Description and quantification of pteropod shell dissolution: 
A sensitive bioindicator of ocean acidification

Nina Bednaršek1,2,3, *, Geraint A Tarling1, Dorothee CE Bakker2, Sophie Fielding1, Anne Cohen4, Alan Kuzirian5, Dan McCorkle4, Bertrand Lézé2, Roberto Montagna2

1 British Antarctic Survey, High Cross, Madingley Rd, Cambridge, CB3 0ET, UK
2 School of Environmental Sciences, University of East Anglia Research Park, Norwich, NR4 7TJ UK
3 University of Nova Gorica, Laboratory for Environmental Research, Vipavska 13, Rožna Dolina, 5000 Nova Gorica, Slovenia
4 Woods Hole Oceanographic Institution, Department of Geology and Geophysics, 266 Woods Hole Rd, Woods Hole, MA 02543
5 Marine Biological Laboratory, 7 MBL Street, Woods Hole, Woods Hole, MA 02543

Please note that this is a copy of the author’s accepted manuscript of the paper. Minor changes may have been made to the text and certain details of the figures during the proof stage.

This document is stored on the NERC Open Research Archive at http://nora.nerc.ac.uk/500554/ and a copy of the final published article can be found at http://dx.doi.org/10.1111/j.1365-2486.2012.02668.x
Description and quantification of pteropod shell dissolution:  
A sensitive bioindicator of ocean acidification

Nina Bednaršek¹,²,³, *, Geraint A Tarling¹, Dorothee CE Bakker², Sophie Fielding¹, Anne Cohen⁴, Alan Kuzirian⁵, Dan McCorkle⁴, Bertrand Lézé², Roberto Montagna²

¹ British Antarctic Survey, High Cross, Madingley Rd, Cambridge, CB3 0ET, UK
² School of Environmental Sciences, University of East Anglia Research Park, Norwich, NR4 7TJ UK
³ University of Nova Gorica, Laboratory for Environmental Research, Vipavska 13, Rožna Dolina, 5000 Nova Gorica, Slovenia
⁴ Woods Hole Oceanographic Institution, Department of Geology and Geophysics, 266 Woods Hole Rd, Woods Hole, MA 02543
⁵ Marine Biological Laboratory, 7 MBL Street, Woods Hole, Woods Hole, MA 02543

*Corresponding author: nina.bednarsek@ung.si, Tel: +386 (0)53315204, Fax: +386 (0)53315296

Keywords: pteropods; ocean acidification; aragonite shell; shell dissolution; dissolution quantification; bioindicator.

Abstract

Anthropogenic ocean acidification is likely to have negative effects on marine calcifying organisms, such as shelled pteropods, by promoting dissolution of aragonite shells. Study of shell dissolution requires an accurate and sensitive method for assessing shell damage. Shell dissolution was induced through incubations in CO₂ enriched seawater for between 4 and 14 days. We describe a procedure that allows the level of dissolution to be assessed and classified into three main types: Type I with partial dissolution of the prismatic layer; Type II with exposure of underlying crossed-lamellar layer, and Type III, where crossed-lamellar layer shows signs of dissolution. Levels of dissolution showed a good correspondence to the incubation conditions, with the most severe damage found in specimens held for 14 d in undersaturated condition (Ω ~ 0.8). This methodology enables the response of small pelagic calcifiers to acidified conditions to be detected at an early stage, thus making pteropods a valuable bioindicator of future ocean acidification.
**Introduction**

Modelling the ocean-carbon cycle under the 'business-as-usual' IS92a scenario, aragonite undersaturation is predicted for the Southern Ocean surface waters by 2100 as a result of anthropogenic ocean acidification (Caldeira and Wickett 2003; Orr et al. 2005) and possibly sooner in wintertime (McNeil and Matear 2008). Pteropods are likely to be the first indicators of where ocean acidification is starting to impact high-latitude pelagic marine communities since they are one of the few pelagic organisms to make their shells entirely out aragonite, a relatively soluble form of calcium carbonate (Orr et al. 2005; Fabry et al. 2008).

