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Dorothy-Ellen A. Renegar  
*Nova Southeastern University, [drenegar@nova.edu](mailto:drenegar@nova.edu)*

Patricia Blackwelder  
*University of Miami, Nova Southeastern University, [pblackwe@nova.edu](mailto:pblackwe@nova.edu)*

Alison L. Moulding  
*Nova Southeastern University, [moulding@nova.edu](mailto:moulding@nova.edu)*

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## Coral ultrastructural response to elevated pCO<sub>2</sub> and nutrients during tissue repair and regeneration

D.A. Renegar<sup>1</sup>, P.L. Blackwelder<sup>1,2</sup>, A.L. Moulding<sup>1</sup>

1) National Coral Reef Institute, Nova Southeastern University Oceanographic Center, 8000 North Ocean Drive, Dania, FL 33004

2) University of Miami RSMAS, 4600 Rickenbacker Causeway, Miami, FL 33149

**Abstract.** Corals and coral reefs have recently experienced widespread decline attributed to anthropogenic pressure on reef systems. Studies have demonstrated that nutrient and pCO<sub>2</sub> stress effect coral growth and calcification, but study of specific effects on coral tissue is lacking. The objective of this research was to examine wound healing in corals and how it is affected by exposure to elevated nutrients and pCO<sub>2</sub>. Coral tissue repair and regeneration during wound healing in *Montastraea cavernosa* and *Porites astreoides* were assessed histologically and ultrastructurally by examining colony fragments exposed to elevated nitrate, phosphate, and pCO<sub>2</sub>. In *M. cavernosa*, tissue repair was facilitated by granular amoebocytes, and the zooxanthellae population size increased under enriched nutrient conditions. In *P. astreoides*, zooxanthellae chloroplasts were markedly abnormal in phosphate-enriched corals, and the concentration of chromophore cells at the healing tissue front was markedly lower under elevated nutrient conditions. The area of wound healed was higher after 14 days under every experimental condition in *M. cavernosa* compared to *P. astreoides*. In both species, phosphate enrichment had the most deleterious effect on repair and regeneration.

**Key words:** coral ultrastructure, tissue repair, pCO<sub>2</sub>, nutrient enrichment.

### Introduction

A growing global population and the close proximity of coral reefs to coastal areas has resulted in increasing anthropogenic pressure on reef systems. In recent history, the implications of environmental change on coral reefs have become progressively more far-reaching. Both eutrophication and global climate change (including increasing atmospheric pCO<sub>2</sub> and temperature) are sources of nonspecific general anthropogenic stress to corals. Other sources, such as over-fishing, sedimentation and turbidity from dredging and beach restoration activities, have deleterious effects on corals (Szmant 2002, Vargas-Ángel et al. 2005). In addition, direct physical damage can result from storms, coral collection, dynamite fishing, blasting, and ship groundings (Curtis 1985, Szmant 2002). Physical damage events, coupled with existing eutrophication stress (a complex process of organic production and accumulation) and changing global climate present a poor outlook for successful natural recovery of reef communities and individual colonies (Szmant 2002).

Elevated nutrient and pCO<sub>2</sub> levels in areas prone to physical damage may contribute to a reduced ability of damaged corals to successfully heal and survive. Significant decreases in calcification rate and/or growth rate have been observed in several species at nitrate concentrations of <5 μM (Tomascik and

Sander 1985, Bell and Tomascik 1993, Marubini and Davies 1996, Renegar and Riegl 2005) and at phosphorus concentrations of >1 μM (Kinsey and Davies 1979, Walker and Ormond 1982, Tomascik and Sander 1985, Ferrier-Pagès et al. 2000, Renegar and Riegl 2005). Increasing atmospheric CO<sub>2</sub> partial pressure (pCO<sub>2</sub>) is predicted to alter ocean surface carbonate saturation, resulting in reduced reef growth (Leclercq et al. 2000, Guinotte et al. 2003, Hughes et al. 2003). The possible effects of low pH and CO<sub>3</sub><sup>2-</sup> (including weaker skeletons and increased erosion) may have a greater impact on net calcification than nutrient enrichment (Kleypas et al. 1999, Marubini and Atkinson 1999).

