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# Response of *Halimeda* to ocean acidification: field and laboratory evidence

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## Abstract

Rising atmospheric $pCO_2$ levels are changing ocean chemistry more dramatically now than in the last 20 million years. In fact, pH values of the open ocean have decreased by 0.1 since the 1800s and are predicted to decrease 0.1–0.4 globally in the next 90 years. Ocean acidification will affect fundamental geochemical and biological processes including calcification and carbonate sediment production. The west Florida shelf is a natural laboratory to examine the effects of ocean acidification on aragonite production by calcareous green algae. Scanning electron microscopy (SEM) of crystal morphology of calcifying organisms reveals ultrastructural details of calcification that occurred at different saturation states. Comparison of archived and recent specimens of calcareous green alga *Halimeda* spp. from the west Florida shelf, demonstrates crystal changes in shape and abundance over a 40+ year time span. *Halimeda* crystal data from apical sections indicate that increases in crystal concentration and decreases in crystal width occurred over the last 40+ years. Laboratory experiments using living specimens of *Halimeda* grown in environments with known pH values were used to constrain historical observations. Percentages of organic and inorganic carbon per sample weight of pooled species did not significantly change. However, individual species showed decreased inorganic carbon and increased organic carbon in more recent samples, although the sample sizes were limited. These results indicate that the effect of increased $pCO_2$ and decreased pH on calcification is reflected in the crystal morphology of this organism. More data are needed to confirm the observed changes in mass of crystal and organic carbon.

## 1 Introduction

Ocean pH and carbonate concentrations are declining, and fundamental chemical balances are changing as a result of ocean $CO_2$ uptake (Kleypas and Langdon, 2006; Doney et al., 2009). Ocean acidification is being studied widely to understand the



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magnitude of changes that are occurring both in physical environments and in biological systems. The impacts of recent (0.1 pH units over the last two centuries) and future shifts in seawater carbonate chemistry on biocalcification and survival rates of marine organisms are pressing topics that require both field and experimental study.

Critical questions include whether (and which) calcifying organisms can adapt to decreasing pH and saturation state. To date, corals, coccolithophorids, pteropods, and shallow-dwelling species of coralline red algae have been shown to reduce calcification rates with decreases in pH (Feely et al., 1988; Fabry, 1990; Langdon et al., 2003; Kuffner et al., 2008) and have been predicted to respond negatively as a result of ocean acidification (Orr et al., 2005). However, the impacts on many organisms have not been established. Some results have been equivocal. Studies on coral calcification records from the previous decades to centuries have found little to no evidence of changes in calcification rates in response to changing ocean chemistry (Lough and Barnes, 1997, 2000; Bessat and Buigues, 2001). Some photosynthetic organisms actually benefit in lower pH seawater (Langer et al., 2006; Iglesias-Rodriguez et al., 2008) by increasing both calcifying and noncalcifying carbon fixation rates (Doney et al., 2009). Therefore, while it is clear that some organisms could be negatively impacted, others may actually benefit from some degree of ocean acidification. The net effect however, will likely change the functioning of many marine and coastal ecosystems.

Calcification in corals is predicted to decrease by 30±18% within 30–50 years (ISRS, 2008). Will calcareous green algae respond similarly?

### 1.1 *Halimeda* responses

Calcareous green algae have demonstrated their versatility in adaptation through their range in habitat from tropical to subtropical/temperate environments and depth-ranges from shallow water to depths in excess of 110 m. One of the most important and well studied genera, *Halimeda* (*Halimedaceae*, *Chlorophyta*) (Fig. 1), has been recognized in the geologic record as prolific sediment producers, in some cases comprising the major portion of reef sands and nearshore algal banks (Milliman, 1974; Neumann and





Land, 1975; Littler, 1976; Jensen et al., 1985). The genus has been documented in the Cretaceous (Hillis-Colinvaux, 1980), and taxa that are either related or identical have been described in the Tethyan Upper Triassic (Flügel, 1988). Previous work indicates that *Halimeda* fragments contribute greatly to mass of carbonate sediment in many atoll-type environments (Hillis-Colinvaux, 1980).

Calcareous green algae such as *Halimeda* and *Udotea* appear to utilize the $CO_2$ produced by calcification to enhance photosynthesis and consequently primary productivity (Borowitzka and Larkum, 1976; Reiskind et al., 1988), indicating that their rates of photosynthesis can be $CO_2$ limited. If this relationship holds, increases in $pCO_2$ of seawater may enhance primary productivity of these organisms by making more $CO_2$ available for photosynthesis, but likely at the expense of calcification. For example, some taxa such as *Halimeda discoidea* Decaisne have deep water varieties (e.g., v. *platyloba* Borgeson) with segments even larger and less calcified than typical *H. discoidea* (Taylor, 1960). James et al. (1999) noted weakly calcified, fleshy morphologies of *Halimeda* from depths of 35–50 m on the southwest Australian shelf, where sediments had a distinctive cool-water character, though larger foraminifers were abundant and some zooxanthellate corals were present. On the other hand, prolific *Halimeda* bioherms occur at similar depths in the Sunda Straits, Nicaragua Rise, and Great Barrier Reef lagoon where clear, oligotrophic surface water permits sufficient light penetration, and local hydrodynamics jet $CO_2$ and nutrient-charged uppermost thermocline waters onto those shelves (Hallock, 2001 and references therein).

