Re-evaluation of using rhodolith growth patterns for paleoenvironmental reconstruction: An example from the Gulf of Panama

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ABSTRACT

Rhodoliths are a proxy data source for understanding environment and climate variation in tropical to arctic oceans. A clear understanding of growth pattern and rate is required to interpret time-series proxy data. Variability in growth on multiple-axes of a single rhodolith has not been extensively studied. This investigation compares algal band counting and dating methods found in the literature, and has implications for detecting biases in branch selection for growth and geochemical analyses. Panamanian rhodoliths (*Lithothamnion* sp.) were collected at the Archipelago de Las Perlas, Gulf of Panama (GOP). Alizarin Red S (ARS) stain was used to study growth rates and banding patterns for 10 branched samples randomly selected for microcosm experiments over a 172-day period. DNA analysis was used to help determine the dominant species present in the sample group and reduce the possibility of inter-sample variation. Samples were overturned every 2 weeks for a multi-axial growth test and were grown under ambient seawater conditions except for the addition of LED lighting. Growth post-ARS staining was

measured along the long (A), intermediate (B), and short (C) axes. Bisected branches show banding correlated with growth rate while banding was highly variable within an individual and between individuals. Average growth band widths in both the pre- and post-ARS stained growth were approximately the same, suggesting that non-environmental factors, such as overturning or biological influences, can cause the formation of growth increments. Differences in growth pattern and rate suggest that careful inspection of growth formation is needed before pursuing rhodolith-based time series proxy research.

Keywords: Rhodoliths; Gulf of Panama; Coralline red algae; Growth rates; Banding; Algeochronology

1. Introduction

Annual or sub-annual band validation in non-geniculate coralline red algae (CA; Rhodophyta: Corallinales) is a fundamental step in developing a paleoenvironmental proxy. This process establishes the timeline from which all other parameters can be assessed when attempting to establish a new proxy for areas of research such as paleoclimatology or paleoproductivity. Most age estimations of CA in this context are dependent on establishing the periodicity of observed bands in the algal skeleton. Growth bands are observable in the two predominant, non-geniculate morphological forms of CA: attached living crusts (encrusting) and free-living (rhodoliths). Rhodoliths in particular are in need of more thorough validation studies within the context of proxy research, mainly due to their unique, rounded to sub-rounded shape that allows for frequent overturning on the seafloor while they grow (Marrack, 1999).

Rhodoliths have increasingly become a source of paleoclimate proxy data over the past 15-20 years (McCoy and Kamenos, 2015; Halfar et al., 2000). Like other biogenic marine proxy sources, such as bivalves and corals, rhodoliths have been shown to accrete annual and sub-annual bands of high-Mg calcite (HMC) which can be used to understand fluctuations in ocean geochemistry (Foster et al., 2013; Kamenos et al., 2009; Halfar et al., 2000). However, the controls on the deposition of these bands may

vary among different species (Blake and Maggs, 2003). Hypothesized causes of band growth include: seasonality expressed through temperature or salinity flux (Kamenos et al., 2012; Rivera et al., 2004; Adey and McKibbin, 1970), light and dark exposure (Fortunato, 2015; Burdett et al., 2011), tidal and lunar cycles (Moberly, 1968; Blake and Maggs, 2003), and growth cessation caused by rhodolith overturning (Cabioch, 1966 via Blake and Maggs, 2003).

Some of the advantages rhodoliths have for paleoenvironmental analysis are their longevity, diversity of habitat across most low to high latitudes, and lack of ontogenetic effects (Kamenos et al., 2008; Foster, 2001). For example, the rhodolith forming coralline alga *Lithothamnion crassiusculum*, with a reported biogeographic presence in the Gulf of California, Mexico and in Italy (van der Heijden and Kamenos, 2015), may live over 100 years based on extrapolation of ^{14}C bomb spike ages relative to the entire length of a rhodolith branch (Frantz et al., 2000). Another example of longevity is found in *Lithothamnion glaciale*, whose distribution extends from the Arctic, throughout the coastal areas of North America (e.g. Alaska, Newfoundland, and Maine), Europe (e.g. Iceland, Ireland, and Norway), Asia (e.g. Japan) and Australia (van der Heijden and Kamenos, 2015). *L. glaciale* has been found to grow for over 60 years using various band counting and geochemical techniques to establish growth models (Kamenos et al., 2012). Additionally, when stacked upon each other on the seafloor under favorable preservation conditions, rhodoliths have the potential to remain relatively *in situ* for over 20 ky (Adey and Macintyre, 1972), thus possibly providing a long-term paleoclimate record. These attributes make rhodoliths worth investigating in order to generate robust paleo-records.

As outlined by Foster et al. (2013) there are three major steps to developing a proxy, particularly for rhodolith research: (1) determine biological and environmental controls on algal growth, (2) validate the proxy using geochemical analyses of the algae and correlate these data to historical changes in the environment, and (3) apply the algal proxy to environmental change using records that pre-date historical data. Before rhodoliths can be used for environmental proxy development, growth and algochronology must be characterized for the population of interest. This includes being able to identify if growth is

continuous and similar on all sides of a rhodolith, determine periodicity of banding, and establish an average growth rate.

There are six main methods that have been used to determine growth rates of both encrusting and rhodolith CA. These include: (1) performing *in situ* (i.e. wild) growth experiments on living rhodoliths to determine how natural environmental parameters (e.g. seasonality) influence growth rate (Steller et al., 2007; Adey and McKibbin, 1970); (2) using ^{14}C values to measure pre-and post-bomb growth (Frantz et al., 2005; 2000); (3) using Mg/Ca to count growth bands (Darrenougue et al., 2013; Schäfer et al., 2011; Halfar et al., 2000); (4) counting observed growth bands based on density changes from light to dark cell rows (Freiwald and Henrich, 1994); (5) using Alizarin Red S (ARS) staining and total growth grown after the ARS stain line during an experiment (Darrenougue et al., 2013, Halfar et al., 2008; Rivera et al., 2004); and (6) averaging the band widths along a 2 mm transect with the assumption that bands are annual (Schäfer et al., 2011). However, it has to be kept in mind that a number of rhodolith studies have found sub-annual banding as well (Foster et al., 2013; Darrenougue et al., 2013; Halfar et al., 2000; Frantz, 1999, Agegian, 1981). There is little to no consensus about which method is the most accurate to determine growth rates. In this study we apply methods 4-6, thus allowing for further comparison to previous rhodolith growth studies that only use one method.