The process by which tissue repair takes place and normal function restored is a complex process that has been described in other calcifying aquatic organisms but remains largely uninvestigated in scleractinians. The mechanism of invertebrate tissue repair, organic matrix production and skeletal deposition has been ultrastructurally studied, for example, in echinoderms and molluscs (Wilbur 1973, Meenakshi et al. 1975, Blackwelder and Watabe 1977). Mollusc epithelial ultrastructure changes dramatically during repair, and the minerals formed differ from normal morphology and mineralogy (Watabe and Blackwelder 1980). In corals, most studies have focused on physical parameters such as lesion or colony size. Lesional

perimeter length is likely the most important factor in regeneration, although the size, shape and location of lesions can be significant (Meesters et al. 1997, Oren et al. 1998, Lirman 2000). Regeneration may be supported by a limited amount of energy related to the extent of damage, an aspect possibly linked to colony size (Bak and Van Es 1980, Lirman 2000).

A more complete understanding of the effects of anthropogenic environmental factors on coral cell biology is essential to reef management and prediction of the capacity for natural recovery. This goal of this study was to examine the process of tissue repair in corals and how it is affected by elevated nutrients and pCO<sub>2</sub>. The target species, *Montastraea cavernosa* and *Porites astreoides*, are important and widespread Caribbean reef corals.

### Materials and Methods

Four colonies each of *M. cavernosa* and *P. astreoides* were acclimated to laboratory conditions. Colonies were cut into 4 cm<sup>2</sup> fragments. A wound (~4 mm wide and 2 cm in length) was created with a rotary tool in each fragment, and fragments were placed in experimental tanks. Experiments were conducted in 20 separate (8 l) flow-through aquaria partially submerged in a water bath to control temperature variation between tanks. Five treatment conditions were maintained, with two tanks and 32 fragments of each species for each treatment: control; nitrate enrichment; phosphate enrichment; nitrate and phosphate, and pCO<sub>2</sub> enrichment. Each set of treatment tanks was continuously supplied with natural seawater from reservoirs dosed at a specific rate. Irradiance was supplied by metal halide lamps (175 watt, 10,000K, photoperiod 12:12).

Elevated mean nutrient concentrations of 10.8 (±0.5) μM NO<sub>3</sub><sup>-</sup> and 4.4 (±0.3) μM P-PO<sub>4</sub><sup>3-</sup> were achieved by addition of KNO<sub>3</sub> and KH<sub>2</sub>PO<sub>4</sub> to reservoirs supplying the appropriate tanks. Nitrate concentration was determined with NECi Saltwater Nitrate Test Kit (SW-NTK). Phosphate concentration was determined utilizing the method of Parsons et al. (1984). Elevated mean pCO<sub>2</sub> concentrations of 1381 (±66) μatm were achieved with a pH controlled CO<sub>2</sub> injected reservoir system described by Reynaud et al (2003). Total alkalinity and pH were used to monitor pCO<sub>2</sub>.

Corals were maintained under experimental conditions for 14 days. Fragments were then fixed in glutaraldehyde fixative solution [2 mL 70% glutaraldehyde in 68 mL cacodylic buffer (2.16 g cacodylic acid in 200 mL of .22 μm filtered seawater)]. Samples were maintained at 4°C in the fixative solution for 1-2 days, rinsed in buffer, and subsequently post-fixed in buffered 1% osmium tetroxide solution (5 mL 4% aqueous osmium

tetroxide in 30 mL of cacodylic buffer) for 1 hour. Samples were again rinsed in buffer and then dehydrated in a graded series of ethanols. Excess skeleton was trimmed and the samples were embedded in Spurr resin. Ultrathin sections were cut (40 to 60 nm thick) using a Sorval MT-2 ultramicrotome fitted with a diamond knife. Sections were retrieved on nitrocellulose and carbon coated 200 mesh copper grids, stained with Reynolds lead citrate and/or 2% uranyl acetate solution, and viewed in a Phillips 300 TEM.