Ocean acidification, and the modifications it induces to carbonate chemistry, will impact the shells of pelagic calcifiers in two ways. Firstly, it will alter the ability of the organism to calcify and secondly, it will dissolve the calcified shell. In terms of calcification, Comeau et al. (2010) found that a decrease in saturation of aragonite in seawater ($\Omega$) decreased the level of calcification in pteropod shells (*Limacina helicina*) significantly, although some calcification still occurred at relatively low levels of saturation. From these results, Comeau et al. (2011) predicted that *Limacina helicina* will become unable to precipitate CaCO$_3$ over much of the Arctic by the end of the century under the IPCC SRES A2 scenario. Also, such predictions do not take into account the effect of aragonite undersaturation on shell dissolution, which may act to accelerate the vulnerability of pelagic calcifiers. For instance, in a pilot study, Orr et al. (2005) noted significant corrosion to the shells of the pteropod *Clio pyramidata* incubated in water undersaturated for aragonite for 48 h.

Shell dissolution has been used as a useful indicator of aragonite undersaturation horizon ($\Omega<1$) in sedimentary studies. For example, Ruddiman & Heezen (1967) determined the percentage of the non-carbonate versus carbonate material to assess dissolution. Almogi-Labin et al. (1986) examined changes on the surface of pteropods shells using light microscopy and categorised...
them into stages, from transparent, indicating an excellent state of preservation, to
opaque/white, indicating corrosion. Opacity under light microscopes was also adopted as an
index of aragonite dissolution by Haddad & Droxler (1996), Gerhardt et al. (2000), Gerhardt &
Henrich (2001), and Manno et al. (2006).

Each of the above methods was developed to assess effects on mainly dead organisms below
the saturation horizon where levels of dissolution are substantial. However, the effects of
modern-day ocean acidification are likely to be small and sub-lethal, probably involving minute
changes to the shell-surface. In such instances, light microscopy may not show sufficient
details to detect such changes and there is a need for more precise and sensitive assessment
methods. Scanning electron microscopy (SEM) can provide the extra resolution required to
carry out such assessments. Nevertheless, the vacuum environment of SEM may otherwise
introduce artefacts unless appropriate preparation techniques are used. A particular hazard in
studying shell microstructure with SEM is the fragility of the thin pteropod shell walls (Bé et
al. 1972). In the *Limacinadae* family, this microstructure consists of underlying thick cross-
lamellar layers and an upper thin prismatic layer that is covered with an organic layer
(periostracum; Fig. 1). It is particularly thin in juveniles and prone to mechanical damage that
may contribute to errors in interpreting damage induced by dissolution. Furthermore, the
periostracum must be removed before SEM analysis, as remnants of this layer prevent an
accurate examination of the shell surface, or be misinterpreted as shell damage.

This article describes a method for preparing pteropod shells and analysing dissolution on them
that is capable of fine resolution and sensitive to the fragility of the specimens. We show that
the technique can reveal the processes involved in the dissolution of pteropod shells under
acidified conditions. We further develop a standardized metric scheme with which to categorise
different levels of dissolution damage. This scheme establishes a benchmark against which the
dissolution status of different pteropod species and populations can be compared. Such
information is essential for an informed debate on the threat to shelled pteropods by ocean
Materials and Methods