Additionally, the GOP offers a unique setting to study long-term change using CA because it is a seasonal upwelling zone that experiences significant shifts in temperature, salinity and nutrient concentration between the upwelling and non-upwelling seasons. It is also a natural system that shows no significant evidence for high nutrient loading from freshwater sources, thus providing a dynamic marine setting to study seasonal nutrient flux and its impacts on productivity (D'Croz and O'Dea, 2007). Given the natural variability in ocean conditions and the potential for rhodoliths to archive ocean chemistry over decadal to centennial time periods, responses to these variables within the algal growth patterns may help in the understanding of productivity over time.

The overall aims of this study were to (1) reduce uncertainty in inter-sample species diversity by conducting DNA and morphological analysis for the sample group, (2) determine band periodicity (e.g. annual, sub-annual) and growth patterns of the GOP rhodoliths, (3) calculate their annual growth rates, and (4) offer further insight into the influences on rhodolith banding and how methodology may influence the interpretation of time series analyses used in paleoclimate applications.

2. Study Site

The Archipelago de las Perlas (Pearl Islands) sample site is located approximately 48 km off the southern Pacific coast of Panama in the GOP (Fig 1). The Archipelago lies within the 50 m bathymetric zone of the shelf, which covers an area of 12,434 km². The rest of the shelf is predominately <100 m deep and covers 24,024 km² (Schäfer et al., 2011). Panama and the GOP experience a dry, upwelling season (Jan-Mar) and a wet, non-upwelling season (Apr-Dec), with variations in SST (15.6 – 30.0°C at 5 m) and sea surface salinity (SSS; 31.7 ‰ – 34.2 ‰ at 5 m) (STRI, 2015). The experiment was located at the Smithsonian Naos Marine Facility (NMF) near Panama City and the Bay of Panama where semi-diurnal tides range up to 3.65 m (Benfield et al., 2005). This tidal regime is also characteristic of the collection site. The collection site was a rhodolith bed off the western shore of Isla San José (Fig 1). Based on ten 10 m transects surveyed by two divers during this study, coverage of the bed was 81% rhodoliths, 13% coral, 5% sand, and 1% other macroalgae with a density of 130.4 rhodoliths/m². Using the Bosence (1983) classification scheme, the predominant shape of the rhodoliths in the bed (n=196) is spheroidal. No previous surveys or published information are known for this bed.

Figure 1. (A) Map of Panama including dive collection site (lower star) and the Smithsonian Naos Marine Facility location (upper star) where the experiment took place. Latitude (7-9°N) and longitude (77-83°W) are indicated by numbers marked on the perimeter; (B) Distribution of rhodoliths on the seafloor at 2-3 m depth; (C) Example of an open-branching GOP rhodolith sample rinsed with freshwater and air dried; (D) Map showing approximate location of dive sample collection, more closely approximated by the starred location.

3. Materials and Methods

3.1 Sample Collection

Rhodoliths were collected in March 2014 using SCUBA diving in 3-4 m of water from the rhodolith bed collection site. Only live (i.e. visibly red/pink), predominantly open-branched rhodoliths were collected (n=250) for the purposes of the growth experiment. The samples ranged in diameter between 2.4 – 4.4 cm; however, larger (> 11 cm in diameter), more rounded rhodoliths do exist at the collection site. The open-branch rhodoliths provided easier access to individual branches within the three axial directions analyzed in this study compared to the denser protuberances characteristic of the larger, more rounded rhodoliths.

Samples were transported in seawater-filled coolers to the NMF where they were placed in covered, outdoor flow-through aquaria with circulation pumps and filtered seawater (Strainrite polyester felt 10 µm pore size filter bag). Samples were placed in the tanks for one week prior to being placed in experimental microcosms. Analogous growth experiments were not able to be performed in the wild due to logistical considerations. Vouchers of the dried samples are housed in the Department of Geological Sciences Microsampling Lab at the University of Alabama.

3.2 Experimental design, staining, and sample preparation

Thirty samples were randomly selected from the live sample collection. If excessive epiphytes resided on the sample, another randomly selected rhodolith was taken from the tank. Samples were stained for 60-hrs in 10 liters of seawater mixed with 15 mg/L of ARS stain (adapted from Darrenougue et al., 2013). Staining took place under continuous LED (Jiangjing; 18W; 170 lux) lightening to help ensure that rhodoliths absorbed the stain. ARS stain was successfully absorbed in previous staining trials and was clearly marked in bisected branches by a bright pink line. This stain was differentiated from all other growth because non-stained growth bands seen in bisection were light or dark shades of white based on differences in HMC density.

Samples were then placed in white plastic bins (13 cm x 24 cm; i.e. microcosms) such that there was one rhodolith per container (Fig 2). No substrate was added. Each container was fed ambient, filtered seawater via a ¼” plastic tube connected to a header tank with ~100% turnover rate every 20 minutes. The header tanks were fed from the flow-through aqueduct system from coastal water near the NMF. The setup, which contained 30 microcosms, was housed completely outdoors under an awning, and was maintained at ambient temperature, salinity, and pH conditions throughout the experiment (172 days; i.e. ~6 months). The setup was overhung by LED (Jiangjing; 18W; 170 lux) lighting on a 12:12 hr cycle. Samples were randomly overturned every two weeks simulating natural, unsystematic movement of the rhodoliths on the seafloor. Overturning was performed when rhodoliths were temporarily removed from the microcosms in order to remove fouling algae from the containers. It is also possible that some rhodoliths experienced little to no overturning during this process.

Figure 2. (A) Microcosm experimental setup at the Smithsonian Naos Marine Facility near Panama City, Panama. (B) Plastic bins used for microcosms. Solid arrow pointing to a HOBO Pendant temperature and light monitor. Dashed arrow pointing to the sample rhodolith.

Out of the rhodoliths that remained alive until the end of the experiment (n=26), 10 were randomly selected for growth analysis labeled here as samples R1-R10. Selected samples were confirmed to be living at the end of the experiment by identifying >90% red epithelial coverage. A minimum of three branches from each sample were broken at their bases from the nucleated rhodolith cores. One to two branches in each of the three primary axes (long [A], intermediate [B], and short [C]) (Fig 3) were broken at their base. A total of 37 branch samples were prepared for the growth analysis. This included replicates along axis B for samples R8 (n=3) and R9 (n=2), as well as axis C for samples R8 (n=2) and R9 (n=2) which were made to conduct intra-axial growth comparisons. Branches were embedded in Epothin epoxy resin with hardener (Buehler), bisected, glued to glass thin sections using CrystalBond, and cut to uniform thickness (1 - 9 mm) using a variable speed diamond-tipped Buehler Isomet 1000 Precision Saw. Care was taken to bisect directly in the middle of the greatest growth extension on each branch. Samples were then polished in three stages using a Buehler Ecomet 3 Polisher. Samples were first abraded using a 360 μm embedded diamond grit plate, and then moved to cloth plates coated in 15 μm , 6 μm and 1 μm diamond pastes. Once polished, samples were hand washed with a brush and soap, ultrasonicated for 2 minutes, and air-dried. Images were generated using geo.TS software and an automated Olympus VS-BX reflected light microscope at the University of Toronto – Mississauga (Canada) Paleoclimate and Geobiology lab. Any remaining organic matter was left in place.