How the calcareous green algae have responded in recent decades as ocean chemistry has begun to change in response to rising atmospheric $CO_2$ and how they will respond to changing seawater conditions are still unknown. Our study combines a retrospective look over 40 years at calcification in *Halimeda* with laboratory experiments to simulate predicted carbonate chemistry under which calcification will occur in the future. This approach allows evaluation of how ocean acidification has affected this green alga and provides a tool for predicting future changes.

In this pilot study, we analyzed two species of *Halimeda* from archived samples collected in the 1960s, 1970s and 1980s, with specimens collected in 2007–2008, to determine if crystal and ultrastructural changes could be observed over the ~40 year time span. In addition, laboratory experiments were performed in which adult *Halimeda* from the west Florida shelf (WFS) were transplanted and grown in laboratory tanks at lower pH levels to document crystallographic change.

## 2 Materials and methods

### 2.1 Historical samples

Apical segments from 22 dried, archived *Halimeda* samples collected in the 1960s through 2008 were prepared for scanning electron microscopy (SEM). The samples came from either personal collections of PH or from the University of South Florida Herbarium Collection. Locations of origin of these samples were used to inform collection of recent living samples. Notably, we used samples from the west Florida shelf, off Tampa Bay, Florida.

### 2.2 Laboratory experiments

Specimens of live *Halimeda* spp. (Fig. 1a) were collected from field sites on the WFS (Fig. 2) or purchased from a dealer. *H. tuna* and *H. opuntia* were used for the laboratory experiments because of their availability. The specimens were obtained from sites with a pH of 8.1. Apical and basal segments (Fig. 1b) used as controls were cut from *Halimeda* plants before the experiments and were rinsed in distilled water for approximately one minute, dried and analyzed via SEM. Only apical data are reported here.

Four adult living *Halimeda* specimens were cleared of visible epiphytes and epizoa. The plants were immersed for 45 min in an Alizarin Red solution, thereby staining the

thalli and facilitating visual identification of new growth. Plants were then rinsed with tank water and transferred into sealed artificial-seawater 37.8 liter aquaria containing autoclaved aragonite sand substrate (Fig. 3). A compact fluorescent light fixture was installed ~12 cm above the sealed acrylic cover. The fixture consisted of two 130 W daylight bulbs (6700 K and 10 000 K), two 130-W actinic bulbs (420 nm and 460 nm), and four LED lunar lights. An automatic timer was used to keep the lights on from 07:00 to 19:00 daily. The tank was maintained at the following parameters: pH=7.5 $S$=35.0; $T$=25.1–26.3°C. A $CO_2$ injection system was attached to the tank, consisting of an Aquacontroller 3 controller/monitor (AC3), temperature and pH probes, $CO_2$ tank and bubble counter, $CO_2$ reactor, and a water pump to maintain the parameters. $CO_2$ was bubbled through the aquaria to adjust the seawater pH from recent levels (pH 8.1) to pH 7.5. Specimens were then allowed to grow for a period of 3 weeks. During this time, additional apical sections were formed and subsequently sampled, rinsed in water from Millipore Elix 10 Water Purification System for less than one minute, dried and prepared for SEM (see below). The glass pH electrode was calibrated to the total hydrogen ion concentration pH scale before initially injecting $CO_2$ and weekly thereafter using a two-point calibration with pH 7.00 $Na_2HPO_4/KH_2PO_4$ buffer and pH 10.00 $NaHCO_3/Na_2CO_3$ buffer. The accuracy of the calibration was confirmed spectrophotometrically (Dickson et al., 2007).

### 2.3 Preparation of apical segments

Living specimens of *Halimeda* sp. were collected, rinsed briefly in fresh water, and prepared for SEM. Apical segments from both archived and recently collected samples were removed from a thallus and placed on a glass plate under a stereomicroscope. Longitudinal sections of the segment were cut from near the center using a scalpel and the section mounted on an SEM stub covered with double-stick tape. When available, a second segment was removed from near the base of the frond. Specimens were sputter coated with gold-palladium for approximately 6 min using a Hummer 6.2 Sputtering system and imaged with a Hitachi S-3500N Variable Pressure scanning electron

microscope. For comparative purposes, utricles were located on the segments and, with split screen, a close-up of interutricle crystals was scanned along with a lower-magnification location of the scan. Photomicrographs were taken of the segments.

