An evaluation of staining techniques for marking daily growth in scleractinian corals

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Abstract

In situ skeletal markers have been widely used to quantify skeletal growth rates of scleractinian corals on sub-annual time-scales. Nevertheless, an evaluation of different techniques, both in terms of their efficacy and potential impacts on the growth process itself, has not been undertaken. Here the effects of exposure to four different dyes (alizarin, alizarin complexone, calcein, oxytetracycline) and isotope spikes (Ba and Sr) on the growth rates of scleractinian corals are compared. Oxytetracycline increased coral growth. Alizarin, alizarin complexone, calcein, and Sr and Ba isotope spikes had no significant effect on coral growth, but polyp extension appeared reduced during exposure to alizarin and alizarin complexone. Calcein provided a more intense fluorescent mark than either alizarin or alizarin complexone. Isotope spikes were challenging to locate using isotope ratio analysis techniques. Thus, calcein appears best suited for marking short-term calcification increments in corals, while a combination of alizarin or alizarin complexone and calcein may be useful for dual labeling experiments as there is little overlap in their fluorescence spectra.

Keywords: alizarin, calcein, isotope, coral, calcification, stain

1. Introduction

Mounting concern about the impacts of climate change, ocean acidification and direct anthropogenic activities on coral reef ecosystems has spurred the need for accurate and precise quantification of rates of skeletal growth of corals and other calcifying organisms, on diurnal through seasonal timescales, in both field and laboratory experiments. The most frequently used measure of skeletal growth rate in corals is based on annual banding, high and low density couplets that together represent one year. While extremely successful and widely used (e.g. Buddemeier 1974; Macintyre and Smith 1974; Cantin et al., 2010), this technique limits measures of growth to timescales of one year or longer, and cannot resolve sub-annual growth responses that are essential for understanding calcification responses to changes in light, temperature, nutrient availability, carbonate ion concentration, photosynthesis and catastrophic events such as storms.

A wide range of approaches have been developed to estimate coral growth on sub-annual timescales, including: alkalinity uptake (e.g. Smith 1973; Jacques and Pilson 1980), changes in buoyant weight (e.g. Davies 1989), and radioisotope incorporation (e.g. Tambutte et al., 1995), direct physical measurement (e.g. Cruz-Pinon et al., 2003), time lapse photography (Barnes and Crossland 1980), laser diffraction (Stromgren 1976; Vago et al., 1997), and the use of various dye, elemental, and isotope spikes. Dye- and isotope-based approaches are commonly used in a range of calcifying organisms and offer many advantages over other approaches. In particular, dye and isotope based marks are easily implemented in field settings, can be used in-situ, offer the ability to mark large numbers of organisms at the same time, and can be used on corals of vastly different sizes – from newly settled polyps to colonies meters across. Due to their ease of detection, dyes are

commonly used to provide a time point marker within the coral skeleton as a means of estimating coral growth or identifying skeleton deposited within a particular time interval (Barnes 1970, 1972; Gladfelter 1983; Cohen et al., 2004; Marschal et al., 2004; Raz-Bahat et al., 2006; Tambutte et al., 2011; Venn et al., 2011). However, one commonly used dye, alizarin, has been shown to negatively affect the growth of corals (Dodge et al., 1984), thus alternative dyes are desirable. Here, four dyes (alizarin, alizarin complexone, calcein, and oxytetracycline) and stable isotope spikes (commonly used to mark fish otoliths (Thorrold et al., 2002; 2006) and bones (Sun et al., 1992)) were used to mark the skeletons of corals to assess if they were effective in marking coral skeletons and if exposure to the dye impacted coral growth. Absorption spectra for each dye in seawater are presented to assist in choosing dyes which do not absorb light in regions of the spectrum which may be of experimental interest. Emission spectra for each dye incorporated into the skeleton are also presented to aid in choosing appropriate filter sets for imaging the dyes with fluorescence microscopy.

2. Methods

2.1. Dyes

One of four dyes was used in each dye incubation to mark the skeleton: alizarin red S (sodium salt – Alfa Aesar 42040 lot E22R017 – referred to as alizarin throughout this manuscript), alizarin complexone (Alfa Aesar A16699 lot E8180A), calcein (Alfa Aesar L10255 lot USLF006789 - this particular lot was soluble in distilled water, suggesting it was in the form of a salt), and oxytetracycline HCl (USB 23659 lot 113648). In addition,

isotope spikes (Ba 135 or Sr 86, purchased as carbonate salts from Oak Ridge National Lab) were used as markers in some incubations.

All dyes and isotope spikes were added as aliquots of stock solutions. Spikes were mixed with sufficient HCl to dissolve the carbonate salt and made up in distilled water to make stock solutions of which 50-125 μl was used per liter of seawater. For Sr isotope spikes, 50 μl of a given stock solution added to a liter of seawater doubled the concentration of that particular Sr isotope. For Ba isotope spikes, 50 μl of stock solution almost doubled the total Ba concentration.

2.2. Coral maintenance

Colonies of the temperate scleractinian coral *Astrangia poculata* were collected and processed as described by Holcomb et al. (2010), except that in addition to colonies, newly settled polyps and their associated substratum were also attached to slides. All slides with corals were suspended vertically in a flow-through aquarium receiving filtered (20 μm) Vineyard Sound seawater. Incoming seawater was heated in the winter, thus corals experienced a temperature range of 14-30 ^{o}C, temperatures at the time of experiments are as specified. Aquaria were aerated to maintain water circulation. Corals were maintained under aquarium conditions for at least one month prior to use in experiments. A mixture of brown and white colonies (zooxanthellate and azooxanthellate colonies) each ~2-5 cm in diameter was used for all treatments. Corals were fed regularly with newly hatched and frozen brine shrimp.

For marking experiments, corals were placed in pre-washed (with fresh and seawater) 1 L PET food service containers with lids (SOLO) containing ~800 ml of water from the source aquarium. Airstones were added to each container and each container bubbled

continuously. Containers were held within a water bath with a temperature similar to that of the source aquarium.

