: none"> <li>• <i>Applies to non-model organisms</i></li> </ul>	<ul style="list-style-type: none"> <li>• <i>Some inhibitors are not specific to a single pathway</i></li> <li>• <i>Action of inhibitors should be verified by a known physiological measurement</i></li> </ul>

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