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Variation in Skeletal Microstructure of the Coral *Galaxea fascicularis:* Effects of an Aquarium Environment and Preparatory Techniques

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Abstract. To compare the crystalline microstructure of exsert septa, polyps of the scleractinian coral *Galaxea fascicularis* were sampled from shallow reef flat colonies, from colonies living at a depth of 9 m, and from colonies kept in a closed-circuit aquarium. Septal crystal structure and orientation was markedly different between corals in the field and in aquaria. In samples collected from deep water, acicular crystals were composed of conglomerates of finer crystals, and skeletal filling was considerably reduced when compared with samples collected from shallow water. Comparisons were also made between septa prepared in sodium hypochlorite (commercial bleach), sodium hydroxide (NaOH), hydrogen peroxide (H_2O_2), and distilled water (dH_2O). Commercial bleach was the most effective solvent for tissue dissolution in investigations of skeletal structure. Samples prepared in NaOH commonly displayed crystalline artefacts, while the use of dH_2O and H_2O_2 was labor intensive and often resulted in unclean preparations. Fusiform crystals were seen only on *G. fascicularis* septa prepared in bleach and on *Acropora formosa* axial corallites prepared in either bleach or dH_2O. We suggest that the mechanical agitation and additional washing necessary for samples prepared in dH_2O, NaOH, or H_2O_2 resulted in the loss of fusiform crystals from these preparations.

Introduction

The use of aquaria to grow and maintain corals for scientific experimentation has become increasingly popular (see Carlson, 1999) and, with the degradation of coral reefs across the globe, it may soon be necessary for experimental corals to be constantly maintained in this way. In the past, keeping scleractinian corals alive under artificial conditions for long periods of time was exceedingly difficult, but advances in filtration, lighting, and water systems have made the propagation of these corals in aquaria much easier. However, little is known about the impact of artificial environments upon scleractinian coral calcification, behavior, growth, and reproduction, with direct comparisons between field and aquarium-maintained corals rare.

Studies of the crystalline and overall skeletal structure of scleractinian corals necessitate the removal of the surrounding epithelia to visualize the $CaCO_3$ skeleton underneath. Many treatments have been used to dissolve the epithelial tissue, including H_2O_2 (Jell, 1974), freshwater (Wainwright, 1963; Johnston, 1979), NaOH (Johnston, 1979; Isa, 1986), and commercial bleach (sodium hypochlorite) (Sorauf, 1972, 1974; Gladfelter, 1982, 1983; Brown *et al.,* 1983; Hidaka, 1988, 1991a, b; LeTissier, 1988, 1990, 1991; Constantz, 1989; Hidaka and Shirasaka, 1992). Unfortunately, the possible effects of these chemicals upon the underlying crystal structure have been ignored. Comparisons between studies are complicated by the variety of methods used to prepare samples and by the broad range of species studied.

In this study, we describe differences in crystal structure, orientation, and patterns of deposition between corals collected from field conditions and closed-circuit aquaria. In addition, we evaluate and compare the suitability of several chemical treatments commonly used to prepare scleractinian coral skeletons for examination with a scanning electron microscope.

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Materials and Methods

Collection and maintenance of Galaxea fascicularis

Colonies of brown and green color morphs of the scleractinian coral *Galaxea fascicularis* were collected at Heron Reef in the Capricorn Bunker Group of the Great Barrier Reef, Australia. Morphs of both colors were obtained at low tide from the reef flat; brown morphs only were collected by scuba divers from a depth of 9 m.

Specimens to be maintained under field conditions were transported in buckets of seawater to Heron Island Research Station, where they were kept in well-aerated, flow-through aquaria in natural seawater at 24–25 °C. Colonies collected from the reef flat were kept in outdoor, sunlit aquaria, with light levels equivalent to those experienced on the reef flat; colonies collected at depth were kept in shaded aquaria, with light levels similar to those at a depth of 9 m in the field.

Specimens to be maintained in an aquarium environment were sealed into plastic bags with a small amount of seawater, and the bags were put into small insulated boxes for transport to La Trobe University, Melbourne. These colonies were kept in a closed-circuit marine aquarium. The aquarium contained natural, well-aerated seawater at 25 °C and was subjected to an illumination cycle of 14 h of light and 10 h of darkness; the light was provided by metal halogen lamps (photosynthetic photon flux density 150 μmol photons m^{-2} s^{-1}). Conditions in the aquarium were monitored regularly: 25% of the water was changed twice weekly; pH was kept between 8.1 and 8.2 and salinity from 900 to 1200 mosmol kg^{-1}. Corals were fed a mixture of brine shrimp and fish weekly.