Pteropod specimens were collected with slowly-hauled fine-meshed nets from various locations within the Scotia Sea, Southern Ocean (see Supplementary Information). Micro- and mesozooplankton sized specimens (mainly juveniles) were collected by vertically integrating the upper 200 m using a vertical Bongo (mesh size 200 µm with an opening of 0.5 m²) and a towed Bongo net (2 nets, with 300 µm and 600 µm mesh sizes). Macrozooplankton sized specimens (adults) were caught in RMT8 and RMT25 trawls (mesh size 4.5 mm). All the organisms used in incubations were alive at the start of the experiments, except for the natural control specimens that were preserved in 70% ethanol immediately. The majority of incubations were carried out on juvenile specimens for between 4 and 14 d at various Ω saturation levels. Mortality at the end of the experiments was low (<10%) and dead specimens were not considered further for SEM analysis. As pteropods are prone to mechanical damage, a key factor when designing the incubation apparatus was to minimise physical interference. For this reason, closed blacked-out systems (2 L sealed borosilicate bottles) were used in which the water was enriched with CO₂ prior to incubation. Enrichment was achieved through the bubbling of different air/CO₂ mixtures (500 ppm, 750 ppm and 1200 ppm) through 0.7 µm filtered sea water until the required omega saturation level was reached. Omega was assessed from measurements of DIC (dissolved inorganic carbon) and total alkalinity (TA) at the start and end of each incubation experiment, which lasted for 4, 8 or 14 d (see Supplementary Materials). Ω values were often different between incubation start and end points (up to ±0.17, Table 1), so only broad categorisations of incubation conditions were possible for comparative purposes, those being: a) supersaturated (Ω > 1.2)  b) transitional (Ω = 0.95 to 1.2) and c) Ω undersaturated (Ω < 0.95; Table 1). The experiments with Ω supersaturated conditions lasted for 8 days while transitional and Ω undersaturated experiments lasted for 14 days. The
incubation bottles were seeded with either live juveniles or adult *Limacina helicina antarctica* or adult *Clio pyramidata forma antarctica*. and incubated for the set period, after which, they were preserved in 70% ethanol. Specimens were also extracted directly from the nets and preserved as above, so as to provide a control for incubation effects.

*Shell preparation for SEM analysis* (Fig. 2): Shell preparation was performed in five major stages: a) removal of abiogenic crystals from the shell surface, b) dehydration, c) mounting on the SEM stub, d) removal of the organic layer and e) sputter coating and SEM analysis.

(a) *Removing abiogenic crystals from the shell surface*: At the time of sample collection from the field, pteropod samples were directly transferred from seawater to 70% ethanol. This resulted in the precipitation of various crystalline structures on the shell surface. To remove crystal deposits from the shell, the samples were transferred from 70% ethanol (in which they were kept from the time of sample collection) to 50% ethanol for 2-3 minutes. They were subsequently transferred to distilled water (3-5 minutes). Two different procedures for removing the precipitated crystals on pteropod shells were tested for their ease of use and efficiency. For the first method, hydrogen peroxide (H$_2$O$_2$) was used to dissolve the crystals. The hydrogen peroxide concentration and the exposure time were adjusted to maintain shell integrity. Samples were treated with two consecutive rinses in 6% H$_2$O$_2$ for 15-20 minutes. Alternatively, Triton-X-100 and slight sonication was used to dislodge surface material. The shells were put into 100-200 ml of 0.5% Triton-X-100 in seawater and sonicated for 5 seconds. The sonication procedure was repeated two to three times at 5 seconds each. In this study, we mainly used the first approach for abiogenic crystal removal which we recommend for future use. Finally, all samples were washed twice in distilled water for a total of five minutes.

(b) *Dehydration*: Dehydration was undertaken using 2,2-Dimethoxypropane (DMP; chemical formula: (CH$_3$)$_2$C(OCH$_3$)$_2$), and 1,1,1,3,3,3-hexamethyldisilazane (HMDS; chemical formula: (CH$_3$)$_3$SiNHSi(CH$_3$)$_3$). Before starting dehydration with DMP, the shells were transferred to 50% methanol for two 5 min washes then transferred to 85% methanol (10 minutes). Complete
tissue dehydration was accomplished by immersion in DMP: two changes at 15-20 minutes each. It was important not to let the shells air dry at this stage, so they were transferred to a 1:1 mixture of DMP and HMDS for about 10 minutes, followed by 100 % HMDS for 20-25 minutes twice. The HMDS was subsequently allowed to evaporate allowing the shells to dry completely (Fig. 2). The moderate vapour pressure and very low surface tension of HMDS allowed the shells to dry without distortion or loss of shell integrity.

(c) Mounting on the SEM stub: Fine brushes were used to mount the dry shells on aluminium SEM stubs using colloidal graphite. Extreme care was needed when manoeuvring the shell with a brush, as this could cause structural damage or even shell collapse. Shells had to be positioned in a dorso-ventral position for the oxygen plasma etching reaction (see below) to expose the maximum surface area, and for qualitative examination. To examine the changes on the growing edge, a proximo-distal position of the shell with the aperture on top was chosen.