### 2.4 Crystal counts and size data

All SEM photomicrographs of *Halimeda* were analyzed for crystal abundances and widths of crystals using ImageJ freeware. A 5 $\mu$m×5 $\mu$m or 10 $\mu$m×10 $\mu$m area was randomly selected, and total crystal counts were performed three times per microphotograph and averaged. The three data sets were then averaged. Widths of crystals were measured using the ImageJ "measuring tool" calibrated to the micrometer scale bar embedded in the image file by the SEM. More than 30 crystals were randomly selected and measured within each square and the measurements averaged. The resulting data set was then averaged for each photomicrograph.

### 2.5 $CaCO_3$ and organic analyses

Total organic, $CaCO_3$, and organic carbon of *H. discoidea* and *H. incrassata* platelets (1960–2008) were analyzed using a Lindberg/Blue Mini-Mite Tube Furnace TF55035A attached to a CM 5015 Coulometer. Apical segments from a plant were combined to achieve approximately 10 mg per sample and ground using a mortar and pestle. An aliquot was weighed for total organic carbon and combusted for approximately 5 min, and $CO_2$ evolved was measured coulometrically. An additional aliquot was taken for inorganic carbon analysis and acidified using perchloric acid, and the $CO_2$ evolved was measured coulometrically. This approach measured the average mass of aragonite and organic carbon per normalized platelet. Because of the destructive nature of this analysis, limited amounts of sample were available for archived material.



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## 3 Results and discussion

### 3.1 Historical data

Scanning electron microscopy of apical platelets from all specimens demonstrated a range of crystal morphologies, from thick blocky forms to longer thinner blades. However, each species showed crystal sizes and abundances specific to themselves. Our data showed that *Halimeda* collected from different locations demonstrated significant crystal-width differences. For example, Fig. 4 shows that within the species *Halimeda incrassata*, crystal width and abundance differences were observed between apical segments from South Florida, Belize, and those collected on the WFS, corroborating findings of Hillis-Colinvaux (1980) regarding interlocality variability.

Image analyses of crystals from apical sections of *Halimeda* spp. from the WFS showed a trend of more abundant crystals in samples taken in 2007 and 2008 than those from older samples from the 1960s and 1970s (Fig. 5a–d). Further, crystals demonstrated an overall decrease of width in more recent specimens collected (Fig. 6) compared to those from the 1960's. Specifically, apical crystals in *Halimeda discoidea* from the 1990's and 2000 showed ~48% decrease in width compared to those from the 1960s, having a mean width of 0.253 $\mu$m, versus a mean width of 0.486 $\mu$m, respectively. *Halimeda incrassata* showed a more dramatic width decrease of ~70%, with average width of 0.247 $\mu$m in recent samples compared to average width of 0.821 $\mu$m from the 1960s.

In terms of crystal abundance (i.e., crystals per unit area), *H. discoidea* from the WFS showed a 39% increase in crystal abundance in apical platelets between 1966 and 2008. *Halimeda incrassata*, again, showed a more dramatic increase than *H. discoidea*, with 62% crystal increase from 1971 to 2008.

In *H. incrassata* specimens, inorganic carbon, as a percentage of sample weight, decreased and organic carbon increased in more recent samples as compared to older samples, while *H. discoidea* showed much smaller differences in both parameters (Fig. 7). These results are intriguing but not statistically significant because of



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the limited number of samples available for analyses.

### 3.2 Laboratory experiments

Laboratory results corroborate data from archived material: apical sections of segments grown in pH 7.5 water have thinner crystal widths than those grown in pH 8.1 water (Figs. 8a–d and 9). *Halimeda opuntia* crystals grown in pH 7.5 water were ~43% smaller, having a mean width of 0.281 $\mu$m compared to a mean width of 0.497 $\mu$m for crystals grown in pH 8.1 water. *Halimeda tuna* crystals grown in pH 7.5 water were ~18% smaller than those grown in pH 8.1, having a mean width of 0.209 $\mu$m compared to a mean width of 0.253 $\mu$m. Conversely, counts of crystals per unit area (point density) of *Halimeda opuntia* grown in pH 7.5 water, were ~66% greater than crystals grown in pH 8.1 water, while point density of *Halimeda tuna* crystals grown in pH 7.5 water was 11% greater than crystals grown in pH 8.1 water.