Figure 3. (A) Diagram illustrating the long (A), intermediate (B), and short (C) axes selected for the multi-axial growth analysis. (B) Example of growth extension during the experiment, or post-ARS (Alizarin Red S stain) growth (scale bar = 1 mm). Dashed line indicates growth axis. (C) Dashed line is a 2 mm transect drawn in the wild, or pre-ARS growth (scale bar = 1 mm). (D) SEM image showing cell density shift between large and small cells making bands visible at the tip of a branch.

3.3 Water and light monitoring

Two HOBO Water Temp Pro v2 (Model U22-001) loggers and three HOBO Pendant loggers (Model UA-002-64) for temperature and relative light level measurements were placed in various microcosms to capture the range in temperature or lighting bias. One Temp Pro v2 logger was placed in the header tank and the other in a microcosm bin. One Pendant logger was located under direct light in a microcosm bin and the remaining two Pendant loggers were positioned under indirect light. All loggers recorded data every hour for the entirety of the experiment.

To convert light meter lux readings to photosynthetic photon flux density (PPFD) to quantify PAR (photosynthetically active radiation), calibrations were performed at the NMF using a Licor sensor (Kipp & Zonen PQS1) to establish a conversion factor (e.g. constant) of 0.374 for the actinic/daylight combined LED light source used (Section 3.2) (Thimijan and Heins, 1983). The conversion equation used was (Eq 1):

$$\frac{\text{Lux}}{\text{Constant}} = \mu\text{mol} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$$

The light comparison was made to help determine the cause of growth variation, but was not an intentional treatment to be tested. Therefore, a qualitative comparison to light exposure was assessed. Rhodoliths were not placed at the exact same distance from the light source due to the constraints of the equipment designs and that only 3 light loggers were available for use. Samples were determined to be either directly under the LEDs (0.5 m) or indirectly under the LEDs (> 0.5 m). In addition to temperature and light, salinity, pH, dissolved inorganic carbon (DIC), dissolved organic carbon (DOC), total dissolved nitrogen (TDN) nutrients (NO_3^- , NO_2^- , PO_4^{3-} , NH_4), and dissolved Fe (DFe) were also monitored

throughout the experiment to identify if fluctuations in the concentrations of these parameters through time significantly influenced or coincided with growth patterns. Salinity was recorded twice a day, while the other parameters were measured on a bimonthly basis to track background conditions. Nutrients were measured at the Dauphin Island Sea Laboratory (AL, USA) on a Skalar San + continuous flow autoanalyzer with wet chemistry modules designed for standard colorimetric techniques for individual analytes (NO_3^- and NO_2^- analyzed using EPA method 353.2, PO_4^{3-} analyzed using EPA method 350.1, and NH_4 analyzed with EPA method 365.3) (Lu et al., 2015). Dissolved organic nitrogen (DON) was calculated as follows (Eq 2):

$$\text{DON} = \text{TDN} - (\text{NO}_3^- + \text{NO}_2^-) - \text{NH}_4$$

DOC was measured using a Shimadzu TOC-V total organic carbon analyzer at the Organic Geochemistry Laboratory at the University of Alabama following the method described in Lu et al. (2015). SUVA_{254} , the ratio of absorbance ($\lambda = 254 \text{ nm} \cdot \text{m}^{-1}$ / DOC concentration) was calculated to determine the aromaticity of DOM, which assists in determining the source of a body of water. DFe was measured at the University of Miami (FL, USA) following the methods of Wu and Boyle (1998) using clean room sampling protocol.

3.4 Species identification

DNA analysis was conducted using one air dried sample selected after experimental treatment (i.e. ARS stain application) to assist in the species identification process and was morphologically analogous to the other samples in the experiment. DNA extraction, PCR (polymerase chain reaction) amplification of *psbA* and *rbcL* gene, and sequencing methods used in the analysis follow Adey et al. (2015). The resulting sequence was used to search for similar sequences already ID in the GenBank data set by BLAST (Basic Local Alignment Search Tool at NCBI webpage; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Morphological features such as cell shape and size, as well as available reproductive features were used to assess taxonomy using Robinson et al. (2013) as a guide for Eastern Pacific rhodoliths and references

used in Schäfer et al. (2011). These features were observed using Geo.TS software, which was used to produce high-resolution histological images, as well as scanning electron microscope (SEM) images. SEM images were generated using gold sputter-coated samples on glass slides mounted with carbon tape and were collected at the University of Alabama Central Analytical Facility (CAF) using a Philips XL-30 SEM.

3.5 Interpretation and measurement of algal growth bands and rates

For samples with replicates in the same axis (e.g. multiple branches from axis B or C in the same sample), growth data were averaged such that each axis had a single measure for comparisons between samples (n=10). An exception to this is Fig 6 where all branches are represented (n=37) to show the variation in band counts for samples that had replicate branches prepared to assess intra-axial variation, and in Fig 7 where the band counts and total growth are compared and all but one outlying branch measurement is included (n=36). At least two researchers performed all band counts and width measurements in replicate.

A high-resolution, contrasted image of each branch was uploaded to ImageJ version 1.50b (<http://imagej.nih.gov/ij/>) where the correct scaling was referenced from geo.TS. The ImageJ measurement tool was used to (1) count the number of growth bands grown in the pre- and post-ARS sections, (2) measure the total distance from the tip to the ARS-line, and (3) measure the width of individual growth bands grown in the pre- and post-ARS sections (Fig 3). All measurements were made along transects parallel to the growth axis.

Growth bands were visually inspected and identified based on the alteration of light and dark calcification. This color variation is due to the difference in the density of the cells. Smaller, more compact cells produce dark bands and larger, less compact cells produce lighter bands (Kamenos & Law, 2010; Halfar et al., 2008). Counts were categorized as pre- or post-ARS growth to try and determine

variation between wild and microcosm growth conditions. Distinct bands were visible in both the wild (pre-ARS) and microcosm (post-ARS) growth.