After fixation and dehydration as discussed above, SEM samples were dried in HMDS, mounted on carbon adhesive covered aluminum stubs, coated with palladium in a sputter coater and examined in an FEI XL-3- ESEM fitted with an Oxford EDS for elemental analysis of the calcified structures.

Histological samples were decalcified after primary fixation in 5% HCl/EDTA solution, dehydrated and embedded in Paraplast®, sectioned and stained with Hematoxylin & Eosin.

### Results

Wound healing and closure was affected by nutrient and CO<sub>2</sub> enrichment. Area analysis of percent wound healing for each treatment was performed using Coral Point Count (Kohler and Gill 2006) (Table 1). In *M. cavernosa*, 15% of the wounded corals for all treatments fully healed after 14 days. In *P. astreoides*, none of the wounded corals fully healed. In both species, phosphate enrichment had the greatest deleterious effect on the percent of wound repaired. The effect of pCO<sub>2</sub> was similar to that of nitrate enrichment alone.

Table 1. CPCe (V 3.5). Mean percent wound healing in each treatment for *Montastraea cavernosa* and *Porites astreoides*.

	<i>Montastraea cavernosa</i>	<i>Porites astreoides</i>
<b>Control</b>	<b>87% ± 11%</b>	<b>24% ± 5%</b>
<b>Nitrate/Phosphate</b>	<b>85% ± 17%</b>	<b>9% ± 13%</b>
<b>Nitrate</b>	<b>79% ± 11%</b>	<b>10% ± 16%</b>
<b>Phosphate</b>	<b>58% ± 26%</b>	<b>2% ± 6%</b>
<b>pCO<sub>2</sub></b>	<b>75% ± 20%</b>	<b>10% ± 11%</b>

*Montastraea cavernosa*. Histological analysis indicated that tissue repair in *M. cavernosa* was characterized by rapid granulation of tissue across the wound site, facilitated by granular amoebocytes. These amoebocytes coalesced to form new tissue at the healing front (arrows) (Figs. 1A & 1C). No histological differences in the coral tissue have thus far been observed between the treatments. However, the zooxanthellae population number appeared to increase in response to nutrient enrichment (Figs. 1B & 1D).

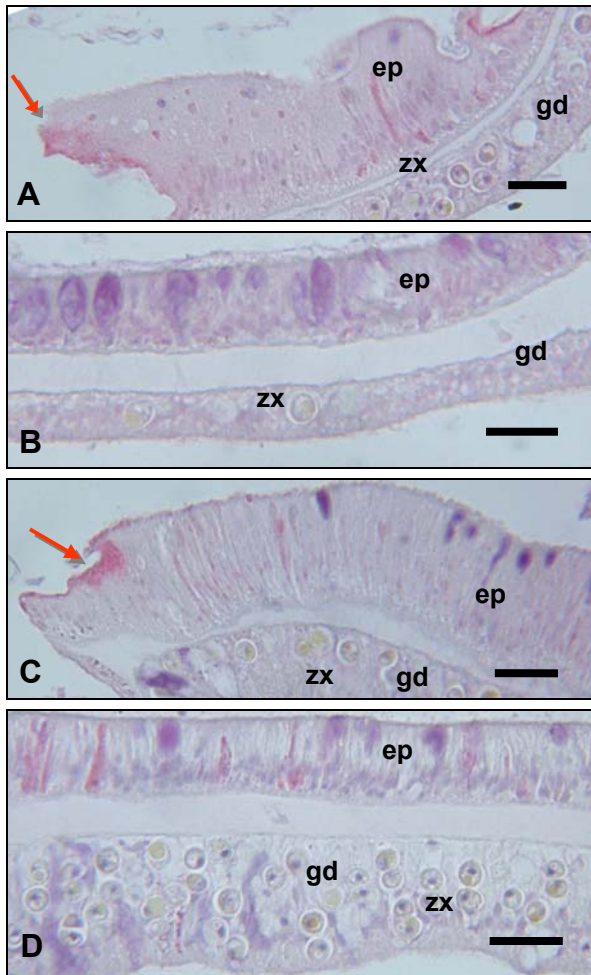


Figure 1. *Montastraea cavernosa*. Histological micrographs. A) Control, B) CO<sub>2</sub> enriched, C) nitrate & phosphate enriched and D) phosphate enriched. ep: epidermis; gd: gastrodermis; zx: zooxanthellae. Scale bars: A, B, C & D = 20 μm.