Sample preparation

Colonies were allowed to recover for at least 2 days after collection before being used in experiments. Individual polyps of *G. fascicularis* were separated from colonies (see Marshall and Wright, 1991) at 1200 h. Five polyps were placed in either commercial bleach (12% NaOCl) or NaOH (5 N) at 60 °C for 30 min; H_2O_2 (10%) for 3 h at room temperature (RT); or dH_2O at RT for at least 24 h, to remove the overlying soft tissue. The resultant corallites were rinsed well in running water and then several times in dH_2O. Any soft tissue remaining upon the exsert septa was removed by agitation and pipetting of dH_2O onto the sample. The corallites were then dried at 60 °C for 24 h. Aquarium-maintained corals were kept in the closed-circuit aquarium at La Trobe University for 26 weeks before polyps (n = 5) from each of two colonies were sampled at 1200 h and prepared in either bleach, NaOH, or dH_2O.

Electron microscopy

Exsert septa were removed from *G. fascicularis* corallites under a dissecting microscope and mounted flat using carbon tape. Samples (n = 50+) were coated with 5 nm platinum and previewed in a JEOL JSM 840A scanning electron microscope at 10 kV. High-resolution imaging was subsequently conducted on a JEOL 6340-F field emission scanning electron microscope at 2 kV. In addition to being mounted flat, septa from colonies growing in shallow field conditions (n = 5) and the aquarium (n = 9) were secured upright in an appropriate substage, with indium foil placed between the sample and the stage to improve conductivity and provide flexible compression. Samples were coated with platinum, then fractured to provide a cross-sectional area. Following fracturing, the samples were re-coated and viewed by high-resolution field emission scanning electron microscopy (FESEM).

Acropora formosa

White-tipped branches (which possess few symbiotic zooxanthellae) were sampled from *A. formosa* colonies at low tide from Heron Reef at 1200 h and immediately placed into bleach (n = 5) for 30 min or into dH_2O at ambient temperature (n = 5) for 24 h, for investigation of the axial corallite. Branches were washed in running water for 24 h before being rinsed in dH_2O and dried at 60 °C for 24 h. Branch tips were secured upright in hollow stubs, using partially polymerized araldite, so that the axial corallite extended about 3 mm above the upper surface of the stub. Polymerization was completed at 60 °C for a further 30 h. Conductivity of the corallites was improved by overlaying the araldite with conductive silver epoxy (ProSciTech) and coating with 10 nm platinum. Samples were viewed in a JEOL JSM 6340-F field emission scanning electron microscope at 1 kV or 2 kV.

Results

Comparison of field and aquarium samples

Marked differences in crystal orientation and deposition were detected between septa from polyps collected from their natural environment (shallow reef flat) and those sampled from polyps that had been maintained in a closed-circuit aquarium for 26 weeks. When sampled from the natural reef environment, septa possessed acicular crystals that remained highly ordered and extended to the outer edge of the growing septa (Fig. 1A). In contrast, septa sampled from aquaria-maintained polyps had small, unordered crystals deposited with little uniformity (Fig. 1B). This pattern of unordered skeletal deposition extended to a depth of 1 $\pm$ 0.06 μm (n = 25). Little variation in this pattern of deposition was observed within or between colonies.

Structural features typically associated with normal *Galaxea fascicularis* septa, such as acicular and fusiform crystals and well-defined fasciculi (Fig. 2A), were rarely discerned on septa sampled from polyps maintained in the closed-circuit aquarium. Instead, much of the septal surface of aquarium-maintained polyps was covered with small (<100 nm in diameter) spherulitic crystals (Fig. 2B). *G. fascicularis* polyps sampled from the closed-circuit aquarium were also typically paler than corals living on the reef flat, and they appeared to have fewer symbiotic zooxanthellae, although this was not quantified.