These observations were then used to assess growth patterns using Methods 4 through 6 (Section 1). Method 4 (band counting) and Method 5 (ARS staining) use the basic rate calculation where $\text{Rate} = \text{Distance/Time}$. So, in the context of this project, this equates to $\text{Daily Growth Rate} = \text{Total Growth}/172$ days. This value is then multiplied by 365 to achieve an annual growth rate, which is most often reported in the literature. For Method 6 (band width average along 2 mm transect), a 2 mm transect was drawn from the ARS line into the pre-ARS growth along the axis of growth. Bands in the pre-ARS (wild) growth were assumed to be annual in order to compare them to the results of Schäfer et al. (2011) who employed this method on GOP rhodoliths of the same size, morphology, and suggested taxonomy (see Section 4.2). Measurements along the 2 mm transect only included complete bands. When measuring band widths in both the pre- and post-ARS growth, continuous growth was assumed given that there was no evidence of growth hiatuses. Hiatuses could be caused by fouling algal growth, breakage (Darrenougue et al., 2013), or microborers leaving holes that require repair. For the pre-ARS growth, the mean algal radius was divided by the average band widths along the 2 mm in order to calculate age (see Schäfer et al., 2011 for method). Diameters of the rhodoliths were measured using calipers at the start of the experiment were used to calculate the radii used in the equation.

3.6 Data analysis

All statistical analyses were performed using the open source tool R version 3.2.2 (<https://www.r-project.org/>) and Microsoft Excel. Data were checked for normality and homoscedasticity using the Shapiro-Wilk normality test and Levene's test, respectively. All data were normally distributed, or were transformed using log or square root transforms as necessary. All significance levels, α , were set to 0.05 for all statistical analyses.

To test the variance of means between the growth categorized by axis (A, B, and C), a one-way ANOVA was performed followed by a *post-hoc* comparison using a Tukey's test. A paired t-test was used to compare the average band widths in the pre- and post-ARS growth. An independent t-test was then used to compare the difference in total post-ARS growth between the samples grouped by type of light exposure (direct versus indirect). The total post-ARS growth is the averaged total growth in mm for branches in all three axes.

4. Results

4.1 Mortality and Alizarin red stain

At the end of the ~6-month period, 87% of the samples were covered in red, living tissue, while the remaining 13% were bleached and not photosynthesizing. The dead samples were excluded from further analysis. In the branches examined for post-ARS growth, 100% of the samples showed a clear ARS line (Fig 3B-C), and only one sample did not grow after the ARS line.

4.2 Species identification

Even when all PCR primer combinations were run as in Adey et al. (2015) (*psbA* and *rbcL*), only a short section of the *rbcL* gene was amplified which included the forward primer F1152 (Gabrielson et al., 2011) and the reverse primer R-rbcS-start (Freshwater and Ruennes, 1994). A short sequence of 283 bp was successfully amplified from the sample. This sequence provided context from which the sample can be identified. The sample was not identical to any sample stored in the GenBank system but related with other members of the subfamily Melobesioideae as *Mesophyllum*, *Synarthrophyton* and *Lithothamnion*.

SEM imaging (n=2) showed secondary pit connections and cell fusions. Clear growth bands were visible in both samples (Fig 3D). Features described for *Lithothamnion* sensu lato pairs well with the open-branching morphology of the rhodoliths herein analyzed and is also the genus described by Schäfer et al. (2011) for the GOP rhodoliths. So, identification was based on DNA sequences, external morphology and features observed using SEM. Samples used in this research can be identified as species of the genus

Lithothamnion within the subfamily Melobesioidea. Species level identification is not possible currently due to the reduced taxonomical information of the genus in the region studied.

4.3 Seawater conditions and nutrients

Seasonal upwelling in the waters near the intake pipe ended around May 1, 2014 based on collected temperature data. Temperature ranged between 24°C to 29°C, and varied between 28°C to 29°C for 90% of the experiment (e.g. the non-upwelling season). Similarly, salinity ranged between 30.5‰ to 35‰, and varied between 31‰ to 32‰ for half of the experiment. The average ambient pH was 8.5 (Fig 4).

Figure 4. Water conditions during the experiment (March 27 – September 19, 2014). Parameters A (salinity) shown with a 10-day moving average for daily measurements. Parameter B (temperature) shown with a 5-day moving average for hourly measurements. Parameter C (pH) was measured on a bi-monthly basis. The dashed line marks the tail-end of the upwelling season.

DOC ranged between 85 – 160 μM (Fig 5). Phosphate (PO_4^{3-}) from 0.6-1.0 μM . Total dissolved nitrogen (TDN), $\text{NO}_3^- + \text{NO}_2^-$, and NH_4 ranged from 9-18 μM , 5-14 μM , and 0.4-8 μM , respectively. SUVA_{254} ranged from 0.00-1.02 $\text{L} \cdot \text{mgC}^{-1} \cdot \text{m}^{-1}$. Dissolved Fe ranged from 117-216 nM. DON was calculated using Eq. 2 and ranged between 0.7-9 μM (Fig 6). The DON for sample event #11 (August 29, 2014) was below detection limits. The overall DON trend across the ~6 months was negative ($R^2 = 0.7$; Fig 5). All monitored and measured parameters mirrored the ambient seawater conditions.

Figure 5. DOM quality and nutrient data during the extent of the 172-day experiment. (A) SUVA₂₅₄; (B) dissolved organic carbon (DOC); (C) total dissolved nitrogen (TDN); (D) dissolved organic nitrogen (DON); (E) ammonium (NH₄); (F) dissolved inorganic carbon (DIC); (G) phosphate (PO₄³⁻); (H) dissolved iron (DFe).

4.4 Multi-axial band and growth rate comparison

Banding was visible in all but one sample, which did not grow beyond the ARS line. In the majority of the samples, bands were distinctly marked by short, dense cells that abruptly transitioned to longer, less dense cells (Fig 3D). A subsection of individual cells was measured to identify this transition. Measured cells (n=81) of the filaments in the light bands ranged from 9-20 µm in length and 5-13 µm in diameter while cells (n=75) in the dark bands ranged from 5-13 µm in length and 4-11 µm in diameter.

4.4.1 Method #4 - Growth band counting

The average number of pre-ARS growth bands counted along the 2 mm transect ranged from 6 to 14 bands per rhodolith. The post-ARS growth (Fig 6) had an overall range of 0 to 20 bands grown per branch during the experiment. These counts were made along the total length of the total post-ARS growth, which ranged from (0.0 – 2.5 mm). Axis A experienced the most growth in 4 of the 10 samples, axis B in 2 of the 10 samples, and axis C in 3 of the 10 samples (Fig 6). Sample R1 experienced near-equal total growth in all three axes and in 5 of the 10 samples, two or more axes had the same amount of bands in each axis. The greatest range in a single rhodolith's post-ARS growth between axes was a 12 band difference in R3 between axis A (13 bands) and axes B and C (1 band each). The number of bands is positively correlated to the total post-ARS growth for samples in the direct lighting group ($R^2 = 0.88$; F

(1, 6) = 36.8; p -value = 0.002; Fig 7; Table 1) and the indirect lighting group ($R^2 = 0.93$; $F(1, 19) = 230.4$; p -value = <0.0001; Fig 7; Table 1).