## 4 Discussion

Extensive research on *Halimeda* has documented structure, crystallography, and chemistry of the organism (Hillis-Colinvaux, 1980; Jensen et al., 1985; Macintyre and Reid, 1995; Ries, 2005). Deposition of aragonite within interutricular spaces (Fig. 10) has been described as a physiological process (Hillis-Colinvaux, 1980; Borowitzka, 1982a, b) that occurs through cellular metabolic processes and photosynthetically induced precipitation, i.e., the removal of carbon dioxide and bicarbonate during photosynthesis raises pH and carbonate saturation to levels where $CaCO_3$ precipitation can occur (Jensen et al., 1985). Indeed, this relationship between calcification and photosynthesis has been demonstrated by a number of researchers (Goreau, 1963; Stark et al., 1969; Borowitzka and Larkum, 1976; Jensen et al., 1985). De Beer and Larkum (2001) recognized that calcification in *H. discoidea* is not regulated by the alga but is a consequence of pH increase during photosynthesis.

Calcification studies on *H. tuna* and *H. cylindrical* have shown that aragonite crystals are deposited in the intercellular spaces (Borowitzka and Larkum, 1976), separated from seawater by the utrical layer. It has been suggested that during photosynthesis the diffusion barrier allows the development of a microenvironment with a high pH suitable for aragonite precipitation. Thus, calcification is thought to be regulated by the morphology of the algae, the uptake of $CO_2$ by photosynthesis, and the nucleation sites on the tissue (Borowitzka, 1989; De Beer and Larkum, 2001). De Beer and Larkum (2001) determined that photosynthesis, respiration, and the light-driven proton pump determine the pH of the intercellular spaces and that the calcification of *H. discoidea* is directly coupled to that localized pH; thus, they predicted that acidification of seawater would decrease calcification (De Beer and Larkum, 2001). On the other hand, Reiskind et al. (1988) showed that *Halimeda* demonstrates increased primary productivity with elevated levels of $CO_2$.

Hillis-Colinvaux (1980) reported that the size and numbers of crystals in the segments vary with species, age of the segment, and from site to site. To constrain potential differences, our study compared only the apical segments of species of *Halimeda* plants from a relatively localized area of the west Florida shelf. Laboratory experiments confirm trends seen in the historical record of apical segments, suggesting that the crystal width and amounts of crystals may be associated with the chemistry of the seawater. Our data showed decreased crystal width and more abundant crystals with decreasing pH. This observation suggests that crystallization events may be initiated and terminated more frequently with increased $pCO_2$.

Some questions that still remain include: Are there more aragonitic plates produced with increasing $pCO_2$? Are the plates weaker with increasing $pCO_2$? Do they more readily become etched in lower saturation states? How do the organic substrates respond? Is there a linear size and density gradient in crystal morphologies associated with seawater pH between 8.1 and 7.5? What is the impact of decreased crystal size and increased abundance on taphonomy and sediment budgets?



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## 5 Conclusions

1. *Halimeda* species showed species specific differences in crystal morphologies (width and crystal abundances) and responses to changing pH/$pCO_2$.

2. Field samples of species demonstrated changes in crystal morphologies over a time frame of <50 years.

3. Similar trends were observed in laboratory studies in which pH was lowered through addition of $CO_2$. Species showed more and thinner crystals per unit area.

4. The thinner, more abundant crystals observed under reduced pH conditions indicates that crystallization events may be initiated and terminated more frequently with increased $pCO_2$.

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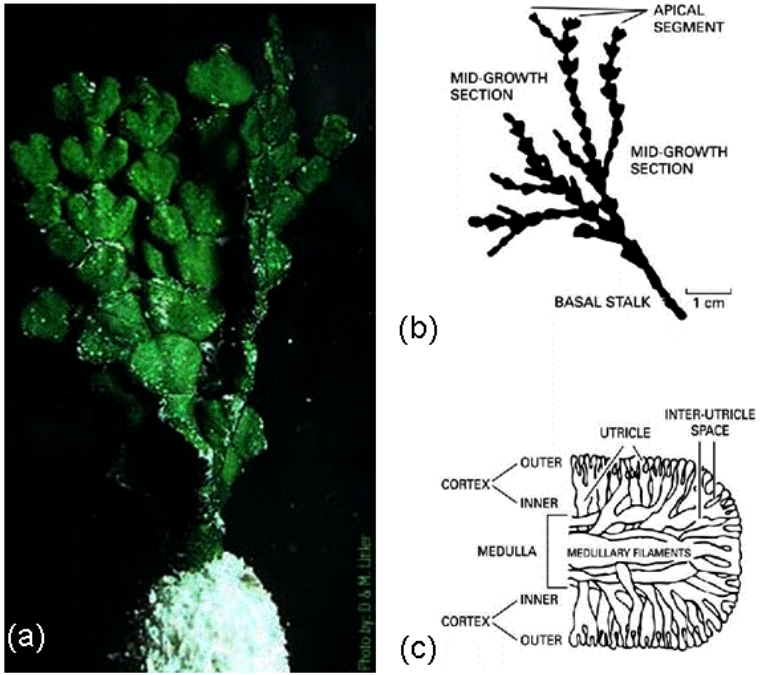


**Fig. 1. (a)** *Halimeda* plant, **(b)** Thallus of *Halimeda* showing where apical segments are located, and **(c)** Longitudinal section through apical segment.