Ultrastructural observations revealed that granular amoebocytes were migrating to and integrating with new tissue at the repairing interface (Figs. 2A & 2B). Newly formed tissue was dense with well-defined cell walls and a distinct granular appearance (Fig. 2C). Zooxanthellae appeared healthy and were in various stages of cell division. Preliminary data indicates few distinct differences in tissue ultrastructure between nutrient, CO<sub>2</sub>, or control treatments.

***Porites astreoides*.** Tissue repair in *P. astreoides* was characterized by an increased concentration of chromophores near the healing tissue front (arrows). This was pronounced in control and CO<sub>2</sub> treatments (Figs. 3A & 3B). In contrast, the nutrient enriched corals appeared to exhibit fewer chromophores near the repairing front (Figs. 3C & 3D), and the gastrodermis was thickened. Vacuolization was observed in phosphate treated tissue, suggesting zooxanthellae degradation (Fig. 3D).

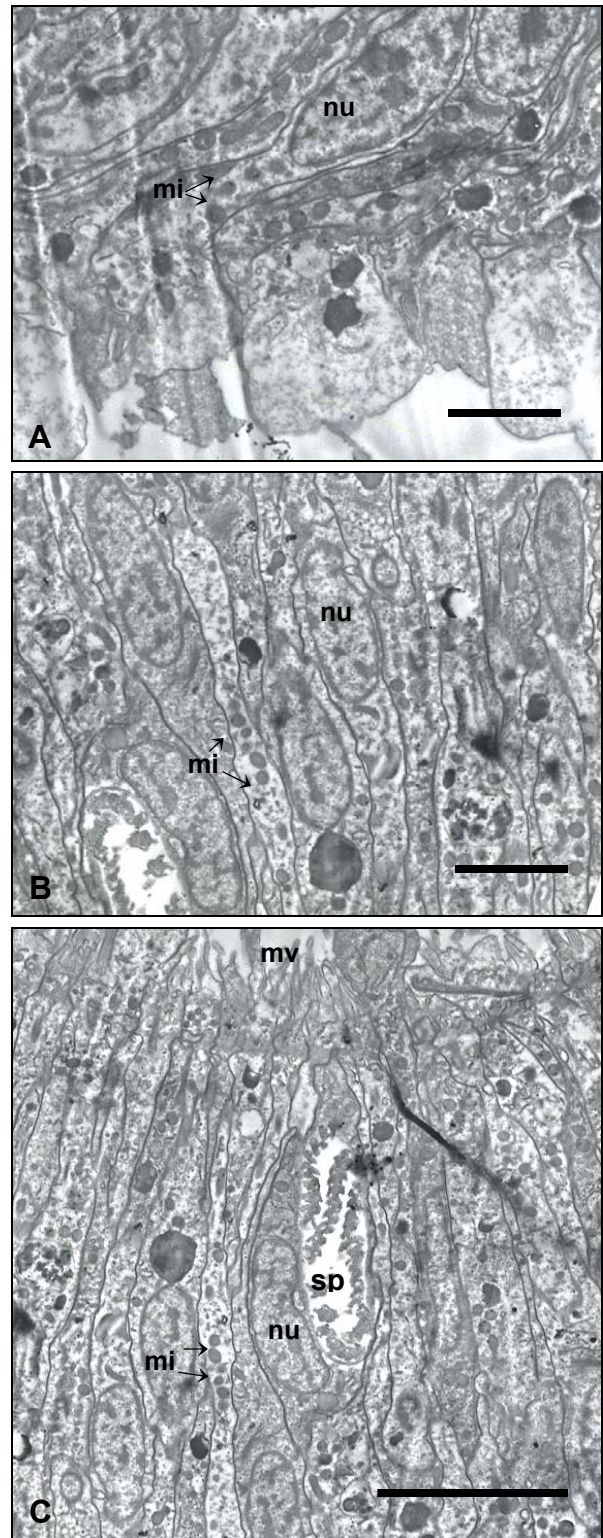


Figure 2. *Montastraea cavernosa*. TEM micrographs. A) Coalescent granular amoebocytes, B) mid-stage tissue formation, and C) late-stage tissue formation. mi: mitochondria; mv: microvilli; nu: nucleus; sp: spirocyst. Scale bars: A = 4 μm, B & C = 5 μm.