In contrast, the crystal structure of septa from deep field-collected *G. fascicularis* colonies was only marginally different from that of septa from shallow field-collected colonies. Acicular crystals of septa sampled from colonies collected from the shallow reef flat appeared as solid, broad crystals tightly packed together (Fig. 3A). However, acicular crystals upon septa collected from deeper water tended to be composed of conglomerates of finer, elongated crystals that were loosely associated (Fig. 3B). This loose assembly gave the appearance of increased porosity, with skeletal filling somewhat reduced. Little structural difference in the other crystal types—nano, lamellar, and fusiform—was noted between septa taken from shallow reef-flat corals and those living in deeper water.

Septum length also varied between polyps from the two field conditions. Septa sampled from polyps growing at depth were considerably longer than those from corallites living on the shallow reef flat (results not shown). We are unsure whether this feature was related to depth or was simply natural morphological variation. No other structural differences were observed between septa sampled from corallites collected from the reef flat and from deeper water.

Chemical treatments

Commercial bleach was the best chemical treatment for digesting soft tissue from corallites of the scleractinian corals *G. fascicularis* and *A. formosa;* dissolution occurred in less than 30 min at 60 °C. Washing samples well in running water, followed by brief rinses in dH_2O, ensured that the septa on the resultant corallites were free of residual tissue (Fig. 4). No intensive cleaning or agitation was necessary. Skeletons prepared in bleach were rapidly cleaned, so the crystalline structure of the septa could be readily observed with SEM. All of the crystal types described by Clode and Marshall (2003) (nano, acicular, lamellar, and fusiform) were clearly observable upon septa prepared in this manner. An example of fusiform crystals is shown in Figure 5.

NaOH was also very effective in removing soft tissue from *G. fascicularis* corallites; tissue was rapidly digested at 60 °C. However, large, crystalline structures regularly observed on septa prepared in NaOH (Fig. 6), were never seen in samples prepared in any other chemical treatment; thus they are probably artefactual. The only way to prevent the appearance of such structures was to ensure that the corallites were washed well after treatment in NaOH, with additional agitation and washing of samples prior to drying. This resulted in septa free from crystalline artefacts. In such cases, nano, acicular, and lamellar crystals were all clearly visible upon the septal surface; however, fusiform crystals were never observed on septa prepared in this manner.

In contrast, the use of dH_2O to remove the overlying soft tissues from *G. fascicularis* septa was difficult, time consuming, and often left corallites unclean. The crystalline surface was masked by overlying material and thus could not be viewed with SEM. Furthermore, polyps immersed into dH_2O secreted excess mucus, which further complicated tissue removal. As a result, many *G. fascicularis* septa prepared in dH_2O were coated in an amorphous material of unknown composition, which completely obscured the crystalline structures underneath (Fig. 7). Clean septa free of tissue remnants and this coating could be obtained by extensive rinsing and pipetting of dH_2O onto the sample. Like septa prepared in NaOH, *G. fascicularis* septa prepared in dH_2O did not possess fusiform crystals.

The perforate nature of *A. formosa* skeletons made treatment with dH_2O much easier and more successful than it was for *G. fascicularis.* Although gentle agitation was necessary to completely remove the soft tissue overlying the axial corallite, the process was considerably less stringent than the rinsing protocol necessary to prepare *G. fascicularis* septa. *A. formosa* axial corallites prepared in dH_2O were clean and free of epithelial remnants following gentle agitation, and fusiform crystals were regularly observed along the primary septa extending into the calyx (Fig. 8).

Tissue dissolution by H_2O_2 was both difficult and highly ineffective (Fig. 9). *G. fascicularis* septa, despite being covered by only a thin layer of soft tissue, took several days to prepare, and the process was exceptionally tedious and labor intensive. Extensive additional agitation and washing was required to produce clean septa, so that the crystalline structure could be seen with SEM. With this treatment, like dH_2O, excessive secretion of mucus added to the difficulty of obtaining suitable preparations. All crystal types, except fusiform crystals, could be readily observed upon septa that had been adequately prepared in H_2O_2 and extensively cleaned with dH_2O.