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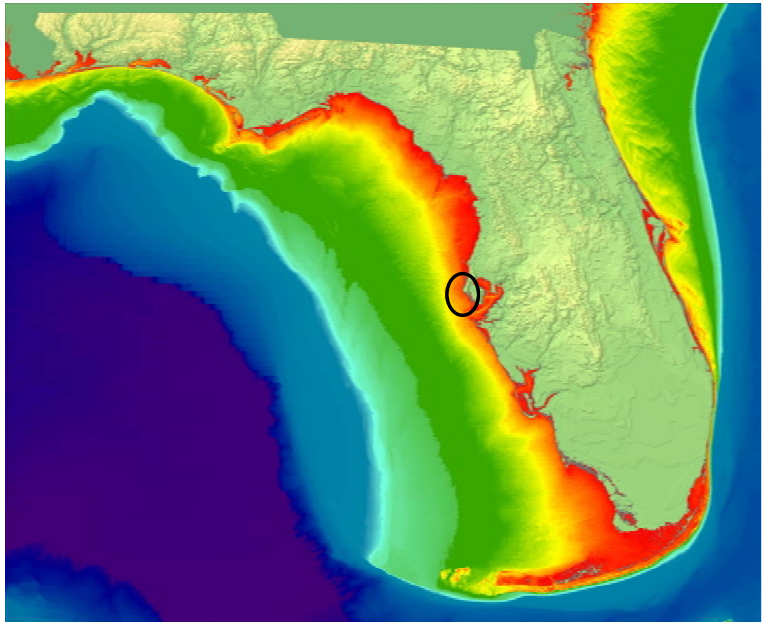

**Fig. 2.** West Florida shelf sample locality (circle).



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**Fig. 3.** Equipment used for laboratory experiments as described in text.



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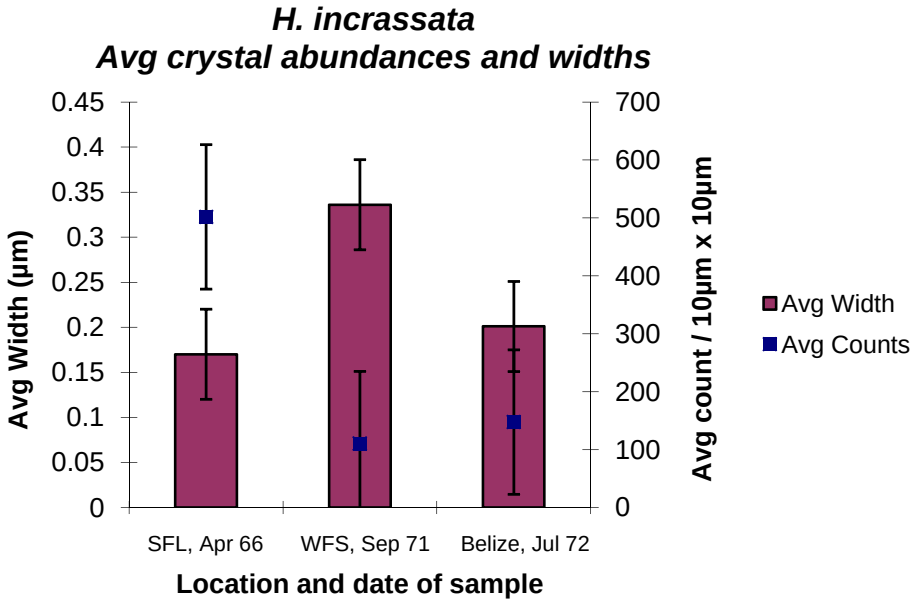


**Fig. 4.** Comparison of apical crystal size of specimens of *H. incrassata* from west Florida shelf (WFS), South Florida (SFL), and Belize. Error bars are 1 standard error of the mean.