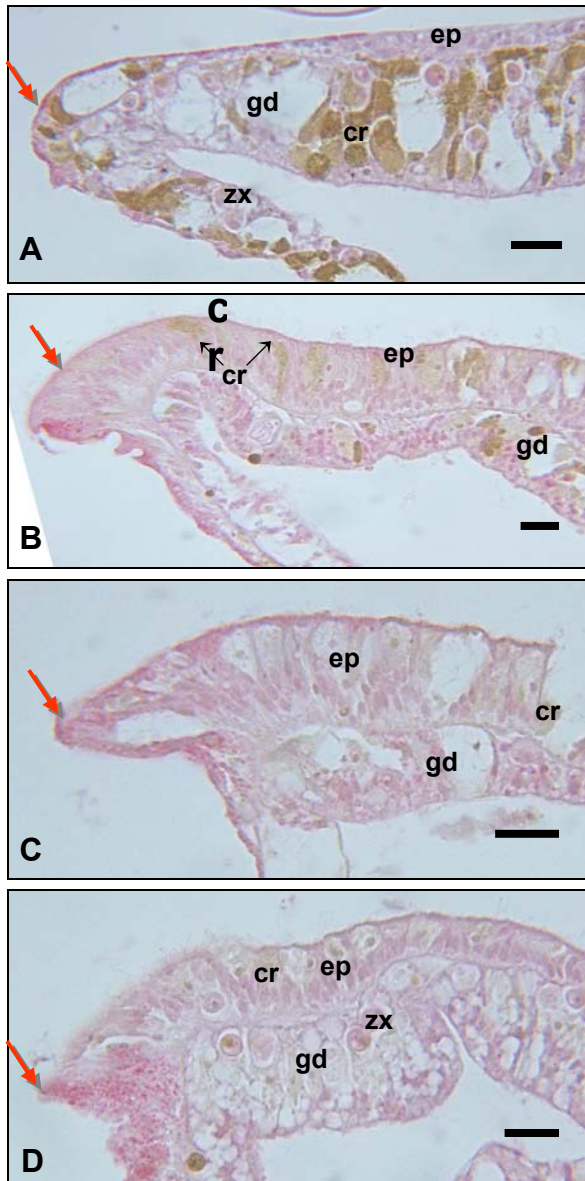


Figure 3. *Porites astreoides*. Histological micrographs. A) Control, B) CO<sub>2</sub> enriched, C) nitrate & phosphate enriched and D) phosphate enriched. cr: chromophore; ep: epidermis; gd: gastrodermis; zx: zooxanthellae. Scale bars: A, B, C & D = 20 µm.

Fine-structural examination (SEM) of the repairing tissue confirmed the presence of chromophores accumulating at the healing tissue front (oval) (Fig. 4A). Ultrastructural examination revealed mature granules within the chromophore cells (Fig. 4B). Abnormal zooxanthellae were observed in the phosphate-enriched corals, with significantly degraded chloroplast lamellae and cellular wall disruption (Fig. 4C). These effects are currently being assessed in greater detail.