Discussion

A comparison of the crystalline structure of exsert septa between colonies growing under aquarium conditions and natural, field conditions has revealed that drastic changes to crystal deposition and skeletal formation may occur in

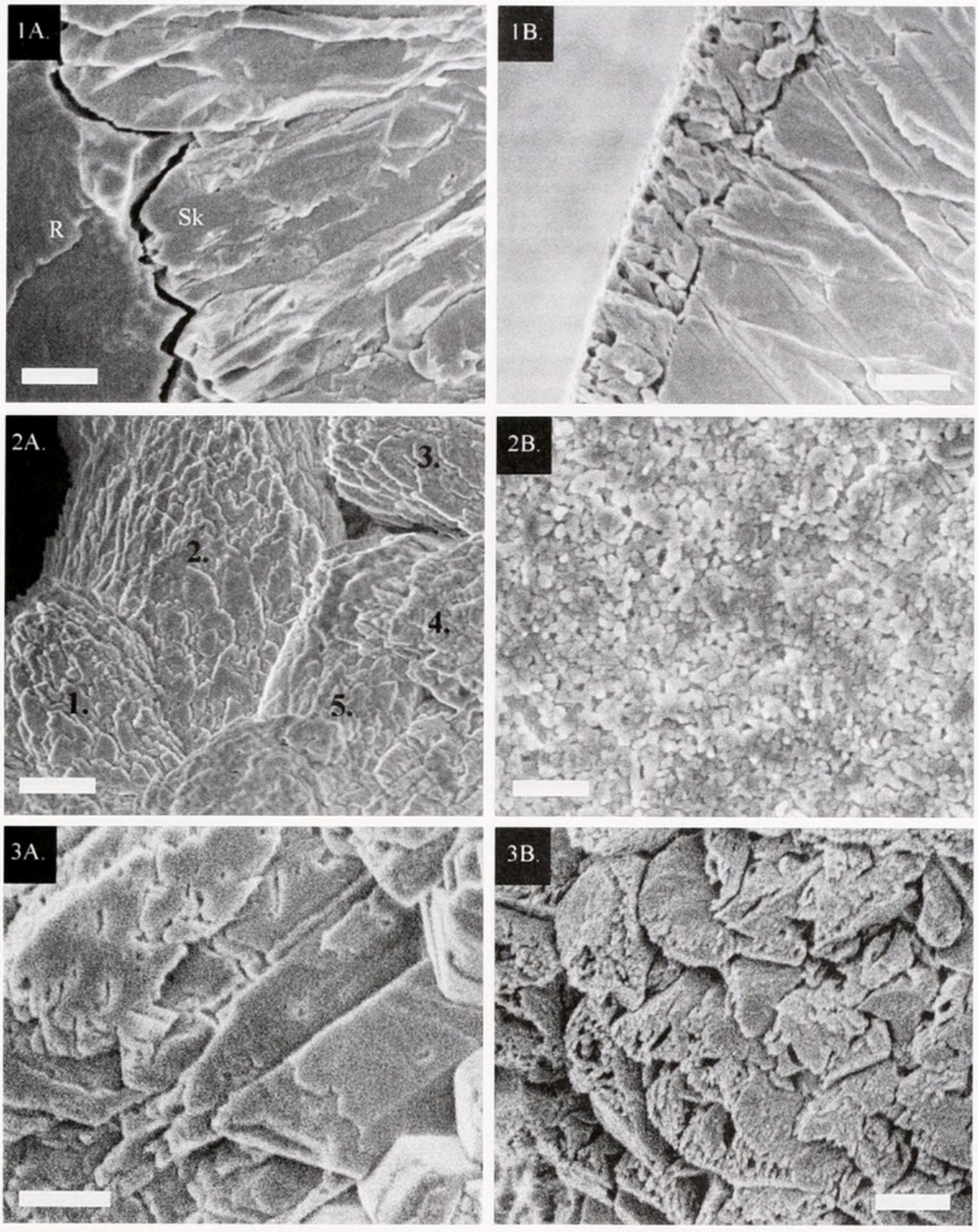

Figure 1. Electron micrographs of cross-fractured exsert septa from *Galaxea fascicularis* corallites sampled (A) directly from the reef flat; and (B) after 26 weeks in a closed-circuit aquarium. Mucus or tissue residue (R) can also be seen in (A) adjacent to the skeleton (Sk). Scale bars: A = 1 μm; B = 500 nm.