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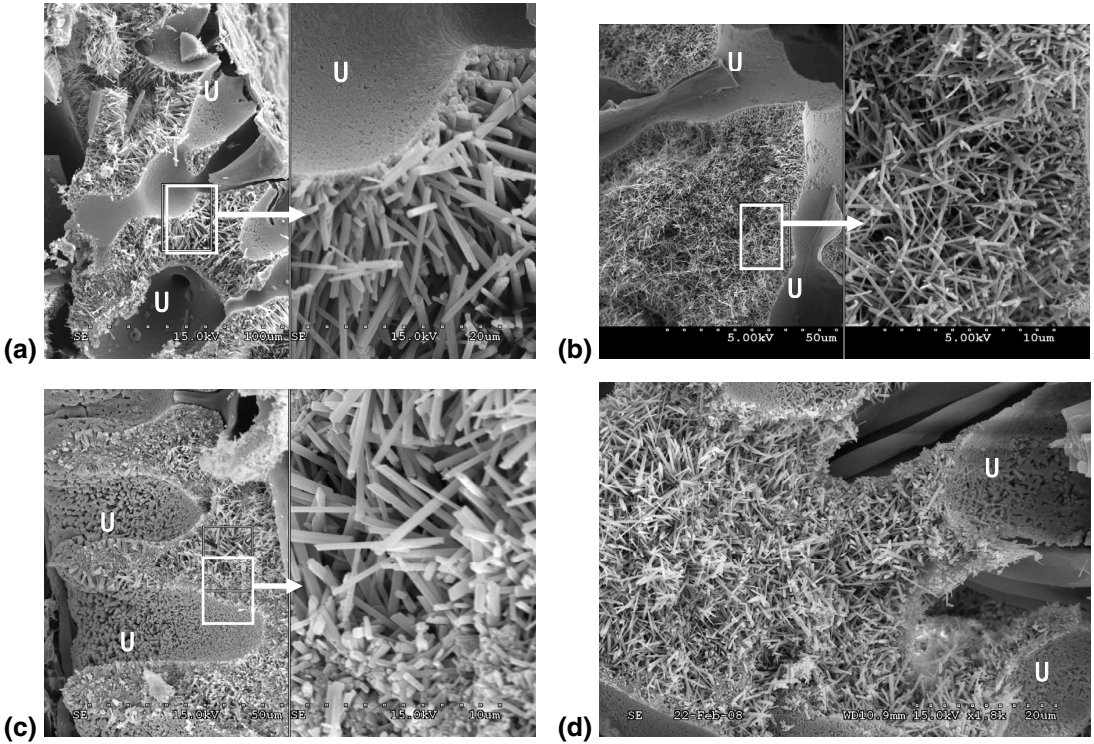


**Fig. 5.** SEM photomicrographs of Halimeda crystals from archived material. **(a)** *H. incrassata*, 1966, **(b)** *H. incrassata*, 2007, **(c)** *H. discoidea*, 1967, **(d)** *H. discoidea*, 2008. U=utricle.



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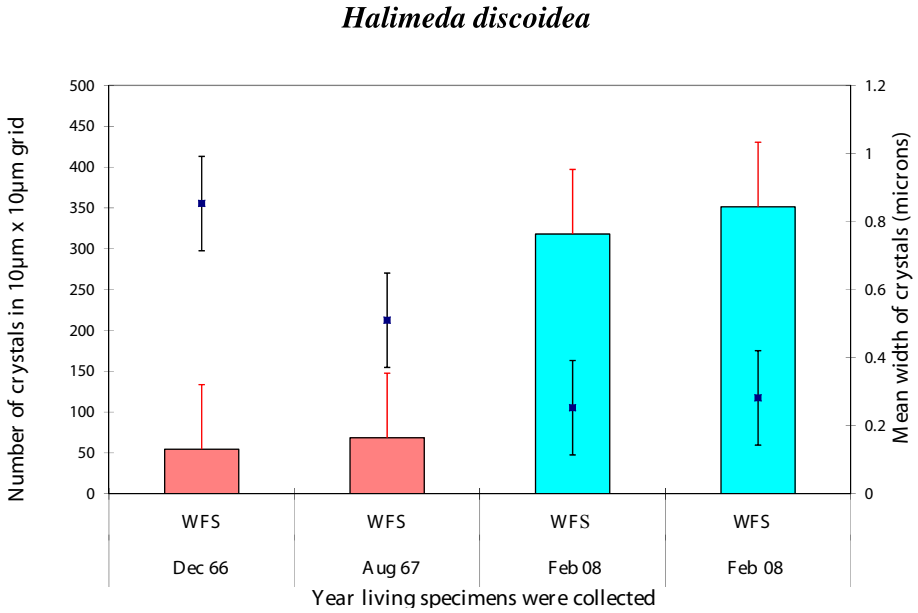


**Fig. 6.** Graph of crystal trends for *Halimeda discoidea* archived samples. Histogram bars indicate number of crystals per unit area. Blue squares represent the mean width of crystals. Error bars are 1 standard error of the mean.