2.3. Dyeing corals

In dye experiments with *A. poculata* (March – Oct. 2009), growth rates were estimated via alkalinity depletion measurements the day before (pre-treatment), the day of (treatment), and the day after (post-treatment) dye exposure. All alkalinity incubations were ~24 hrs in duration, covering a full light-dark cycle. The temperature range was 25-26 ^{o}C. For each treatment 4-7 corals were used, each in an individual incubation container. At the same time as dye treatments, additional corals not exposed to dye were also measured to control for day-to-day variations in growth. Incubations were carried out in 1 L PET food service containers: ~800 ml (actual amount weighed to 0.01 g) of water from the source tank was added to each container, and a coral added. Containers with no coral added were used to estimate background changes in alkalinity. Irradiance (PAR – measured with a diving-PAM underwater quantum sensor (WALZ)) ranged from 10-40 μmol photons/m2/s with a 12 hr light dark cycle (white colonies were incubated under the lower end of the range of light levels, brown colonies under the higher light levels – a similar light gradient was present in the source tank due to different corals being at different distances from the light bulbs or being closer to the ends of the light bulbs which produce less light than the center). Light was provided by two T5-HO bulbs (10000 K, 54 w).

Alkalinity samples were taken from each container ~1 hr after the corals had been added and again at the end of the incubation. Waiting 1 hr after the addition of the coral to take

124 the first sample was intended to allow the coral to recover from any handling stress and
125 thus avoid capturing any temporary changes in calcification. Salinity (Hach conductivity
126 probe – read to 0.1, accurate to ~1) and pH (NBS scale, Thermo-Orion ROSS
127 8165BNWP electrode, read to 0.1 mV) were measured at the end of each incubation for
128 every container, as well as at the start of incubations for a subset of the containers.
129 Aragonite deposition was assumed to be the only process affecting alkalinity, with 2 mol
130 alkalinity consumed per mol of $CaCO_3$ deposited. This may under-estimate calcification
131 as any ammonia released by the coral will increase the alkalinity of the solution (e.g.
132 Jacques and Pilson 1980). Alkalinity depletion rates were corrected for evaporation
133 (based on the change in container mass), and for background rates measured in containers
134 containing no slides. Background alkalinity consumption rates were invariably low, with
135 the highest rates being <10% of coral rates.
136 Final dye concentrations were as follows: 2.7-3.2 mg/kg alizarin (added as ~0.2 ml of
137 stock solution/L, pH not adjusted, but pH declined <0.01 upon dye addition), 8.6-8.8
138 mg/kg alizarin complexone (added as ~1 ml of stock solution/L with sufficient NaOH to
139 dissolve, pH declined ~0.03 upon dye addition), 9.5-10 mg/kg calcein (added as ~0.8 ml
140 of stock solution/L, pH of the stock solution was not adjusted, thus pH declined ~0.03
141 upon dye addition), 24-26 mg/kg oxytetracycline (added as ~0.3 ml/L of stock
142 suspension, pH adjusted with NaOH, no measureable pH change upon addition).
143
144 *2.4. Isotope spikes*
145 Marking corals with isotope spikes was carried out as a part of long term growth
146 experiments (see Holcomb et al. 2010, 2012); data from control corals included in those

147 experiments are presented here. Isotope experiments were carried out using two different
148 isotopes, ^{86}Sr and ^{135}Ba, with 6-16 corals for each treatment. Marking with ^{86}Sr was
149 carried out in much the same manner as used for dye experiments, with 60 μl of an ^{86}Sr
150 solution added to ~800 ml seawater and corals incubated for two days. Growth was
151 estimated from changes in buoyant weight (per Holcomb et al., 2010) for the 5 months
152 prior to and the month following the isotope spike. Corals were held at one of two
153 temperatures - ~19 or ~26^{o}C throughout that six month period.
154 Spikes with ^{135}Ba were carried out in a flow-through aquarium system as used by
155 Holcomb et al. (2012). Each reservoir used to supply water to individual aquaria was
156 spiked with 81 μl ^{135}Ba solution/L seawater. Individual aquaria received spiked seawater
157 for a period of two days: unspiked seawater was then added to the reservoir, diluting the
158 spike ~80%, and each subsequent day the remaining spike was diluted by an additional
159 ~60%. Buoyant weights were measured for the two months prior and one month
160 following isotope exposure using a Sartorius G803S balance, aquaria were held at either
161 16 or 24 ^{o}C throughout this period.
162
163 *2.5. Alkalinity*
164 Alkalinity samples were taken in pre-cleaned glass or plastic scintillation vials with screw
165 top lids and foamed polyethylene liners (Wheaton). Samples were stored refrigerated for
166 no more than 1 month prior to measurement. Alkalinities were measured via titration
167 with 0.01 N HCl containing 40.7 g NaCl/L using a Metrohm Titrando 808 dosimat and
168 730 Sample Changer controlled by Tiamo software to perform automated normalized
169 Gran titrations of 1 ml samples. Duplicate samples were run and additional replicates run

if measured values differed by more than 4 μmol/kg. Certified seawater reference material supplied by the lab of Andrew Dickson (Scripps Institution of Oceanography) was run each time samples were run.

2.6. Spectra

2.6.1. Absorbance spectra

To estimate the potential effect of the presence of the dye on the light spectra received by the coral, the absorbance spectra of each dye in seawater was measured using an Ocean Optics USB4000 spectrophotometer configured for measuring the visible spectrum with a blue filtered (SCHOTT glass BG-34 filter) tungsten light source (LS-1) and a 1 cm cuvette. In addition to the dyes used for the coral experiments, the absorption spectra were also measured for other lots of calcein (from Alfa Aesar, Invitrogen, and Sigma) and oxytetracycline (Acros). Different lots of calcein were found to be highly variable in their appearance and solubility – some being readily soluble in distilled water, while others requiring addition of NaOH to dissolve. Even when purchased as a sodium salt, not all lots were soluble in distilled water. Thus when preparing calcein stock solutions the manner of preparation will depend greatly on the lot of calcein used. It may be possible to make up the solution directly in water, or it may require the use of a base, such as NaOH or $NaHCO_3$ (per Wilson et al., 1987) to solubilize the calcein; it is advisable to check the pH of calcein stock solutions and adjust as needed before use.