Figure 2. Electron micrographs of typical growth surfaces of *Galaxea fascicularis* exsert septa sampled (A) from the reef flat; and (B) after maintenance in a closed-circuit aquarium for 26 weeks. Scale bars: A = 500 nm; B = 300 nm. Distinct clusters of similarly oriented acicular crystals, known as fasciculi, are clearly evident in septa sampled from the reef flat (A; clusters numbered 1–5) but were not observed on septa sampled from the aquarium (B).

Figure 3. Electron micrographs detailing the different nature of acicular crystals at the growing edge of exsert septa of *Galaxea fascicularis* sampled (A) on the reef flat; and (B) at a depth of 9 m. Scale bars: A = 250 nm; B = 400 nm.

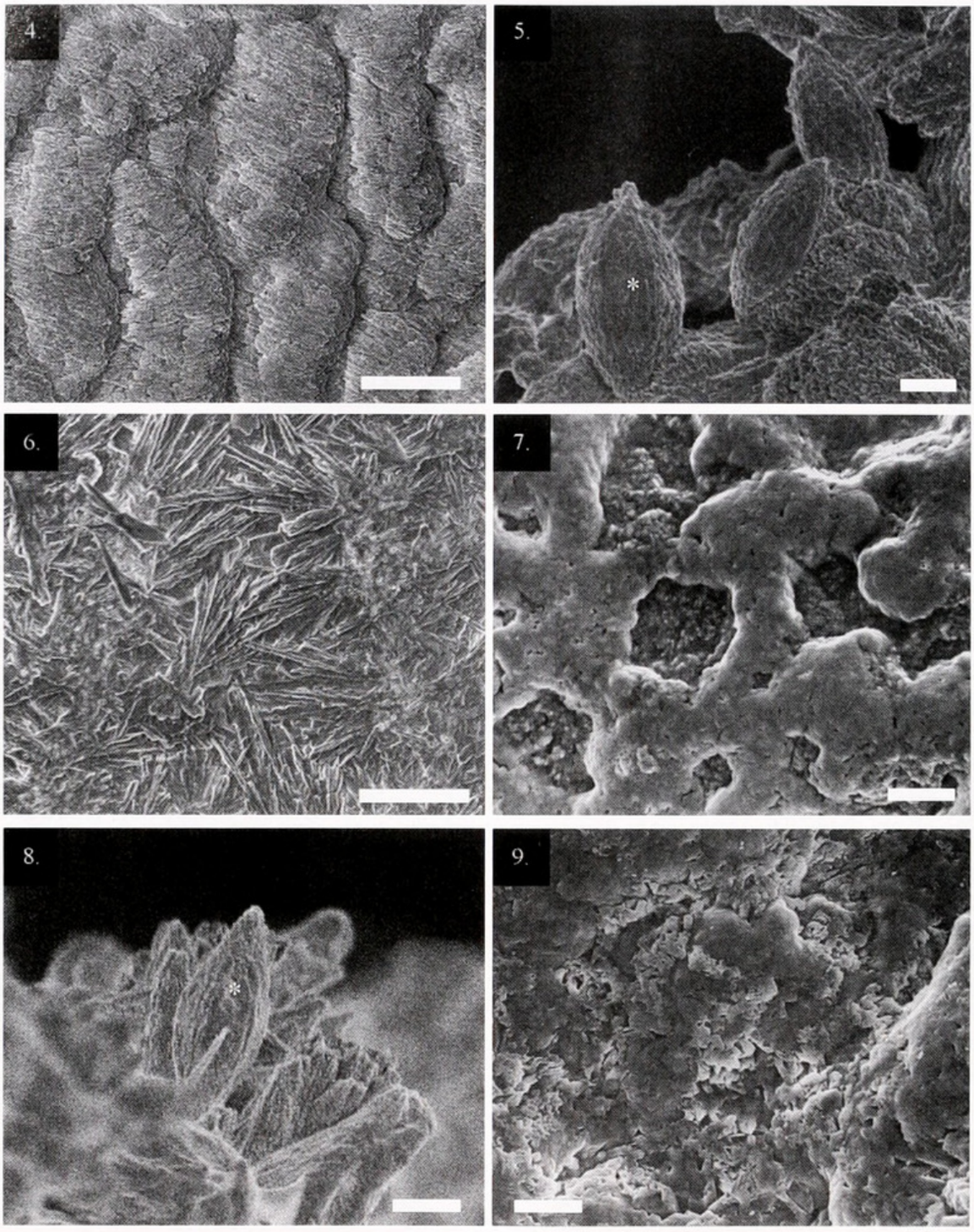

Figure 4. Electron micrograph detailing the crystalline structure of a *Galaxea fascicularis* exsert septum that was readily observed following tissue digestion in commercial bleach. Scale bar = 10 μm.