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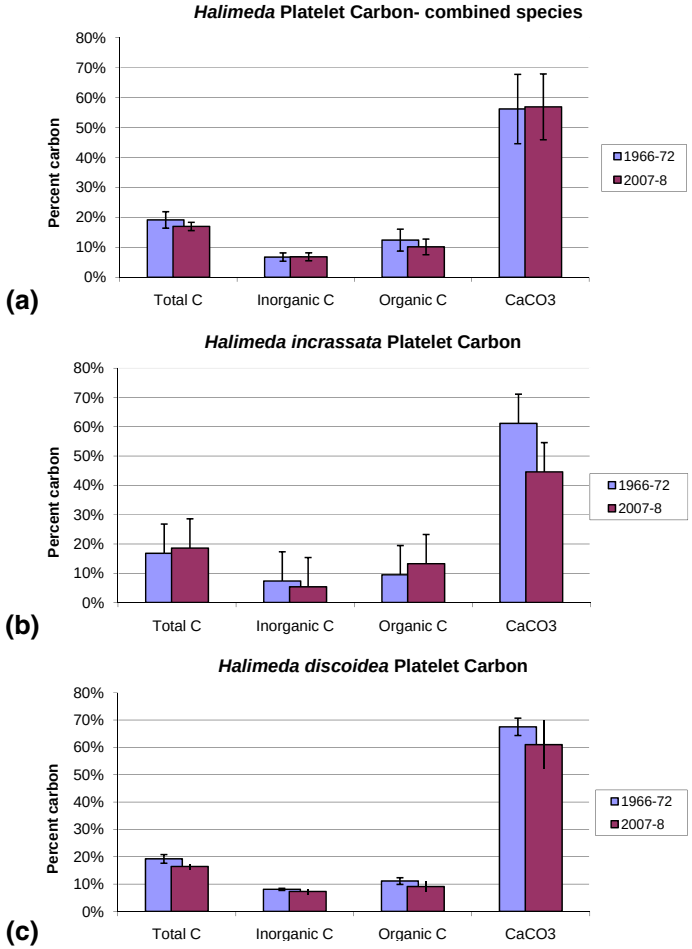


**Fig. 7.** **(a)** *Halimeda* spp. platelet carbon from archived samples. **(b)** *H. incrassata* and **(c)** *H. discoidea* platelet carbon. Error bars are 1 standard error of the mean.



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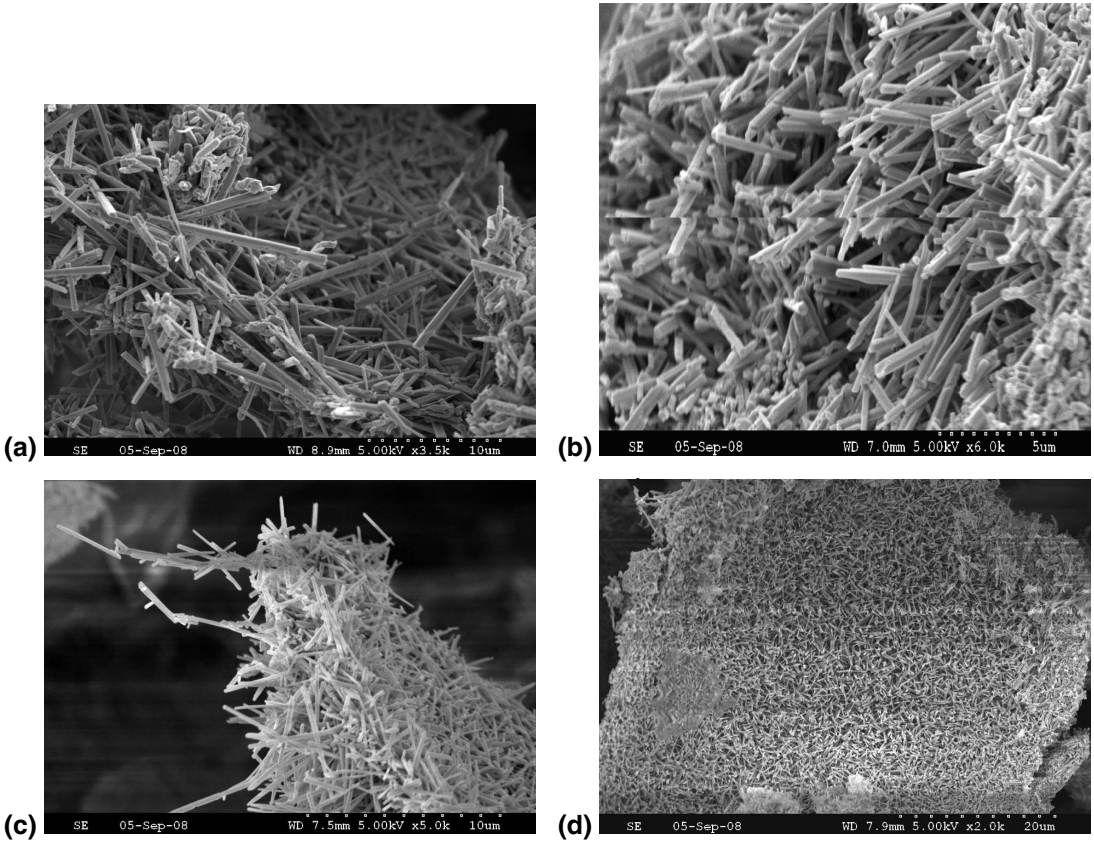


**Fig. 8.** SEM photomicrographs of *Halimeda* crystals from laboratory experiments. **(a)** *H. opuntia* pH 8.1, **(b)** *H. opuntia* pH 7.5, **(c)** *H. tuna* pH 8.1, and **(d)** *H. tuna* pH 7.5.