2.6.2. Emission spectra

192 A Leica TCS SP5 confocal microscope at the Centre Scientifique de Monaco was used to
193 measure the emission spectra of each dye incorporated into the coral skeleton. Polished
194 sections of dyed coral skeletons were prepared using standard methods with water or
195 mineral oil used to suspend polishing compounds (e.g. Holcomb et al., 2009).
196 Fluorescence was excited using one of three lasers: a 543 nm He/Ne laser, a 488 nm Ar
197 laser, or a 405 nm diode laser. The resulting fluorescence spectra were captured with the
198 confocal microscope.
199
200 *2.7. Statistics*
201 All data for dye comparisons were expressed as relative growth rates - the ratio of the
202 post treatment growth rate to the pretreatment growth rate - for statistical and plotting
203 purposes (see Holcomb et al., 2012 for discussion of this normalization approach).

204 $$relative\ growth = \frac{\frac{\Delta\ mass\ \ post\ treatment}{\Delta\ time\ \ post\ treatment}}{\frac{\Delta\ mass\ \ pre\ treatment}{\Delta\ time\ \ pre\ treatment}}$$

205 Rates were further corrected for day to day changes in calcification by dividing by the
206 average relative growth rate of untreated corals run at the same time. Similar patterns
207 were observed for both brown and white colonies, thus data were pooled.
208 Differences among treatments were detected using a Kruskal-Wallis test (Systat 9), and if
209 warranted, nonparametric multiple comparisons were performed to compare treatments to
210 controls per Zar (1984). For isotope treatments at different temperatures (Fig. 2), growth
211 rates were normalized to starting mass; a sign test (Zar 1984) was used to test whether the
212 post isotope treatment growth rate differed from the pre-treatment rate at each
213 temperature.

3. Results

All dyes used gave detectable marks in the skeleton and all corals survived exposure. Growth rates of *A. poculata* were reduced during exposure to all dye treatments except oxytetracycline; rates during exposure were 84%, 77% and 70% of initial rates for alizarin, alizarin complexone and calcein respectively (Fig. 1). Growth rates during oxytetracycline treatment were higher (114%) than initial rates. Following dye exposure, growth rates returned to near pre-treatment rates – 99%, 125%, and 115% of initial rates for alizarin, alizarin complexone and calcein respectively. Growth rates following oxytetracycline exposure, however, were significantly ($p<0.01$) higher (168%) than pretreatment rates. Exposure to isotope spikes had no measurable effect on coral growth (Fig. 2)

Absorption spectra (Fig. 3) measured in seawater showed peak absorbances for oxytetracycline at ~380 nm, calcein at ~486 nm, alizarin at ~540 nm, and alizarin complexone at ~550 nm. Different lots of calcein and oxytetracycline had similar spectra, despite differences in solubility and appearance.

Emission spectra (Fig 4) of dyes incorporated into coral skeletons showed peaks at ~550 nm for oxytetracycline when excited at 405 nm, ~520 nm for calcein when excited at 488 nm, ~610 nm for alizarin and ~630 nm for alizarin complexone when excited at 543 nm.

4. Discussion

All dyes employed in this study proved effective in marking coral skeletons. However, consistent with previous reports of alizarin negatively affecting growth (Dodge et al.,

1984), growth rates tended to be slightly lower following exposure to alizarin (Fig 1), and, though not quantified, polyps appeared less expanded during exposure to alizarin. Oxytetracycline significantly increased coral growth rates (Fig. 1C). Though it is not known why oxytetracycline increases growth rates, corals, such as *Astrangia poculata*, are often host to a wide range of endolithic organisms which erode the skeleton from within (e.g. Tribollet et al., 2009). Oxytetracycline may negatively impact some of the boring organisms and thus could decrease dissolution rates, thereby increasing net calcification. Calcein, alizarin complexone and isotope spikes had no measurable effect (Fig. 1,2), and for calcein and isotope spikes, there was no apparent difference in polyp behavior during exposure.

Isotopes

A few studies have used stable isotope markers in calcium carbonates (Thorrold et al., 2006; Houlbreque et al., 2008; Holcomb et al., 2009; Ries et al., 2010), but this approach is not common due both to the expense of the spikes and to the difficulty of detecting the spikes once in the skeleton, which generally requires specialized instruments such as secondary ion or laser ablation mass spectrometers. In principle, however isotope based techniques for marking coral skeletons offer several advantages. The use of isotopes with low natural abundances can allow a relatively small change in the concentration of a given element to yield a large change in the isotopic composition. Isotope spikes had no measurable effect on coral growth (Fig. 2), and since the elements used occur naturally in the skeleton and surrounding seawater, a small change in their concentration would not be expected to affect growth. Radio-isotopes have been used (Bonham 1965; Knutson et

al., 1972; Marshall and Wright 1998) and the detection means are relatively accessible. However, the risks associated with artificially spiking corals with radioisotopes, and regulatory concerns, may make their use undesirable. The use of stable isotopes avoids these problems, and, since there are multiple stable isotopes available for many of the elements found in coral skeletons, it is possible to introduce distinct isotopic markers at different time points. Our results suggest that even with specialized instrumentation, dyes are still more convenient than stable isotopes for mapping calcification at high spatial resolution over large areas of the skeleton (see supplementary figures). Depending on which salt is purchased (e.g. carbonate or chloride), isotope spikes may require dissolution in acid and pH adjustment prior to use.

Dyes

Dye based methods have the potential to change growth due to the introduction of a foreign substance (e.g. Ibsen and Birkedal 2010), and due to their inherent absorption of light, they will change the light spectrum (Fig 3) received by the coral during the incubation, potentially affecting growth (e.g. Kinzie et al., 1984). Dyes have the potential to have long-term effects on the reflected light spectrum and thus may alter an important source of light for photosynthesis (e.g. Falkowski et al., 1990; Kuhl et al., 1995; Enriquez et al., 2005). Despite their limitations, dyes also offer many advantages. A range of dyes are available, so it is possible to introduce multiple time markers, and the dyes are all visualized with widely available techniques – visible light (alizarin, alizarin complexone) or fluorescence (alizarin, alizarin complexone, calcein, oxytetracycline)

microscopy (see supplemental figures 1,2), and dye methods require relatively little specimen preparation.

