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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, and 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.

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 the 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; Cantin et al., 2010; Macintyre and Smith, 1974), 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. Jacques and Pilson, 1980; Smith, 1973), 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; Cohen et al., 2004; Gladfelter, 1983; Marschal et al., 2004; Raz-Bahat et al., 2006; Tambutte et al., 2012; 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 –

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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 that it was in the form of 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 °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 h in duration, covering a full light–dark cycle. The temperature range was 25–26 °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 was 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 to 40 μmol photons/m^2/s with a 12 h light dark cycle (white colonies were incubated under the lower end of the range of light levels, and 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 (10,000 K, 54 W).

Alkalinity samples were taken from each container ~1 h after the corals had been added and again at the end of the incubation. Waiting 1 h after the addition of the coral to take the first sample was intended to allow the coral to recover from any handling stress and thus avoid capturing any temporary changes in calcification. Salinity (Hach conductivity probe — read to 0.1, accurate to ~1) and pH (NBS scale, Thermo-Orion ROSS 8165BNWP electrode, read to 0.1 mV) were measured at the end of each incubation for every container, as well as at the start of incubations for a subset of the containers. Aragonite deposition was assumed to be the only process affecting alkalinity, with 2 mol alkalinity consumed per mol of $CaCO_3$ deposited. This may under-estimate calcification as any ammonia released by the coral will increase the alkalinity of the solution (e.g. Jacques and Pilson, 1980). Alkalinity depletion rates were corrected for evaporation (based on the change in container mass), and for background rates measured in containers containing no slides. Background alkalinity consumption rates were invariably low, with the highest rates being <10% of coral rates.

Final dye concentrations were as follows: 2.7–3.2 mg/kg alizarin (added as ~0.2 ml of stock solution/l, pH was not adjusted, but pH declined <0.01 upon dye addition), 8.6–8.8 mg/kg alizarin complexone (added as ~1 ml of stock solution/l with sufficient NaOH to dissolve, pH declined ~0.03 upon dye addition), 9.5–10 mg/kg calcein (added as ~0.8 ml of stock solution/l, pH of the stock solution was not adjusted, thus pH declined ~0.03 upon dye addition), 24–26 mg/kg oxytetracycline (added as ~0.3 ml/l of stock suspension, pH was adjusted with NaOH, no measureable pH change upon addition).

2.4. Isotope spikes

Marking corals with isotope spikes was carried out as a part of long term growth experiments (see Holcomb et al., 2010, 2012); data from control corals included in those experiments are presented here. Isotope experiments were carried out using two different isotopes, ^{86}Sr and ^{135}Ba, with 6–16 corals for each treatment. Marking with ^{86}Sr was carried out in much the same manner as that used for dye experiments, with 60 μl of an ^{86}Sr solution added to ~800 ml seawater and corals were incubated for two days. Growth was estimated from changes in buoyant weight (per Holcomb et al., 2010) for the 5 months prior to and the month following the isotope spike. Corals were held at one of two temperatures — ~19 or ~26 °C throughout that six month period.

Spikes with ^{135}Ba were carried out in a flow-through aquarium system as used by Holcomb et al. (2012). Each reservoir used to supply water to individual aquaria was spiked with 81 μl of ^{135}Ba solution/l of seawater. Individual aquaria received spiked seawater for a period of two days: unspiked seawater was then added to the reservoir, diluting the spike to ~80%, and each subsequent day the remaining spike was diluted by an additional ~60%. Buoyant weights were measured for the two months prior and one month following isotope exposure using a Sartorius G803S balance, and the aquaria were held at either 16 or 24 °C throughout this period.

2.5. Alkalinity

Alkalinity samples were taken in pre-cleaned glass or plastic scintillation vials with screw top lids and foamed polyethylene liners (Wheaton). Samples were stored refrigerated for no more than 1 month prior to measurement. Alkalinities were measured via titration with 0.01 N HCl containing 40.7 g NaCl/l using a Metrohm Titrando 808 Dosimat and a 730 Sample Changer controlled by Tiamo software to perform automated normalized Gran titrations of 1 ml samples. Duplicate samples were run and additional replicates were 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