(d) Removal of the organic shell layer using oxygen plasma etching: The samples had to be completely dry and oriented to expose the maximum surface area prior to etching. A BIO-RAD RF plasma barrel etcher PT7150 was used with a forward power of \(\approx 200\) Watts and a reflected power of \(\approx 5\) Watts. The shells were typically etched for between 10-30 minutes, depending on the power of the etcher.

Adult shells are less fragile than juvenile shells, hence less sensitive preparation procedures were sufficient. For instance, for rapid removal of abiogenic crystals, it was possible to use a stronger solution of hydrogen peroxide (30 % H$_2$O). This concentration was also reasonably efficient at removing the periostracum, although it did not perform as well as the plasma etching. Two sequential immersions in 30% H$_2$O$_2$ for 10-12 minutes each were required to remove the abiogenic crystals and most of the overlying organics. Transfer to 100 % HMDS for 25-30 minutes was sufficient for complete dehydration and drying.

Categorization scheme for pteropod shell dissolution: Analysis of the SEM photos enabled observation of the shell surface and identification of shell dissolution; notably pitting, cracks,
and areas where aragonite crystals were absent or porous. Different stages in the level of
dissolution were recognisable and so a scheme was devised that categorised these stages into
three types (Type I, II, III) which are described in detail in a later section.

*Application of scheme to incubated specimens:* The categorisation scheme above was applied in
a semi-quantitative manner using image segmentation analysis to assess the extent of each
dissolution type over the shell surface. Analyses were performed only on those pteropods that
were successfully maintained in the experimental conditions, therefore excluding mortality as a
factor of variability. Overall, 50 animals were examined; 20 animals from the natural
environment, 9 from the supersaturated incubations, 11 from the transitional incubations (Ω
~1), and 11 from the undersaturated incubations. For each individual, 15-20 SEM photographs
were generated across the shell surface area, which amounted to a total of 750 SEM
micrographs, on which image the segmentation analysis was performed. This procedure is
described in Supplementary Materials. Statistical differences in dissolution levels between
incubations were determined with a Kruskal-Wallis 1-way ANOVA on ranks followed by
Dunn’s Method to determine which pairwise differences were significantly different (p<0.05).

**Results and Discussion**

*Shell preparation method* The shell preparation method proved to be non-destructive, providing
specimens with clean and intact shell surfaces, thus limiting the occurrence of methodological
artefacts and rendering an intact crystalline layer suitable for further SEM examination. Each of
the steps is considered in further detail below:

(a) *Removal of abiogenic crystals and bacteria:* The transfer of samples from seawater to 70 %
ethanol caused rapid water removal and the precipitation of phosphate, calcium and sodium
chloride (NaCl) crystals on the shell surface. Without applying the shell preparation method,
the crystals covered much of the shell surface, and made it impossible to examine it for
evidence of dissolution (Fig. 3 shows various abiogenic crystals). Phosphate crystals occurred in the struvite (Fig. 3a), rosette (Fig. 3b) or in triple phosphate forms (Fig. 3c). Sodium chloride formed cubes (Fig. 3b). In addition, bacteria from seawater attached to the surface and in some cases, completely covered the shell (Fig. 3d). Therefore, removal of abiogenic crystals with H$_2$O$_2$ was necessary before SEM examination.

An alternative approach to minimise or avoid the precipitation of abiogenic crystals on the shell surface during sample fixing would have been to place samples in a low ethanol grade (50 %), followed by subsequent, transfer to higher concentrations (up to 70%) in gradual steps. This method allows dilution of the salts in the high concentrations of seawater and their subsequent removal. Samples should not be fixed in buffered formalin for dissolution studies, because formalin dissolves aragonite crystals.

(b) Removal of the organic layer: Although extremely thin, the outer organic layer is fully embedded within the surface crystals of the shell. This periostracum must be removed before SEM examination for two reasons: i) microstructural changes of the aragonite crystals can otherwise be obscured and ii) disintegration of organic remnants during coating for SEM can lead to misinterpretation of dissolution in the subsequent analysis or obscure signs of shell dissolution. Juvenile shells are especially fragile and prone to mechanical damage and disintegration, thus an inappropriate method could easily damage these delicate shells. A common way to etch the surface of SEM samples is acid hydrolysis that denatures surface proteins and thus, eliminates the periostracum. However, acid treatment is unsuitable for shell dissolution studies because it dissolves aragonite crystals. Therefore, oxygen plasma etching was found to be a non-destructive and efficient procedure for removing the periostracum from delicate pteropod shells.