Alizarin

Alizarin has a long history of use for marking coral skeletons and has been used on a wide range of species (e.g. Barnes 1970, 1972; Cohen et al., 2004; Trotter et al., 2011). Alizarin is readily available as a sodium salt making solution preparation simple and reducing the need for pH adjustment. We have used various lots of alizarin and the dye appears fairly consistent lot to lot. The pink color of alizarin incorporated into the skeleton is readily distinguished from the unstained skeleton under visible light. Alizarin can be detected with fluorescence microscopy too, and its absorption and emission spectra are sufficiently different from those of calcein and oxytetracycline that it can potentially be used for dual labeling experiments. The pink-purple color of water containing alizarin allows its presence to be readily verified.

Unfortunately, alizarin can also negatively impact the growth of corals (e.g. Dodge et al., 1984). Our own observations suggest that 12 hr exposures to 10 mg alizarin/L seawater can kill some species of corals and reduce polyp extension in other species yet have no effect on still others (we have since used <5 mg alizarin/L seawater and at this dye concentration have not observed any mortalities for a range of species). Alizarin will precipitate out of seawater if present at high concentrations (Barnes 1972), thus when adding concentrated stock solutions the seawater must be vigorously mixed to prevent the formation of precipitates.

Alizarin complexone

Alizarin complexone is similar in many respects to alizarin, with the advantage that it has not been found to reduce the growth of any coral species, although our results suggest it may reduce polyp extension in *Astrangia poculata*. Unfortunately, alizarin complexone is not as easy to distinguish from unmarked skeleton using transmitted light microscopy – the purple coloration of alizarin complexone provides nowhere near the contrast of alizarin. However, it can be seen readily with fluorescence microscopy (see supplementary materials), with a spectrum similar to that of alizarin. We have only used a single lot of alizarin complexone; for that lot a base must be used to get the alizarin complexone into solution, and pH adjustment is advisable.

Calcein

Calcein does not appear to affect coral growth and has been recommended over alizarin and Sr marking for some shellfish species (Riascos et al., 2007; Herrmann et al., 2009), though it may affect the growth of some organisms (Thebault et al., 2006). The effect of calcein on the incorporation of Sr and Mg into calcite has been investigated, and it has been found not to significantly affect incorporation of these elements (Dissard et al., 2009). The brilliant yellow-green color of calcein in seawater makes it easy to detect when it is present. The formation of precipitates was not observed to be a problem, making it convenient to inject a concentrated stock solution into bags surrounding corals for field marking. Fluorescence microscopy is required to detect calcein in the skeleton as the yellow-orange color of aragonite containing calcein is difficult to see, while the intense fluorescence of calcein is easily detected. Unfortunately there appears to be

considerable variability between suppliers, and for certain suppliers, lot-lot variability in the composition of the material sold as calcein. So depending upon the lot, base may be needed to dissolve the calcein or it may be readily soluble in distilled water, and pH adjustment may or may not be needed.

Oxytetracycline

Oxytetracycline suffers from relatively low solubility at seawater pH making preparation of concentrated stock solutions more difficult, and if used without pH adjustment, it will reduce pH. The increase in growth observed following oxytetracycline exposure suggests that it affects the coral holobiont, and should be used with caution.

Of the dyes used, calcein appears to be the most satisfactory for marking coral skeletons. Calcein had no detectable effect (negative or positive) on coral growth and no obvious effect on tissue expansion, it is readily available, and relatively soluble (in alkaline solutions). The brilliant yellow-green color of the water following its addition makes it easy to verify it is present in field settings, and its strong fluorescence signal allows it to be easily detected. Additionally, calcein has the potential to be used with alizarin or alizarin complexone to carry out dual marking studies.

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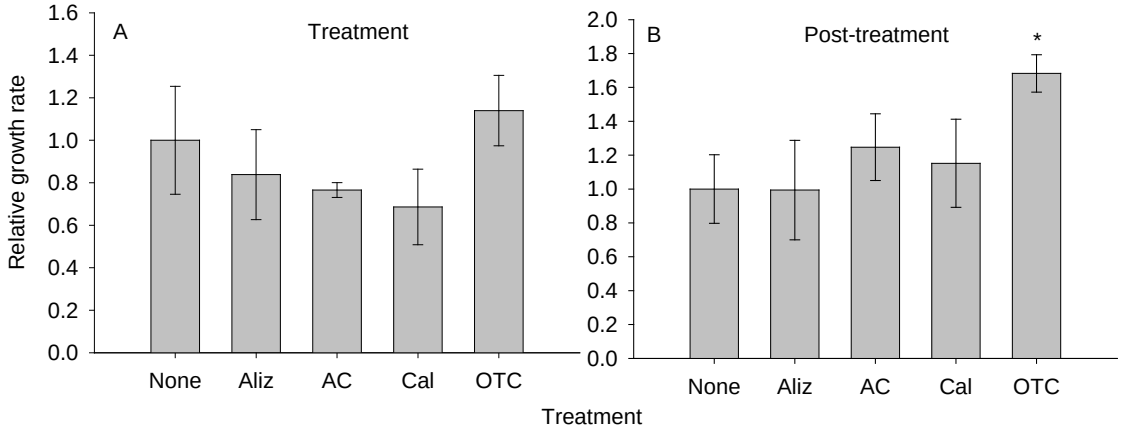
Figure Legends

Figure 1. Relative growth rates of corals versus treatment based on changes in alkalinity depletion measured during dye exposure (A), and following dye exposure (B). Bars represent average values for 11 untreated corals, 7 for alizarin, 4 each for alizarin complexone and calcein, and 5 for oxytetracycline. Treatments are as follows: None = untreated control, Aliz = alizarin, AC = alizarin complexone, Cal = calcein, OTC = oxytetracycline. Bars are means, error bars are standard deviation. The treatment significantly ($p<0.01$) different from control (OTC in B) is indicated by "*".