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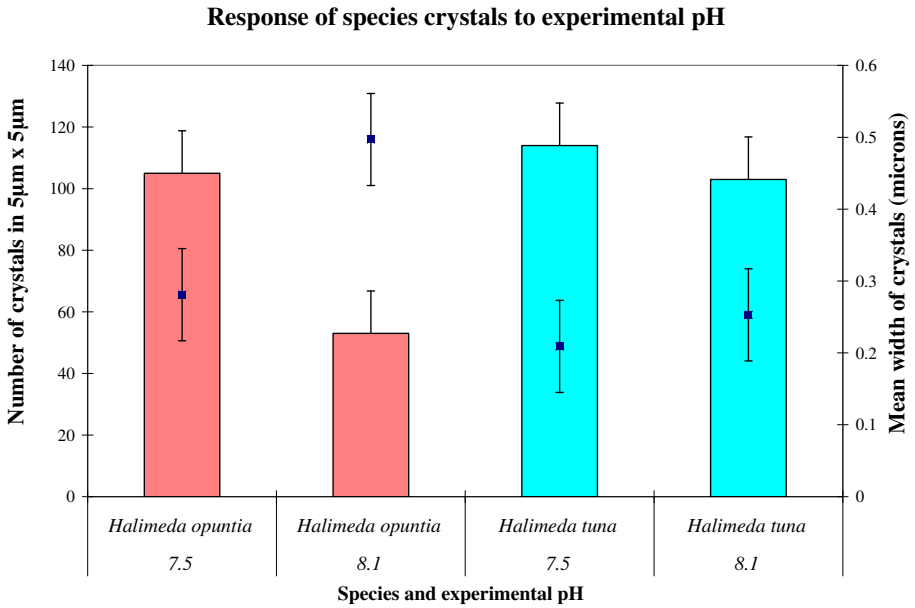


**Fig. 9.** Graph of crystal trends for laboratory experiments. Histogram bars indicate number of crystals per unit area. Blue squares represent the mean width of crystals. Error bars are 1 standard error of the mean.



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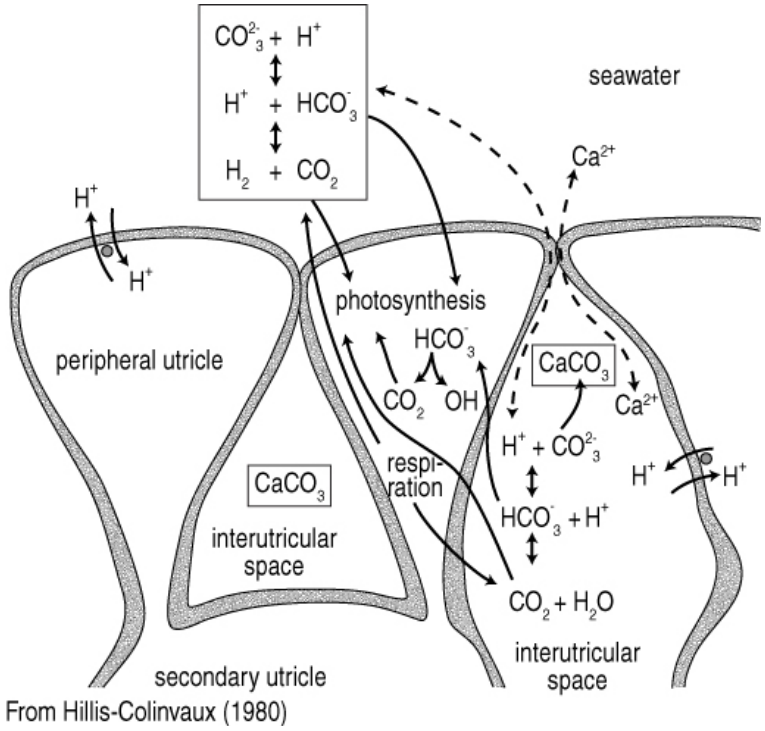


**Fig. 10.** Model for biomineralization in *Halimeda* from Hillis-Colinvaux (1980).



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