(c) Preparation artefacts: Drying of the samples and mounting them on SEM stubs were the steps with the greatest potential to cause mechanical damage to the shells. Despite taking the utmost care, mechanical damage (e.g. cracks, pitting, broken growing edges) mostly originated
from handling while mounting the samples on SEM stubs. It was nevertheless possible to
distinguish this type of damage from the effects of dissolution when scrutinised under SEM. In
addition, in shells that were not properly dehydrated, the vacuum force during coating for SEM
resulted in the collapse of the organic matrix (Fig. 4a,b), and cracked and chipped growing
edge (Fig. 4c), and pitted surfaces (Fig. 4d). Occasional damage to shells, particularly at the
growing edge, occurred most frequently in juveniles that had been incubated in Ω
undersaturated water, where dissolution had weakened the shells. Hence it was important to dry
fully and remove the organic layer prior to coating. DMP and HMDS were used to avoid these
artefacts. DMP did not cause any deterioration or physical change to specimens (Maser &
Trimble 1977). HMDS dried the organic meshwork and stabilized the complex protein
structures of the shell without changing or destroying any structural features. This was achieved
by decreasing surface tensions during drying with HMDS; otherwise damage to fragile
structures within the shell would occur. HMDS is an inexpensive, easy to handle, and time-
saving alternative to critical point drying.

The methodology used for live juveniles was equally effective for adult *L. helicina ant*. Adults
were rare in our samples but were easier to work with than juveniles, since they are larger to
handle and their shells are more compact. This allowed certain preparation steps to be
modified. For example, H₂O₂ could be applied in higher concentrations and for longer
durations to remove the abiogenic crystal precipitation. This reduced or totally eliminated the
time required to remove the organic layer by oxygen plasma etching. Additionally, dehydration
steps could be shortened (for instance, through eliminating 85 % methanol and double
immersion in DMP), as long as the HMDS step was long enough to attain proper water
removal.

The method also worked effectively for *Clio pyramidata f. ant.*, despite differences in the shell
structure compared to *L. helicina ant*. The shell microstructure of *Clio pyramidata f. ant.* is
helical with a prismatic layer that is a few microns thick and covered by a thin organic layer
(Bé and Gilmer 1977). With the organic layer removed, the crystal structure of the prismatic layer was revealed (Fig. 4e). For samples exposed to high CO$_2$ levels, SEM observations revealed initial (Type I; Fig. 5), intermediate (Type II; Fig. 6) and advanced (Type III: Fig. 7) dissolution levels on the shell.

The process of dissolution and categorization scheme for shell dissolution. Dissolution of the aragonite crystals on the shell surface and growing edge was assessed by comparing SEM images of pteropods exposed to high CO$_2$ levels with those of controls. The progression of shell dissolution was divided into four stages based on the level of shell damage: No dissolution, and Type I, II and III (Table 2). A pteropod shell taken from ambient seawater with supersaturated conditions for omega typically had a smooth, sleek shell surface (Fig. 4e) and the shell structure was compact with very little intracrystalline porosity. Type I dissolution corresponded to a mild degree of dissolution defined by small effects on the upper prismatic layer and initial exposure of aragonite crystals. As a result of dissolution, intact heads of the aragonite crystals appeared as 'cauliflower heads' (Fig. 5a,b). There were only rare deep intrusions into the crossed-lamellar layer but the shell had become porous, less compact, and hence more fragile than an intact shell.

The next level of dissolution was categorised as Type II dissolution. It was represented by the partial disappearance of the prismatic layer. The crossed-lamellar layer had been exposed, but not affected by dissolution (Fig. 6b). Progressive roughening of the shell surface induced by dissolution substantially enhanced the rate of shell dissolution, as first suggested by Acker & Byrne (1989). Shell porosity had increased, and the shell surface had numerous, large damaged patches across the shell surface (Fig. 6a).