Figure 2. Growth rates normalized to skeletal dry weight for *Astrangia poculata* specimens treated with isotope spikes at different temperatures. At 26 ^{o}C and 19 ^{o}C, corals were treated with ^{86}Sr, 16 corals were used at 26 ^{o}C, 6 at 19 ^{o}C. At 24 ^{o}C and 16 ^{o}C, corals were treated with ^{135}Ba, 10 corals were used at 24 ^{o}C, 8 at 16 ^{o}C. Black bars are pre-treatment rates, gray bars are post treatment rates, values are means, error bars are standard deviation. Post-treatment growth rates did not significantly ($p<0.01$) differ from pre-treatment rates.

Figure 3. Absorption spectra for each dye in seawater. Alizarin (Aliz) 6.6 mg/kg, alizarin complexone (AC) 4.9 mg/kg, calcein (Cal) 8 mg/kg, and oxytetracycline (OTC) 26 mg/kg.

Figure 4. Fluorescence emission spectra from coral skeletons (*Porites* and *Goniastrea*) containing different dyes, as well as background spectra taken on adjacent unstained regions of the skeleton, with fluorescence excited using different wavelengths. A. 405 nm excitation. B. 488 nm excitation. C. 543 nm excitation (some spectra also include 488 nm excitation). Note, the portion of the spectra within 10 nm of the excitation wavelength(s) has been deleted for clarity. Spectra are shown for OTC = oxytetracycline, BKG = background, taken on an unstained region of the coral, Cal = calcein, AC = alizarin complexone, CalAliz = calcein and alizarin staining the same region, Aliz = alizarin, Cal Aliz AC = calcein, alizarin, and alizarin complexone staining the same region.



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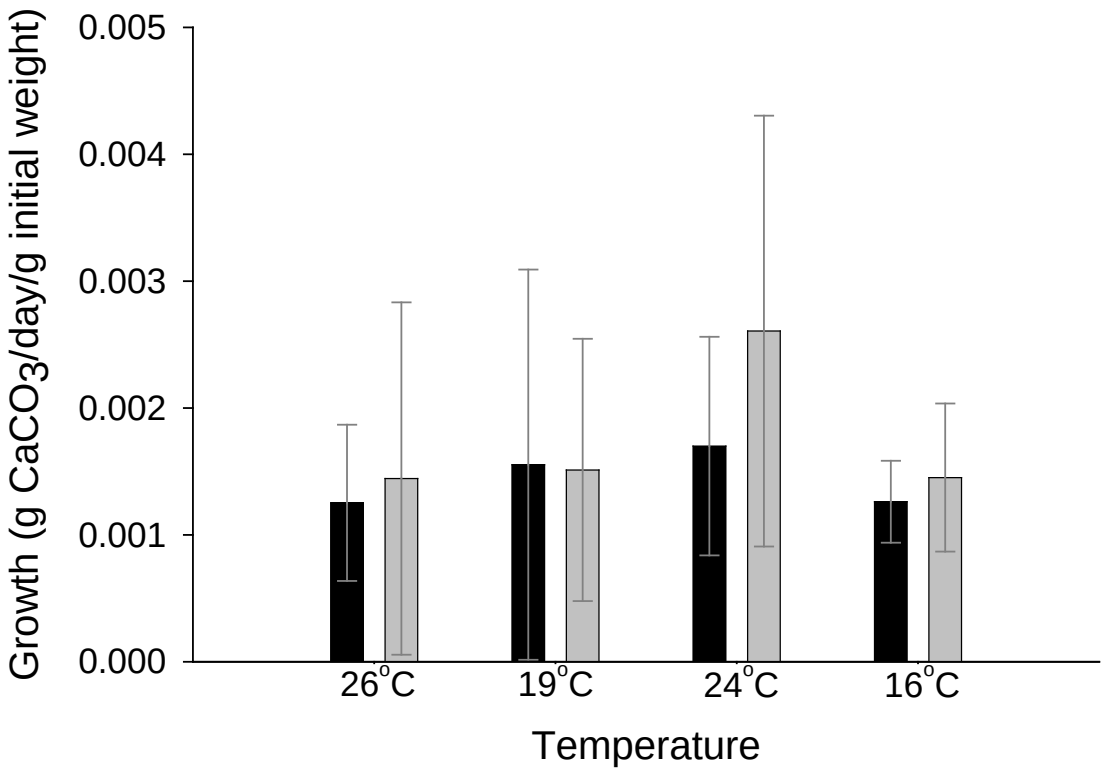