Figure 6. Post- ARS growth band counts categorized by axis for samples R1-R10 and position relative to light source (n=37). Sample R8 contains 3 branches that were band counted for axis B and 3 branches for axis C. Axis C3 has a band count of 0. Sample R9 shows 2 branches for both axes B and C. Multiple branches were prepared and analyzed as replicates to assess intra-axial variation in growth. Gaps between axes in some of the samples indicate no replicates were generated for that sample.

Figure 7. Positive correlation in the total number of bands grown in each branch and total growth for the post-ARS growth ($n = 36$). One outlier was removed in axis C of sample R3, which had a total length of 1.5 mm for one band. The banding was likely not distinguishable in thick section and more bands were likely present than what was visible. Samples in direct light had an $R^2 = 0.88$; p -value = 0.002. Samples in indirect light had $R^2 = 0.93$; p -value = <0.0001. Error bars represent standard error.

4.4.2 Growth rate calculations and chronologies

4.4.2.1 Method #5– ARS Staining

Using the total amount of growth measured after the ARS line, the mean growth rate for all of the sampled branches was 0.005 mm d^{-1} , which equates to 1.84 mm yr^{-1} . Rates varied by axis, with axis A showing the highest rate at 2.27 mm yr^{-1} , and axes B and C rates both calculated to be 1.62 mm yr^{-1} . The average band width for the post-ARS growth in all samples was $0.18 \pm 0.07 \text{ mm}$. The total amount of growth, or extension, by axis was statistically not significantly different between the three axes ($F(2, 27) = 0.555$; p -value = 0.58; Table 1) (Fig 8).

Figure 8. Annual growth rate comparison between the long (A), intermediate (B), and short (C) axes for ten rhodoliths regardless of light exposure (e.g. direct vs indirect) ($n = 30$; ANOVA; p -value = 0.58). Samples with replicates in axes B and C were averaged to compare all axes equally in a 1:1:1 relationship. The branch from sample R8 that did not grow any bands is not included here.

464 **Table 1.** Summary of results from statistical analyses and band counting.

	Groups Used for Analysis	Statistical Test	Light Exposure	R²	Test Statistic (df)	p-value	Conf. Interval	n	Min Bands	Max Bands
(1)	Total post-ARS stained growth (mm)	Liner Regression and F-test	Direct	0.88	$F = 37 (1,6)$	0.002	NA	9	1	13
	and Number of band count(s) per branch		Indirect	0.93	$F = 350 (1,26)$	<0.0001	NA	27	1	20
(2)	Total post-ARS growth (mm)	One-way ANOVA		NA	$F = 0.555 (2, 27)$	0.58	NA	30	NA	NA
	Growth grouped by axis	Tukey's Test								
	<i>post hoc</i>									
	comparison									
	Axis B - Axis A			NA	NA	0.636	95%	10	1,1	20,18
	Axis C - Axis A			NA	NA	0.639	95%	10	1,1	15,18
	Axis C - Axis B			NA	NA	1.000	95%	10	1,1	15,20
(3)	Pre- and Post- ARS band widths	Paired t-test		NA	$t = -3.804 (27)$	0.000371	95%	28	1,1	17,20
(4)	Total post-ARS growth (mm)	Independent t-test		NA	$t = -4.334 (16)$	0.000256	95%	30		
	Growth grouped by direct or indirect light placement								1,1	13,20

Multiple branches from the same axis in the same sample were analyzed in two samples (R8 and R9). Intra-axial variation for these samples is summarized in Table 2. The greatest variation was among three branches selected from axis C of sample R8 in which one of the three branches grew 18 bands at a rate of 4.36 mm yr⁻¹, one grew 1 band at a rate of 0.25 mm yr⁻¹, and the third branch did not grow after the ARS stain was applied (Fig 9). Post-ARS growth rates for each rhodolith are shown in Fig 10.

Table 2. Summary of intra-axial variation in annual growth rate for samples R8 and R9 for axes that had replicates made. Both samples were grown under indirect lighting conditions.

Sample	Axis (n)	Mean Growth Rate (mm yr ⁻¹)	SD
R8	B (n = 3)	3.22	0.23
R8	C (n = 3)	2.00	1.99
R9	B (n = 2)	1.14	0.74
R9	C (n = 3)	0.20	0.09

Figure 9. Three branches collected from axis C in R8 (indirect light) showing the variation in the number of bands grown within the same axis. Solid arrows indicate the position of the ARS line. Dashed line represents growth axis along which band counts were made. (A) Branch grew 18 bands; (B) branch grew 1 band; (C) branch grew no bands. The line below the ARS line in Image A is a post-band count laser ablation (LA-ICP-MS) scan line. All scale bars = 1 mm.

Figure 10. Post-ARS growth rates averaged for all axes for each rhodolith sample (n=10). Post-ARS growth rates were calculated using the total time of the experiment (i.e 172 days). See Section 3.5 for calculation details. Error bars show standard error. The outlier data for axis C in sample R3 is included in the average of R3 so that three axes are represented in each sample.

4.4.2.2 Method #6 – Band width averaging along 2 mm pre-ARS transect and age calculation

Measuring the band width along the 2 mm transects resulted in an average growth rate (aka average band width) for pre-ARS growth of $0.18 \pm 0.7 \text{ mm yr}^{-1}$. Taking Method 6 one step further, Schäfer et al. (2011) assumed an annual banding periodicity and estimated the ages of the rhodoliths by dividing the mean rhodolith radius by the average band width along the 2 mm transect (Schäfer et al., 2011). In our study this resulted in rhodoliths (n=37) ranging in age from $40 - 122 \pm 28 \text{ yrs}$ at the time the experiment began. Error in age estimations for this study is reported as standard deviation ($SD=1\sigma$). These values compare

well with Schäfer et al.'s age range (n=3) of 55 ± 17 yrs to 93 ± 28 yrs using band counting, and uranium/thorium (U/Th) mass spectrometry dates that ranged from 50 ± 15 yrs to $120\text{-}145 \pm 20$ yrs.