As the dissolution process continued, an extended degree of porosity and dissolution of the crossed-lamellar layer was evident over the shell surface (Fig. 7b). In the case of Type III dissolution, the crystals of the crossed-lamellar layer had been partly eroded and became
thicker and chunkier in appearance (Fig. 7c). Since shell dissolution was extensive with a loosely organised crystal structure, shell integrity had been lost and the shell was prone to fragmentation and increased frailness (Fig. 7a).

Dissolution at the growing edges The dissolution categorization scheme for the shell surface was also applicable to the growing edge. At the onset of dissolution, the intact crystal structure (Fig. 8a) was replaced by 'cauliflower heads' indicating Type I dissolution (Fig. 8c). In the most severe cases, dissolution transformed the crystals from long aragonite rods present on an intact shell (Fig. 8b) to thick, short, loosely arranged crystals with eroded lateral edges of Type III dissolution (Fig. 8d). As a result, the growing edge had become less rigid and more fragile.

Semi-quantification of shell surface dissolution Image segmentation allowed the extent of the three different categories of shell dissolution to be estimated in a semi-quantitative manner. There were statistically significant differences in dissolution levels between natural and incubated specimens and also between different incubations treatments (Kruskall-Wallis 1-way ANOVA on Ranks, $H = 29.15$ to $35.38$, $P<0.001$; Fig 9). After incubation in supersaturated conditions ($\Omega \sim 1.65$) for 8 days, roughly half of the shell surface was covered with Type I dissolution (Fig. 9a). Given that very little dissolution was observed in natural field samples extracted from supersaturated ambient conditions, we considered such dissolution to be an incubation effect and used as a baseline with which to compare against other incubations. Type I dissolution was also present on shells incubated in transitional conditions for 14 days but covered 80% of the surface area of each shell (SD=15) with a remaining 15% (SD=10) covered with Type II dissolution. In shells incubated in undersaturated conditions for 4 d, Type II dissolution covered 26% of the shell (SD=6; Fig. 10) and there was evidence of Type III dissolution, although only over 3% of the shell surface (SD=3). In specimens incubated for 14 d in undersaturated conditions, Type III dissolution was present across one third of the total
shell surface (31% coverage, SD=6; Fig. 9a). The type and extent of dissolution was consistent across pteropods incubated in similar conditions as evidenced by the low standard deviations (Fig. 9a).

The fact that Type I dissolution occurred when the organism was held in supersaturated conditions for 8 days and the organic layer was intact raises questions about the role of the organic layer as a protection against dissolution. Clark and George (1999) postulated that the organic layer would have to be severely altered or destroyed before the crystal structure of the shell would be affected by chemicals, i.e. acidic attack. Similarly, Vermeij (1987) regarded a thick periostracum as a molluscan adaptation for slowing down dissolution. A microstructure with a high organic content provides high resistance to dissolution by shrouding the crystals (Harper 2000). However, while Glover & Kidwell (1993) regarded the organic layer as a protection against dissolution, they also acknowledged the possibility that the organic layer could enhance dissolution by promoting growth of acid-releasing microbes. We propose that the periostracum does not provide protection from acidified waters.

Altogether, the development of a reliable method by which to prepare pteropod shells for SEM analysis has allowed us to describe in detail the process of how shell-surface dissolution progresses from its initial stages to severe levels of damage. It has also allowed us to develop an easily applicable categorization scheme for types of shell dissolution and subsequently, a means of obtaining a semi-quantitative assessment of the extent of different types of dissolution on pteropod shells, with the assistance of some custom-made freeware for image segmentation analysis (see Supplementary Materials). With appropriate calibration, these developments will in turn allow levels of dissolution to be quantified and contribute to studies of oceanic carbon budgets. Establishing functional responses between aragonite saturation state and the extent of dissolution in pteropod shells is the next important step in this process. Such functions are an important aspect of not only considering the biogeochemical consequences of the dissolution of calcifiers but also their future viability in acidifying oceans. Based on their demonstrated
response to subtle changes in carbonate chemistry, this study suggests that the monitoring of pteropod shell dissolution can serve not only as a proxy of saturation state in the past (Gerhardt & Henrich 2001), but also as a sensitive bioindicator of modern day ocean acidification. Wider application will also increase our ability to assess the impact of these changes on other pelagic calcifiers.