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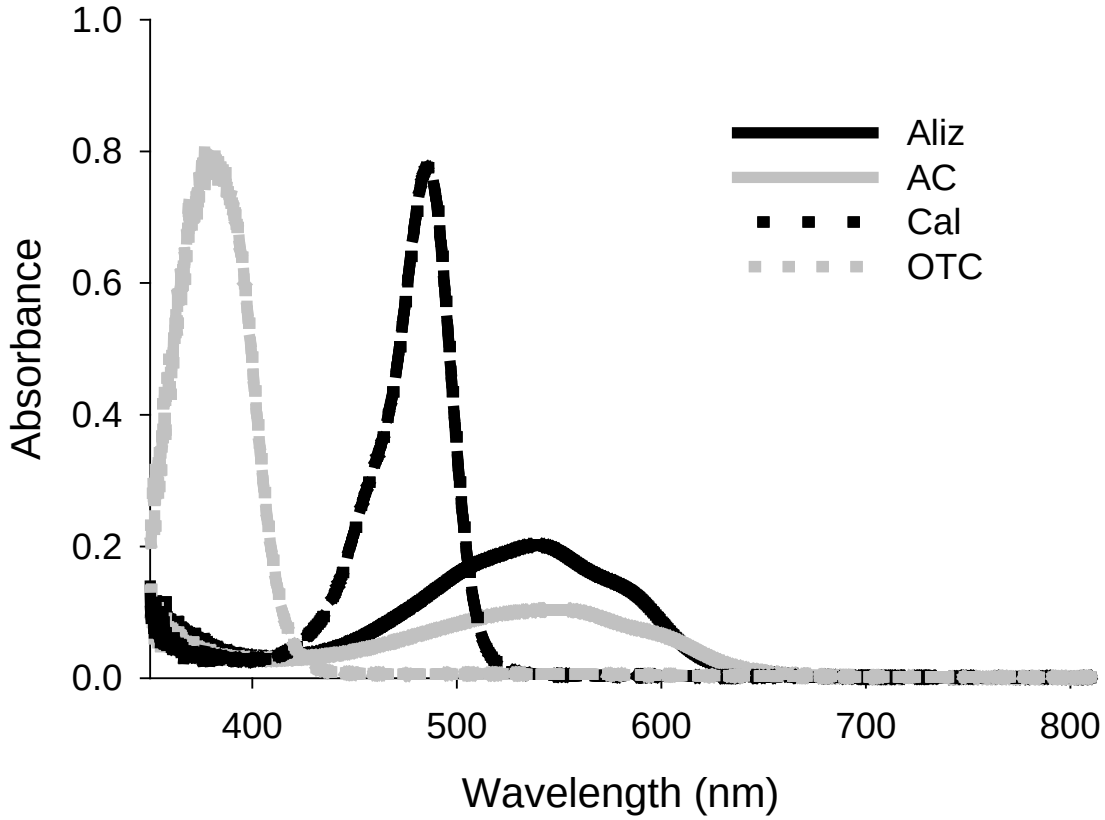

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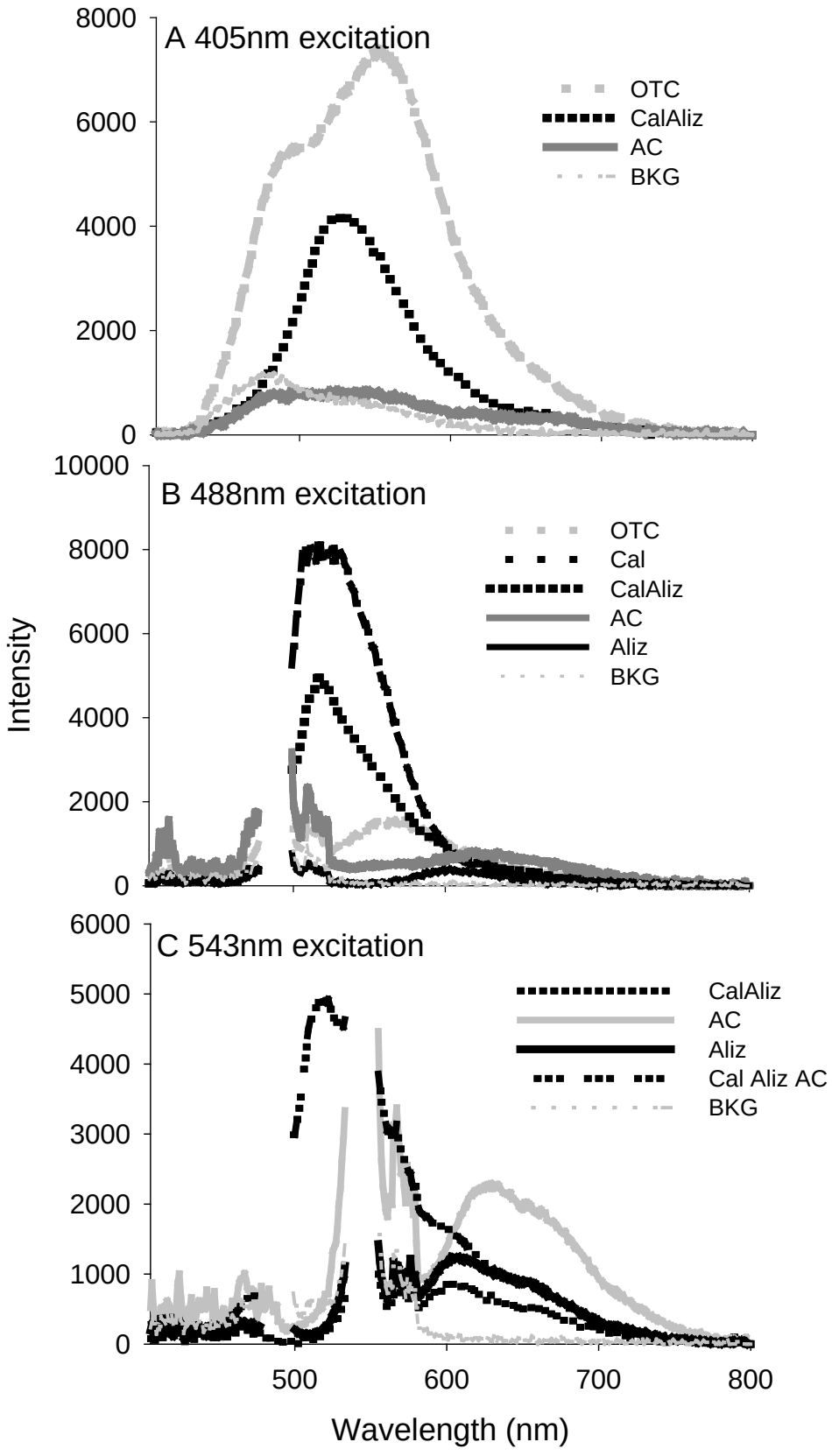
A 405nm excitation
OTC
CalAliz
AC
BKG
B 488nm excitation
OTC
Cal
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AC
Aliz
BKG
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CalAliz
AC
Aliz
Cal Aliz AC
BKG
Intensity
Wavelength (nm)