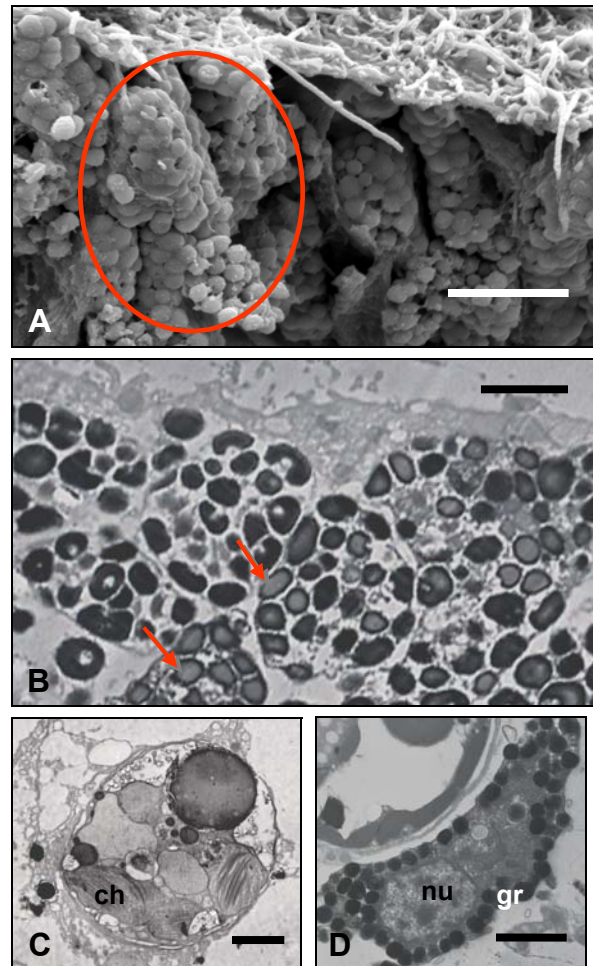


Figure 4. *Porites astreoides*. A) SEM micrograph, chromophore cell accumulation in healing tissue. TEM micrographs, B) mature granules in chromophores, C) phosphate-enriched zooxanthellae and D) mast-type cell in early stage tissue repair. ch: chloroplast; gr: granule; nu: nucleus. Scale bars: A = 5 µm, B, C & D = 2 µm.

## Discussion

Tissue repair in *M. cavernosa* was characterized by granulation of new tissue across the wound site, facilitated by coalescent granular amoebocytes. This is similar to observations in gorgonians (Meszaros and Bigger 1999). As little organic matrix was observed associated with the calicodermis near the repairing front, the wound healing strategy of this species appears to emphasize rapid wound closure and formation of new tissue before calcification resumes. The percentage of wound repair was highest in the controls, and lowest in the phosphate treatment. Zooxanthellae concentration appeared higher near the healing front in the nutrient treatments compared to the control or CO<sub>2</sub> treatments. The variation in zooxanthellae concentration is likely due to the interaction between regeneration energy demands and the presence of limiting nutrients, as overall increases in zooxanthellae concentrations have been found in

wounded gorgonians compared to non-wounded (Meszaros and Bigger 1999). This aspect is currently being studied in greater detail.

In contrast to *M. cavernosa*, the wound repair strategy of *P. astreoides* appeared to involve rapid sealing and reorganization of the tissue and continuation of calcification, with closure achieved by recalcification across the wound. This is supported by the marked effect of nutrients and pCO<sub>2</sub> on wound closure rate in this species. Accumulation of chromophores at the healing tissue front was notable in the control and CO<sub>2</sub> treatments, in contrast to the few seen adjacent to the tissue front in the nutrient treatments. Interestingly, the ultrastructure of some chromophores was similar to vertebrate mast cells (Fig. 4D) (Porter and Bonneville 1974). Chromophores may have many functions, including involvement in tissue repair and the coral immune response. For example, differences in chromophore ultrastructure have been observed in healthy vs. diseased *P. astreoides* (Kaczmarsky, pers. com.).

The synergistic effect of nitrate and phosphate combined appeared to be antagonistic in both species. Similar observations have been made regarding growth rate and calcification in other coral species. The effect of nitrate and phosphate combined on the growth rate of *Acropora cervicornis* was additive at low concentrations (5 μM NO<sub>3</sub><sup>-</sup> and 2 μM P-PO<sub>4</sub>), and antagonistic at high concentrations (10 μM NO<sub>3</sub><sup>-</sup> and 4 μM P-PO<sub>4</sub>) (Renegar and Riegl 2005). This effect may be a consequence of disruption of the coral-zooxanthellae relationship resulting from the stress of and energy requirements for regeneration and repair (Meszaros and Bigger 1999).

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