Acknowledgements

Thanks go to the officers and crew of the RRS James Clark Ross for their support during the cruise JR177, from which the live material was obtained and experiments were conducted. NB was supported by the FAASIS (Fellowships in Antarctic Air-Sea-Ice Science), a Marie Curie Early Stage Training Network with grant number MEST-CT-2004-514159. GT and SF carried out this work as part of the DISCOVERY 2010 and ECOSYSTEM programmes at BAS.
References

Acker JG. and Byrne RH (1989) The influence of surface state and saturation state on the
dissolution kinetics of biogenic aragonite in seawater. *American Journal of Science, 289*, 9,
1098-1116.

preservation and stable-isotope record of the Red Sea. *Palaeogeography, Palaeoclimatology,
Palaeoecology, 52*, 2-4, 195-211.

Bé AWH, Gilmer RW (1977) A zoogeographic and taxonomic review of euthecosomatous
London.

Bé AWH, MacClintock C, Currie DC (1972) Helical shell structure and growth of the pteropod
*Cuvierina columnella* (Rang) (Mollusca, Gastropoda). *Biomineralization Research Reports, 4*,
47-79.

Berner RA and Morse JW (1974) Dissolution kinetics of calcium carbonate in sea water; IV:

Byrne RH, Acker JG, Betzer PR et al. (1984) Water column dissolution of aragonite in the


International Conference on Pattern Recognition, 4*, 150–155.
Clark GR II (1999) Organic matrix taphonomy in some molluscan shell microstructures. 

*IEEE Transactions on pattern analysis and machine intelligence*, 24, 5, 603-619.


Manno C, Sandrini S, Tositti L et al. (2006) First stages of degradation of *Limacina helicina*


Figure captions

Figure 1: SEM image illustrating the microstructure of the shelled pteropod *Limacina helicina* ant. An organic layer covers a prismatic layer and an underlying crossed-lamellar crystal layer.

Figure 2: Method for the shell preparation of juvenile *Limacina helicina antarctica* stored in 70% ethanol.

Figure 3: Abiogenic precipitation and contamination with bacteria on the shell surface of juvenile and adult pteropods. a) phosphate crystals in the struvite form (crystal rods) and NaCl salts (squares); b) phosphate crystals in rosette form; c) triple phosphate crystals; d) bacterial contamination with phosphate crystals in rosette form.

Figure 4: Artefacts on shells of juvenile pteropods caused by inadequate shell preparation: a, b) a partly collapsed organic matrix; c) crushed and chipped growing edges generated by mechanical damage or high vacuum exposure; d) pitted surfaces not caused by dissolution; e) the correct shell preparation method leaves the shell and growing edge intact and, with the organic layer removed, the crystal structure is revealed.

Figure 5: Type I dissolution: Aragonite crystals are missing and ‘cauliflower heads’ have appeared (encircled) (Fig. 5a), the porosity has increased (Fig. 5b).

Figure 6: Type II dissolution: Larger areas of the shell surface are covered by dissolved patches,
where dissolution is protruding deeper (Fig. 6a), affecting the prismatic (P) layer, and exposing
the crossed-lamellar (CL) layer (Fig. 6b).

Figure 7: Type III dissolution: Increased shell frailness (Fig. 7a) as a result of much of the shell
surface being affected by dissolution (Fig. 7b), with extensive Type III dissolution causing
large gaps in the prismatic layer and exposing the crossed-lamellar layer (Fig. 7c).

Figure 8: The growing edge of juvenile pteropods: a) intact growing edge covered by the
organic layer; b) intact crystals within crossed-lamellar layer of the growing edge; c) Type I
dissolution - increased porosity and appearance of ‘cauliflower heads’ in the prismatic layer; d)
Type III dissolution - aragonite crystals in the crossed-lamellar layer are thicker and shorter as a
result of dissolution.