Figure 5. Electron micrograph of fusiform crystals (*) evident along the lateral edge of a *Galaxea fascicularis* exsert septum prepared in commercial bleach. Scale bar = 1 μm.

Figure 6. Electron micrograph of crystalline artefacts commonly observed on *Galaxea fascicularis* exsert septa prepared in NaOH. Scale bar = 1 μm.

Figure 7. Electron micrograph of amorphous remnants that regularly covered the crystalline surface of *Galaxea fascicularis* exsert septa prepared in distilled water. Scale bar = 5 μm.

Figure 8. Electron micrograph of an *Acropora formosa* axial corallite prepared in distilled water, showing fusiform crystals (*) upon a primary septum. Scale bar = 1 μm.

Figure 9. Electron micrograph of tissue-like remnants that regularly remained attached to *Galaxea fascicularis* exsert septa after preparation in hydrogen peroxide. Scale bar = 5 μm.

corals maintained for long periods in closed-circuit aquaria. In addition, preparing such samples in commercial bleach may result in the complete loss of fusiform crystals from the skeletal surface.

Comparison of field and aquarium samples

It is evident that calcification in scleractinian corals can be severely affected by changes in the surrounding environment. Much recent attention has focused upon how major changes in environmental conditions such as atmospheric CO_2 levels and water temperature affect coral calcification (Done, 1999; Kleypas *et al.,* 1999; Pittock, 1999). Our results indicate that even relatively small modifications to the surrounding environment, particularly those that are artificially generated, may significantly alter the pattern of crystal deposition and the rate of calcification in captive corals. Variability in the growth rate of aquarium-kept corals may be influenced by, and dependent upon, what are often considered minor details, such as water quality and movement, food availability (Mortensen, 2001), and subtle changes in light intensity and wavelength (see Carlson, 1999).

Acicular crystal growth on the exsert septa of *Galaxea fascicularis* is probably continuous, as evidenced by their lack of crystal substructure (Clode and Marshall, 2003). However, septa sampled from aquarium-maintained polyps did not exhibit this pattern of crystal deposition. Instead, small, randomly oriented, spherulitic crystals covered the entire septal surface. As a result of the discontinuous, unordered growth, skeletal porosity was likely to increase. When compared with those growing in their natural environment, a variety of scleractinian corals show a significant reduction in skeletal porosity following containment within an aquarium (see Carlson, 1999).

The fact that the region of disoriented growth in *G. fascicularis* extends to an approximate depth of only 1 μm in septa sampled from corals kept in aquaria for 26 weeks suggests that calcification and growth rates of these corals may be significantly reduced, with drastic changes in calcification patterns also expected. In many branching scleractinian corals, gross colony morphology changes dramatically following maintenance in aquaria (see Carlson, 1999), with colonies developing unnatural morphological traits.

In addition to directly affecting the physical processes of coral calcification, suboptimal conditions may also invoke changes indirectly through stress responses. Colonies maintained under aquarium conditions appeared to lose a large proportion of their symbiotic algae. Loss of zooxanthellae from gastrodermal cells in times of stress is common to many zooxanthellate scleractinian corals (Hoegh-Guldberg and Smith, 1989; Brown *et al.,* 1995), and calcification rates can be greatly reduced through the induced expulsion of these symbionts (Goreau, 1959). Biomineralization anomalies also occur in symbiont-bearing foraminifera exposed to stressful environmental conditions (Toler and Hallock, 1998).

These results raise concerns about the validity of experiments, particularly those investigating calcification rates, *etc.,* with corals kept in closed-circuit aquaria, where artificially modified or unnatural environments may have induced atypical patterns of calcification. Marked differences in skeletal organization between corals sampled from natural and artificial environments highlight the importance of accurately imitating natural conditions in closed-circuit aquaria where experimental corals are to be maintained.