554

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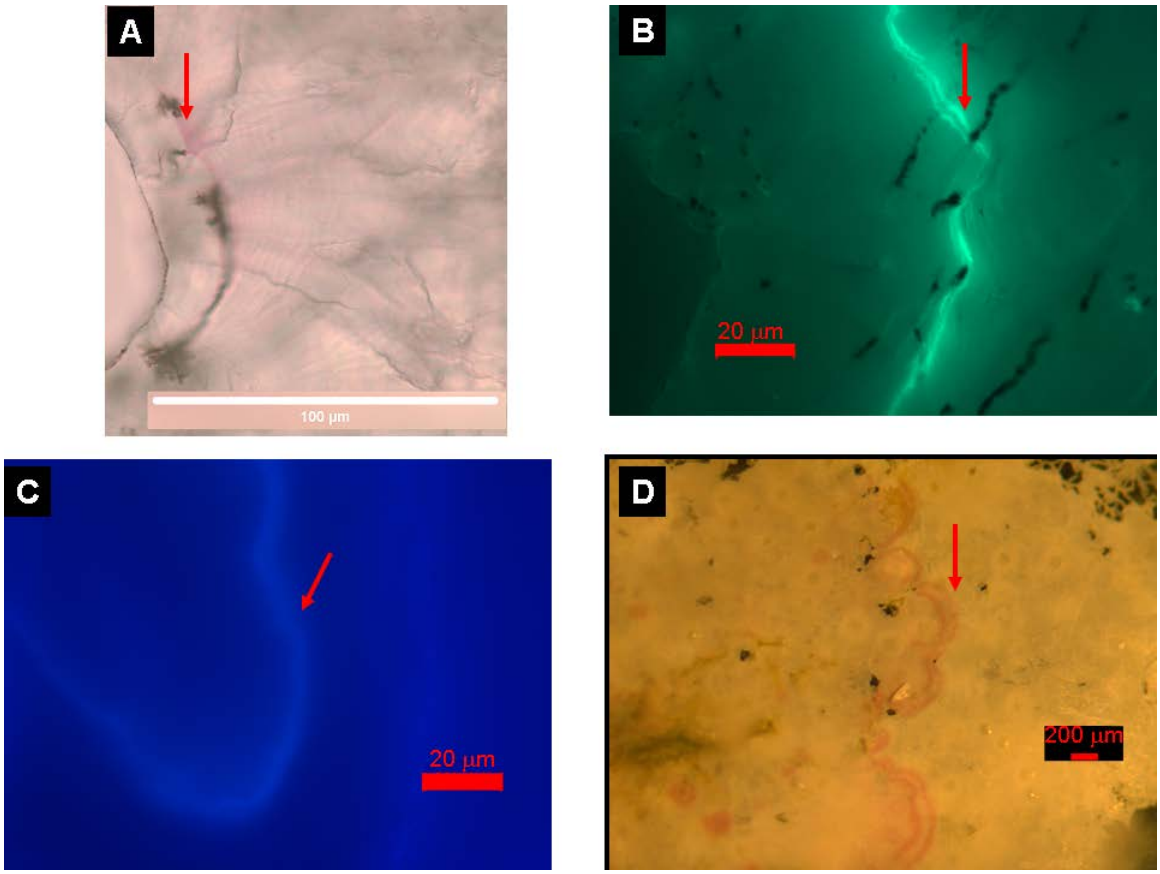
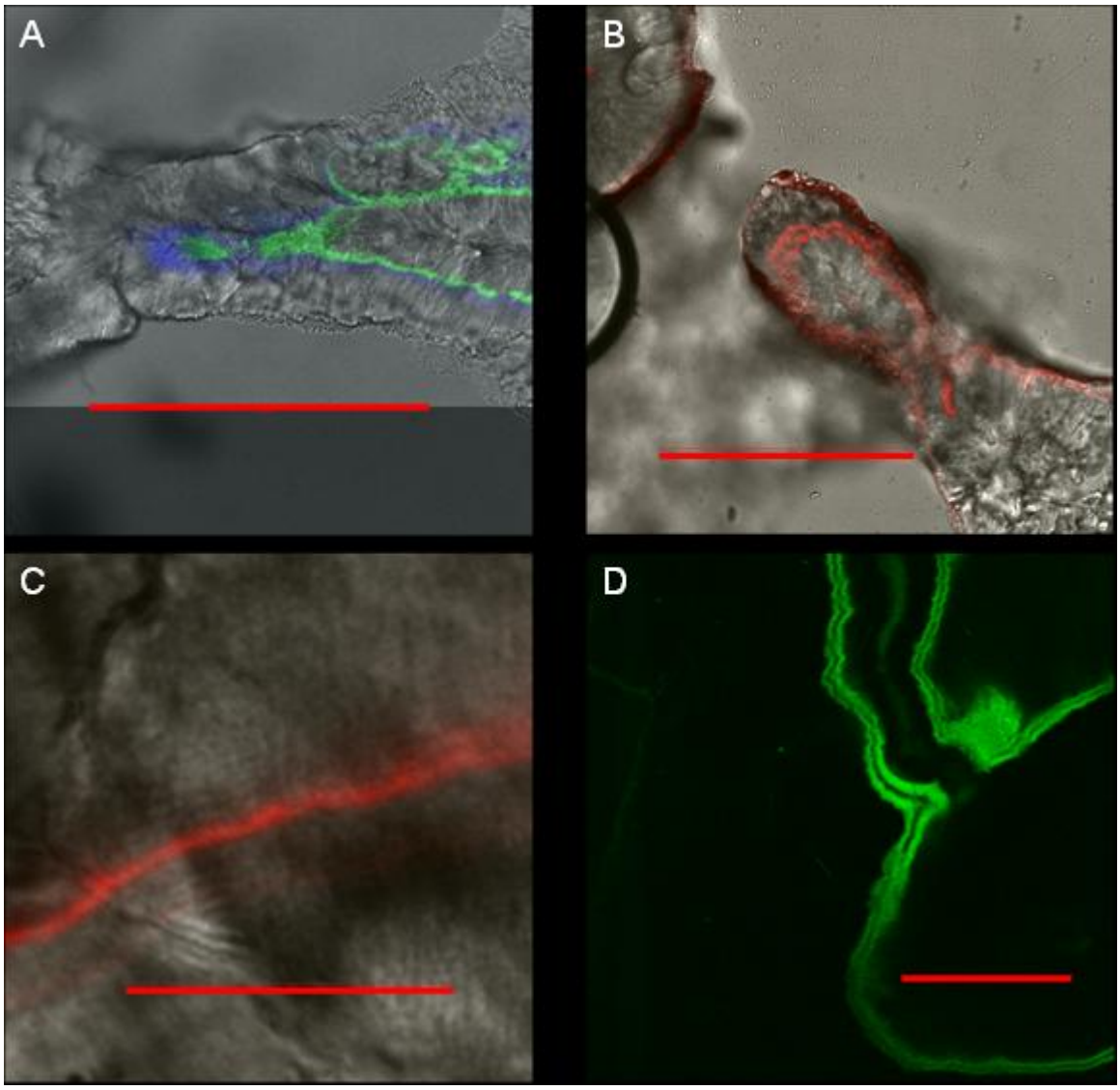
Supplemental Materials: Example stain lines and ion-probe tracks.