Figure 9: Bar graph: Correspondence between the extent of dissolution and the $\Omega$ saturation
state within incubations showing mean (SD) levels of dissolution according to environmental
conditions (natural or incubated at different levels of $\Omega$ saturation). Matrix: dissolution types
showing significant pairwise differences between treatments. ‘x’ denotes that no significant
differences were found; ‘0-diss.’ denotes no detectable dissolution.
Table 1: Conditions of incubations to which live juvenile pteropods were exposed (Ω supersaturation, N=9; near-saturation, N=11 and Ω undersaturation for 4- and 14 days; N=11). Incubations were done in parallels. The value depict the mid-point between the values measured at the start and end of the incubation (± values depict the difference between the start and end measurements). Carbon chemistry parameters derived using Matlab CO2sys, total pH scale, Mehrbach refit by Dickson and Millero.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Salinity</th>
<th>Phosphate (µmol/kg)</th>
<th>Silicate (µmol/kg)</th>
<th>Temp (deg C)</th>
<th>TA (µmol/kg)</th>
<th>DIC (µmol/kg)</th>
<th>pH7</th>
<th>CALCULATED PARAMETERS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Natural control</td>
<td>33.86</td>
<td>1.33</td>
<td>12.5</td>
<td>2.9</td>
<td>2290.6±3.7</td>
<td>2123.0±61.9</td>
<td>8.13±0.13</td>
<td>318±111</td>
</tr>
<tr>
<td>Supersaturation (experimental control)</td>
<td>33.83</td>
<td>1.71</td>
<td>8.0</td>
<td>4.0</td>
<td>2360.3±3.4</td>
<td>2211.5±12.7</td>
<td>8.07±0.03</td>
<td>387±24</td>
</tr>
<tr>
<td>(8 days)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Near-saturation</td>
<td>33.83</td>
<td>1.80</td>
<td>12.5</td>
<td>4.0</td>
<td>2316.8±1.3</td>
<td>2245.0±14.9</td>
<td>7.83±0.05</td>
<td>690±75</td>
</tr>
<tr>
<td>Undersaturation (4 days)</td>
<td>33.82</td>
<td>1.15</td>
<td>10.1</td>
<td>4.0</td>
<td>2323.1±8.9</td>
<td>2295.5±5.4</td>
<td>7.70±0.01</td>
<td>940±27</td>
</tr>
<tr>
<td>Undersaturation (14 days)</td>
<td>33.83</td>
<td>1.80</td>
<td>12.5</td>
<td>4.0</td>
<td>2330.3±6.4</td>
<td>2298.3±0.0</td>
<td>7.73±0.01</td>
<td>883±12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2: Summary of the main features of each dissolution type

<table>
<thead>
<tr>
<th>Dissolution type</th>
<th>Description</th>
<th>$\Omega$ value when first observed</th>
</tr>
</thead>
</table>
| **Type I**       | • First indices of slightly increased porosity  
                  • Aragonite crystals within upper-prismatic layer affected by dissolution with ‘cauliflower heads’ present  
                  • In small extent present in natural environment  
                  • Widespread at decreased $\Omega$ supersaturated state |
| **Type II**      | • Increased porosity  
                  • Damage patches more extensive and numerous  
                  • Prismatic layer partially/completely dissolved, crossed-lamellar layer exposed  
                  • In small extent at Near-saturation $\Omega$ state with (transition between $\Omega$ super- and undersaturation occurs)  
                  • In larger extent in undersaturated conditions |
| **Type III**     | • Damage extent and porosity resulting in less compact crystal structure  
                  • Compromised shell integrity and extreme frailness  
                  • Dissolution within crossed-lamellar layer with crystals thicker and chunkier  
                  • At near-saturated $\Omega$ conditions  
                  • Larger, more extensive patches in the $\Omega$ undersaturated ($\Omega$~0.8) and progressing with the time of exposure |
Figure 1:

- Organic layer
- Crossed-lamellar layer
- Prismatic layer
Figure 2:

- Sample transfer to 50% ethanol
- Transfer to distilled water
- Short rinses with 6% H₂O₂
- Washing off with distilled water twice for 5 minutes
- Sample transfer to 50% methanol for 5 minutes twice
- Sample transfer to 85% methanol for 10 minutes
- Sample transfer to DMP for 15-20 minutes twice
- Transfer to 50-50% DMP-HMDS for 10 minutes
- Sample transfer to 100% HMDS for 20-25 minutes twice
- Leave the sample to dry completely
- Plasma etching to remove organic layer
- Coating and SEM examination
Figure 3a:

Figure 3b
Figure 4e:
Figure 8a:

Figure 8b:
Proportion of shell surface dissolution

Figure 9