4.5 Light exposure influence on growth

The average output from both LED strips throughout the course of the experiment was 175.5 ± 7 lux (16.3 ± 0.66 lumen/ft²). This was calculated to be $469.25 \mu\text{mol} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$. The Pendant placed under the direct light had an average output of $469.25 \mu\text{mol} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$ (175.5 lux; 16.3 ± 0.23 lumen/ft²) and the Pendants under the indirect light averaged $494.92 \mu\text{mol} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$ (185.1 lux; 17.2 ± 0.25 lumen/ft²) and $448.93 \mu\text{mol} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$ (167.9 lux; 15.57 ± 0.23 lumen/ft²). Since only 3 Pendants were available to measure the light across the entire microcosm setup, values for each exposure to individual rhodoliths and their branches are best estimates and based on limited data. The total post-ARS growth of the indirect group (M=1.0; SD=0.75) was significantly more than the growth in the direct group (M=0.44; SD=0.52); (t (16) = -4.3342, p -value = 0.0002; Fig 11; Table 1).

Figure 11. Comparison of post-ARS growth in samples that were placed directly under the LEDs (n=9) and those placed indirectly under the LEDs (n=21). The total post-ARS growth of the indirect group

($M=1.0$; $SD=0.75$) was significantly more than the growth in the direct group ($M=0.44$; $SD=0.52$); (t (16) = -4.3342, p -value = 0.000256). Bold lines in the center of the boxes represent medians, bars of the whiskers indicate maximum and minimum values, and the circle indicates one outlier found in the direct light group. This is the same outlier that was removed from the regression analysis shown in Fig 7.

5. Discussion

The possibility that different rhodolith species were examined in the experiment is present; however measures were taken to eliminate this uncertainty by conducting DNA analysis and observing morphological features. Although species level identification for validation studies is optimal but could not be achieved in this study, the paleontological record is often restricted to similar morphological features for identification and rarely able to identify past the genus level due to compromised preservation. Therefore, our taxonomic identification is comparable for the purposes of paleoenvironmental applications using rhodolith samples from the fossil record.

5.1 Banding

For GOP rhodoliths to be used as a viable environmental proxy data source, the periodicity of their banding needs to be identified in order to link growth to time. Typically, annual banding is the preferred increment in other long-lived proxy sources, but alternatively a known sub-annual band frequency, such as seasonal or sub-monthly bands could be used (Darrenougue et al., 2013). The results of this study show that bands that physically resemble what are often considered “annual” bands may be deposited at much shorter time intervals. Furthermore, the Panama rhodoliths produced highly variable numbers of bands, including different numbers of bands within the same axis of a rhodolith, between multiple axes within a rhodolith, and between rhodoliths in the same population as evidenced by the post-ARS growth. Given this diverse variation, our understanding of algochronology for the purposes of time-based environmental reconstructions needs to be reassessed. Some researchers (e.g. Burdett et al., 2011) have attempted to

account for this variance by averaging growth from three branches of a rhodolith, while others have expanded their methodologies by averaging growth in larger numbers of branches (Darrenougue et al., 2013 [n=43]; Blake and Maggs, 2003 [n=35 per treatment]). However, there is no consensus on how to reconcile large variation in banding periodicity.

Generally, growth banding in many organisms is correlated to environmental or geochemical records to understand the band periodicity (e.g. Frantz et al., 2005). Temperature is commonly assessed as such a parameter. For macroalgae, temperature is known to affect growth (Adey, 1970; Kamenos and Law, 2010) and is often associated with inducing and determining the width of banding in CA, particularly species that grow in temperate to arctic regions (Halfar et al, 2013). In previous work, Schäfer et al. (2011) suggest banding in the GOP rhodoliths is caused by temperature and salinity shifts induced by seasonal upwelling. Since the GOP does have a fairly extreme swing in temperature for a tropical environment (15.6 °C – 30.0°C), a seasonal temperature trigger seems plausible. Although our experiment did not span an entire year to capture this seasonal shift, growth occurred and produced sub-annual bands (up to 20 in one branch) physically similar to those described as annual in other studies. The sub-annual bands that grew in the GOP rhodoliths during the ~6 month experiment do not appear to align with the monitored seawater parameters, which included temperature, salinity, and pH (Fig 4). Temperature ranged between 24°C to 29°C, and varied between 28°C to 29°C for 90% of the experiment. Similarly, salinity ranged between 30.5‰ to 35‰, and varied between 31‰ to 32‰ for half of the experiment. Ambient pH averaged 8.6 and remained in a range that favored growth based on the observation that growth occurred in 97% of the examined branches. It is possible that growth was reduced at the onset of the experiment when upwelling conditions were still in effect (lower temperature, higher salinity, and lower pH) but without an ARS line or other growth marker between the seasonal shifts, it cannot be determined. Overall, though, daily or monthly oscillations in these parameters did not appear to induce banding given that there is no consistency in the number of bands within a single rhodolith that mimics these instrumentally recorded frequencies.

Interestingly, the average band widths in the pre- and post-ARS growth bands are nearly the same (0.15±0.13 mm and 0.18±0.07 mm, respectively). This is also the case when the band widths are separated by light regimes, whereby the three samples grown under direct light have an average band width of 0.14±0.6 mm (one outlier branch removed) and the remaining 7 samples under indirect light had an average band width of 0.15±0.15 mm. This means that the band widths produced in the experiment are near identical to those produced in the wild regardless of light exposure, even though more variation is seen in the indirect group. It also suggests that despite environmental differences (aka wild versus microcosm conditions), the algae grew approximately the same number of cell rows per band which suggests that non-environmental factors can cause growth banding in at least some circumstances. If environmental factors are not the sole control on these variables, perhaps there is a biological influence on the maximum number of cell rows grown that is independent of annual cyclicity. This result also brings into question whether the bands are indeed annual or sub-annual. The method (Method #6 – band width average along 2 mm transect) used to determine the pre-ARS ages was dependent on the average band width measurements and the overall size of the rhodolith, along with the assumption that bands were annual so that Schäfer et al.'s (2011) method could be employed and results from this study compared to theirs. The results of this comparison showed that the experimental growth produced sub-annual banding that mimics the growth structure of bands grown in the wild, therefore conflicting with the notion that wild bands must be annual. In general, both band periodicities can be present in rhodoliths as was demonstrated by Halfar et al. (2000) using elemental maps to observe first and second order banding in rhodoliths (*Lithothamnium glaciale*) from Newfoundland, Canada. However, methodologies in this phase of the project excluded the use of elemental mapping to determine geochemical band signatures that may be present. SEM imaging and other imaging techniques, which are commonly employed for growth pattern assessment, did not reveal second order banding in the samples collected for this experiment. Cells clearly oscillated between dense and less dense bands in the same pattern in both the pre- and post-ARS growth. Observations of sub-annual banding, regardless of the method used to identify them, complicate the interpretation in the periodicity of GOP rhodolith banding and suggest that the method

employed to determine alveochronology for a paleoclimate application can have a significant impact on the outcome.