Transmitted light images were acquired using a Nikon Eclipse E 600 Polarizing microscope equipped with a Spot Insight color CCD camera. Fluorescence images were acquired using a Zeiss Axio Imager Z1 microscope with an Axiocam HR camera at the Marine Biological Laboratory Center for Microscopy or with a Leica TCS SP5 confocal microscope at the Centre Scientifique de Monaco. With the Axio Imager, calcein images were taken using a Zeiss 38HE filter set, oxytetracycline images were taken using filter set 2. Confocal microscopy images (Fig. 2) were taken using a 543nm He/Ne laser for excitation of alizarin complexone and alizarin, calcein was excited at 488nm with an Ar laser, oxytetracycline was excited with a 405nm diode laser. Corals were exposed to dyes for two 12 hr periods 12 hr apart, specific details regarding the times of stain addition, skeletal structures and environmental conditions will be part of a forth coming paper addressing the timing of formation of different portions of the skeleton in different species in relation to environmental parameters.

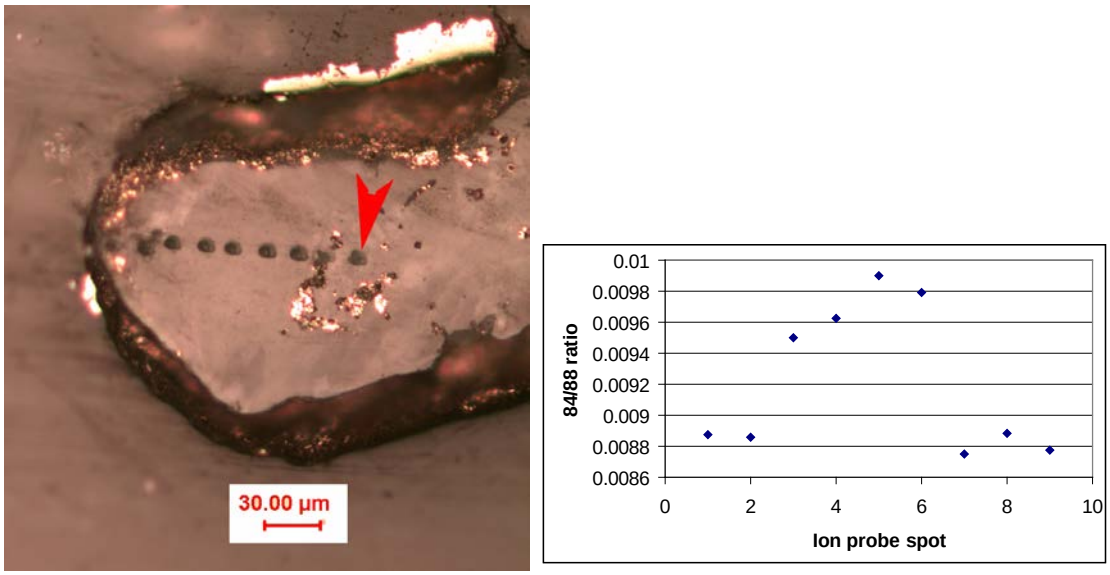
An example series of ion-microprobe spots and associated isotope ratios is shown in Figure 3 (see Holcomb et al., 2009 for details on specimen preparation and measurement). The coral shown was exposed to ^{84}Sr for a day, which, based on dye based estimates, should lead to the formation of an ^{84}Sr enriched band a few microns thick at a growing septal tip, however only a slight ^{84}Sr enrichment was found, suggesting that enriched points may have corresponded to thinner septal thickening deposits. Whether the failure to detect more enriched regions was due to low growth rates in the regions chosen for measurement, or the measurement points missing the center of a labeled band is unknown, however it does illustrate the limitation of using discreet point measurements for detecting narrow, potentially heterogeneous isotopic markers in coral skeletons.



Supplementary figure 1. Light micrographs of stained specimens. A. *Porites* coral stained with alizarin imaged with transmitted light. B. *Goniastrea* coral stained with calcein (fluorescence image). C. *Goniastrea* coral stained with oxytetracycline (fluorescence image). D. Coralline algae (growing adjacent to a coral) stained with alizarin (specimen not polished, imaged with a Nikon dissecting scope). In each figure, an arrow points to the stain line.



Supplementary figure 2. Confocal microscopy images of stained specimens. A. *Porites* specimen stained with calcein (green) followed by alizarin and alizarin complexone (blue). B. *Porites* stained with alizarin complexone. C. *Goniastrea* stained with alizarin. D. *Goniastrea* stained with oxytetracycline. A,B,C show overlay images of fluorescence and transmission channels, D shows fluorescence only. Scale bars are 100 μm, except in C in which the scale bar is 25μm.



Supplementary figure 3. Ion probe measurement spots (dark round dots) and associated ion probe count ratios measured in an ^{84}Sr spiked *Astrangia poculata* specimen. Isotope ratios are plotted in the same order (left to right) as the spots appear on the skeleton. The last (ninth) spot is indicated with a red arrow.