Comparison of the crystal structure of corals living under shallow- and deep-water field conditions revealed only minor differences in septal microstructure. The principal difference in crystal structure between polyps from the two different water depths was that acicular crystals of samples from 9 m were much more finely structured and appeared to have a higher porosity than their counterparts from the reef flat. Skeletal "filling," or secondary thickening (Gladfelter, 1982), was also clearly reduced in septa sampled from corallites collected from the deeper water. A reduced calcification rate because of lower light intensities would account for the differences between septa sampled from the two environments. Further research into the calcification rates of such colonies would help to clarify the cause of the differences, as would an accurate determination of porosity.

Chemical treatments

Despite large variation in skeletal preparatory methods, commercial bleach has been the solvent used most often for digestion of scleractinian coral tissue. Our results confirm that commercial bleach is highly effective in dissolving the soft tissue of the corals *G. fascicularis* and *A. formosa,* particularly when it is heated to 60 °C. Excellent skeletal preparations for SEM can be rapidly obtained with relatively little effort and without discernible deleterious effects upon skeletal microstructure, even if corallites are incubated in bleach for up to 12 h (P. Clode, S. Howe, and A. Marshall, La Trobe University, unpubl. data).

We initially believed that the fusiform crystals we saw on *G. fascicularis* corallites prepared in bleach were artefacts, because they were not present in other preparations. However, the finding that *A. formosa* axial corallites prepared in dH_2O also possessed these crystals ruled out the possibility of a chemically induced origin. We conclude that the additional cleaning of corallites with jets of water and mechanical agitation, which was necessary for corallites prepared in either NaOH, H_2O_2, or dH_2O, removed the fusiform crystals from exposed growing edges.

In *A. formosa* corallites prepared in both bleach and dH_2O, fusiform crystals were observed along the primary septa extending into the calyx, areas protected by the wall of the corallite. Furthermore, the widely spaced skeletal elements and highly perforate nature of the skeleton, which is typical of white-tipped *A. formosa* axial corallites (Oliver, 1984), made the removal of soft tissue easier and the need for extensive cleaning unnecessary. Fusiform crystals observed on axial spines of *Acropora cervicornis* (Gladfelter, 1982) were not seen in *A. formosa.* It is possible that the fusiform crystals on these exposed areas were lost during preparation.

The suitability of NaOH for dissolving soft tissue from *G. fascicularis* corallites is unquestioned, with activity at 60 °C highly effective. However, generation of artefacts and the loss of fusiform crystals from skeletal preparations, which was also noted by Isa (1986) in *Acropora hebes* axial corallites prepared in NaOH, limits the applications of this chemical for coral skeleton preparation.

The use of dH_2O to remove soft tissue was thought to be ideal for maintaining crystal structure, due to the low water solubility of $CaCO_3$ and the unlikelihood of artefact generation. However, we found the method to be time-consuming and difficult, and thus impractical for use with many species of corals. For perforate corals such as *A. formosa,* dH_2O can be effectively applied to remove much of the soft tissue. However, without extensive rinsing, many *G. fascicularis* septa prepared in dH_2O remained coated in a film of unknown material that completely obscured the skeletal structure beneath. The lack of any discernible features in this film suggest that it was not epithelial in nature, but was perhaps either a thin layer of mucus that was subsequently removed in well-washed preparations or a remnant of the mesogloea, as suggested by Hidaka (1991a).

Similarly, the use of H_2O_2 to investigate the skeletal microstructure of *G. fascicularis* exsert septa was inappropriate and labor intensive. As with dH_2O and NaOH, additional cleaning and agitation of samples resulted in the loss of fusiform crystals from the skeletal surface. An additional disadvantage is that H_2O_2 may begin to actively dissolve the $CaCO_3$ skeleton of scleractinian corals (Mitsuguchi *et al.,* 2001).

Our investigations provide an overview of chemical treatments and their suitability for dissolving soft tissue from coral skeleton. We have found that the suitability of these chemicals is species-specific. For example, NaOH is not suitable for use with the azooxanthellate scleractinian corals *Tubastrea faulkneri* and *Dendrophyllia* sp. or the zooxanthellate scleractinian coral *Seriatopora hystrix* (A. Marshall and P. Clode, La Trobe University, unpubl. data). These findings may help to explain why several different techniques have been used in the past and may also account for some of the contradictory results that can be found in the literature on the skeletal and crystal structure of corals.

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