5.2 Influence of nutrient and light availability on growth

Although there may not be a direct connection to CA growth, nutrients were examined in order to rule out this influence on growth considering the experiment site's proximity to potential terrestrial runoff sources that could cause excess nutrient loading, in addition to influences caused by natural upwelling. Because nutrients availability is fundamental to productivity (Paytan, 2009), indirect influences on CA growth from nutrient flux may include reduced photosynthetically induced calcification. This could be caused by a blockage to sunlight exposure when phytoplankton blooms occur during nutrient loading from terrestrial runoff or seasonal upwelling. Nutrient availability is one of two essential controls on primary productivity, the other being light exposure (Paytan, 2009). In the GOP, seasonal upwelling occurs and brings higher concentrations of limiting nutrients and colder water to the surface water during the upwelling season (Jan-Mar), compared to the nutrient-depleted, warmer waters in the non-upwelling season (Apr-Dec) (D'Croz and O'Dea, 2007). Increases in nutrient loading and availability to CA are thought to increase CA abundance, as was seen in the Miocene when rhodalgial facies outnumbered coral facies (Littler and Littler, 2013; Halfar and Mutti, 2005). In this scaled-down experimental setting, no significant pattern in nutrients emerges to explain increased or decreased algal growth (Fig 5). DFe was monitored in order to determine if fluctuations in its delivery impacted growth patterns. DFe remained fairly consistent throughout the experiment (Fig 5). No evidence points to a direct impact by the DFe concentration on growth band formation, as is the case with the remaining nutrients monitored. Negative trends in DOC and DON (Fig 5) are associated with the salinity shift from more saline to less saline seawater as the upwelling season transitioned to the non-upwelling season. The SUVA₂₅₄ values are used to characterize the quality of the dissolved organic matter (DOM) in the water. The results shown in Fig 5 indicate that the water is oceanic in nature and that there is no signal for terrestrial inputs of DOM, which can be associated with increased growth from increased nutrient loading. Even if there was slight

variation in the nutrient delivery from one microcosm to the next, there was still variable axial growth within a single rhodolith, which was exposed to equal nutrient loading within its microcosm and thus helps eliminate the influence of tank effects. Furthermore, seawater cycled through a microcosm approximately every 20 minutes and would have not had the opportunity for the rhodolith to deplete available nutrients. Therefore, nutrients likely played a minor role in growth variation and are not a plausible source of growth band periodicity in this experiment.

In addition to nutrient availability, the amount of sunlight available to an alga's surface could impact growth and may induce banding. This has mainly been associated with encrusting CA (Halfar et al., 2011; Adey, 1970) but has implications for rhodoliths as well. In *Lithothamnion glaciale* from Loch Sween, Scotland, Burdett et al. (2011) showed that greater winter cloud cover resulted in reduced summer calcification during the same year. Blake and Maggs (2003) reported that temperate species *Phymatolithon calcareum*, *Lithothamnion coralloioides* and *L. glaciale* grew faster at 10 m depths than at 5 m depths, and suggested that diffuse irradiance increased growth compared to direct irradiance at shallower depths. In our experiment light exposure did not significantly vary, with the exception of natural day and night cycling. The average measured PPFD to quantify PAR was $469.25 \mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$, which falls in the PPFD range of $300\text{--}500 \mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ measured in shallow rhodolith beds in other tropical regions (Semese et al., 2009). Thus, extreme shifts in light exposure cannot be invoked to explain the observed banding. However, variation in some of the stained rhodoliths appears to be related to the axial position of the rhodolith in reference to a light source, and whether the light is directly or indirectly over the growth surface. For instance, one of two HOBO Pendants averaged more output than the one Pendant directly under the LED lamp, and yet samples still grew on average 40% faster in the indirect grouping. This further suggests that growth variation has to do with the axial position, and not necessarily the light intensity of the light source. This could also mean that the rhodoliths grew more rapidly with the aid of LED lamps, and that the direct placement allowed for an exposure that was too intense and instead stunted growth. Despite the rhodoliths' adaptation to grow under relatively high-light conditions (Adey

and Macintyre, 1973) as observed in the shallow, 2-3 m deep collection site, growth can be stunted from light exposure.

Further evidence of light influence may be taken from the multi-axial variation along with the effects of overturning. In this experiment rhodoliths were randomly overturned approximately 13 times, thus altering the orientation of direct exposure. Darrenougue et al. (2013) suggested that the greatest growth extension would be in the longest axis (A) relative to the shortest (C), and that overturning could be a major contributor to the variability in growth within a single rhodolith as the orientation of axis A changes. This could be because axis A has the most exposed surface area to the light source compared to axis C, allowing for more extensive growth in the longest axis. In the wild, Axis A is also likely never to be fully buried given the geometry of how the rhodolith will move on the seafloor. In our experiment the overall average total growth in axis A was 1.1 mm, compared to axes B and C with an average total of 0.8 mm. However when the total new growth results are separated by light regimes, this relationship reverses. Axis A's average new growth was 0.79 mm (direct) and 1.19 mm (indirect). Axis B had an average total growth of 0.14 mm (direct) and 1.0 mm (indirect), and axis C's averaged total were 0.67 mm (direct) and 0.80 mm (indirect). In cases where axes B or C grew the most bands or total growth, this may have been due to a more preferential position relative to the light source compared to axis A. These observations may have implications for wild samples that get overturned, and have different axes exposed at different times, therefore creating a situation that could cause variable or disrupted growth. Variable growth results in this study could also be related to the fact that there was no sediment or partial burying in the microcosms, which would be present in the wild. Instead, the microcosm bins may have created more reflective surfaces for the light to interact with the rhodoliths. Additionally, when compared to wild conditions, the rhodoliths also sat in about 13 cm of water and were ~0.5 m from the light source compared to 2-3 m below the water column in their natural habitat. This may contribute to the variance in banding and overall total extension.

The question then becomes, which axis is showing the complete growth history? It would seem that they all are; the amount of growth recording the history is just different within the same rhodolith, or growth is inconsistent. For time series analyses, this is a challenge that has not been well-addressed. The answers could range from generating reconstruction methods that use a large number of branch samples and averaging techniques to adding multiple individuals to an analysis to increase overall sample size to suggesting that GOP rhodoliths are not a suitable proxy data source for complete time series reconstructions. The latter may indicate that GOP rhodoliths may be more useful for assessment of general environmental conditions.

5.2 Growth rates and age

The growth feature that correlates the most with the post-ARS growth rates was the number of bands grown (Fig 7). Band widths in both the pre- and post-ARS growth averaged about the same, supporting that banding is systematic and that experimental conditions resembled the natural setting. Thus, if more growth (extension) occurs, it is typically because a greater number of bands was grown. The overall average experimental growth rate of the post-ARS growth was 1.84 mm yr^{-1} using Method #5 (ARS staining) compared to the pre-ARS rate of $0.18 \pm 0.07 \text{ mm yr}^{-1}$ using Method #6 (band width average along 2 mm transect). Obviously, these rates are not directly comparable given the annual assumption of growth band periodicity in the latter method. However, the point is that near-identical band widths were formed in the same branch both pre- and post-ARS, and yet depending on which method is employed, very different growth rates are determined. Both methods cannot be correct, and so there is a potential outcome here that suggests that the pre-ARS bands are indeed sub-annual in at least some cases, and not annual, thus making the Schäfer et al. (2011) method invalid in our sample population.

The range in post-ARS growth rates, though, is quite significant. The highest post-ARS growth rate was 5.35 mm yr^{-1} for a single branch (axis A; indirect), and the lowest rate was 0.1 mm yr^{-1} (axis B; indirect and axis A; direct). The total intra-axial growth rate variation for axis B (indirect) was $2.39 \pm 1.31 \text{ mm yr}^{-1}$ and for axis C (indirect) it was $0.87 \pm 1.56 \text{ mm yr}^{-1}$ supporting that indeed there is faster growth in axis B

compared to C, but the variation based on standard deviations is quite high (Table 2). The average rate of 1.84 mm yr⁻¹ is not out of the range of possibility for CA based on other published studies, particularly tropical rhodoliths. Table 3 shows a wide range of growth rates of CA (both free-living and encrusting) around the world, with samples of *Lithophyllum margaritae* from the Gulf of California (Mexico) exceeding 2.0 mm yr⁻¹. The majority of CA have growth rates <0.4 mm yr⁻¹. The range in rate between the branches shows that if only a few branches are selected for growth analysis, particularly the longest in axis A, a potentially biased timeline may be rendered. Additionally, growth rates and associated axial biases maybe better understood using Mg/Ca ratios due to the observations by Moberly (1968) that a faster growing (less dense) portion of a band incorporates higher amounts of Mg than a slower growing (more dense) portion of a band. One could extrapolate this further and suggest that branches with multiple bands could have more Mg/Ca cycles than what temperature could explain, thus supporting that light or other non-environmental influences control growth, as we suggest in this study.

The suggested ages of the rhodoliths prior to the start of the monitoring experiment are between 40 and 122 ± 28 years based on Method #6 (band width average along 2 mm transect; Schäfer et al., 2011). This estimation assumes that the pre-ARS (wild) bands are annual. This contradicts the experimental results based on post-ARS growth. Either the assumption of annual banding is invalid, or the post-ARS growth was accelerated due to the microcosm environment and bands were produced that do not correspond to any identified environmental cycle.

One line of evidence that supports that the pre-ARS bands are annual is that the age range based on band counting aligns with Schäfer et al.'s (2011) U/Th ages. This dating method was outside the scope of this project, but gives backing to the idea that wild rhodoliths generate annual bands, which could be induced by seasonal upwelling in the GOP. It should be noted that U/Th ages in CA should be interpreted with caution, due to the open-system behavior of U/Th sometimes exhibited in CA (Linge et al., 2008). This can cause the CA ages to appear older than they actually are (Linge et al., 2008), which is a particularly acute problem in U/Th dating of very young samples if the CA uptake Th²³². The result that challenges

734 this accuracy of the U/Th age data is the band width averages being nearly the same for the pre- and post-
735 ARS growth.

736 **Table3.** Summary of CA growth rates from around the world (modified from Darrenougue et al., 2013).

Species	Algal Type	Location	Growth Rate (mm yr ⁻¹)	Chronological Method	Source
<i>Lithothamnion crassiusculum</i>	Rhodolith	Gulf of California (Mexico)	0.6±0.1 0.6	Radiocarbon dating ARS	Frantz et al. (2000) Rivera (1999)
<i>Lithothamnion muelleri</i>	Rhodolith	Gulf of California (Mexico)	0.25-0.45	Mg/Ca, δ ¹⁸ O	Halfar et al. (2000)
<i>Lithothamnion glaciale</i>	Rhodolith	Newfoundland (Canada)	0.60; 0.87	ARS	Rivera et al. (2004)
		Loch Sween (Scotland)	0.25-0.45	Mg/Ca, δ ¹⁸ O	Halfar et al. (2000)
		Subarctic	0.15-0.17	ARS, Mg/Ca, major banding	Kamenos et al. (2008)
<i>Lithophyllum orbiculatum</i>	Crustose	Boreale	0.2		Adey and Vassar (1975)
<i>Lithothamnion</i>	Rhodolith	Gulf of Panama (Panama)	0.16	Mg/Ca, major banding	Adey and Vassar (1975)
		Gulf of Panama (Panama)	1.84	ARS, major banding	Schäfer et al. (2011)
		Gulf of Chiriqui (Panama)	0.17	Mg/Ca, major banding	This study
<i>Lithophyllum margaritae</i>	Rhodolith	Gulf of California (Mexico)	2.9 ± 0.66	Buoyant weight, total alkalinity, ARS	Schäfer et al. (2011)
<i>Clathromorphum circumscriptum</i>	Crustose	Subarctic	0.2		Steller et al. (2007)
<i>Clathromorphum nereostratum</i>	Crustose	Aleutian Islands (USA)	0.30 ± 0.03 0.35 (0.19-0.65)	Radiocarbon dating Major banding, U/Th dating	Adey and Vassar (1975)
<i>Clathromorphum compactum</i>	Crustose	Gulf of Maine (USA)	~0.5	Mg/Ca	Frantz et al. (2005)
		Newfoundland, Quebec (Canada)	0.3 ± 0.08	Mg/Ca	Halfar et al. (2007)
<i>Phymatholithon calcareum</i>		Isle of Arran (Scotland)	0.13-0.119	ARS, Mg/Ca, major banding	Chave and Wheeler (1965)
		Strangford Lough (N Ireland)	0.92	ARS	Halfar et al. (2011)
		Ria de Vigo (Spain)	~0.9	Monthly, in situ growth measurements	Kamenos et al. (2008)
<i>Phymatolithon polymorphum</i>	Crustose	Boreale	0.3		Blake and Maggs (2003)
<i>Hydrolithon reinboldii</i>		Tahiti (French Polynesia)	0.37	ARS	Adey and McKibbin (1970)
<i>Sporolithon durum</i>	Rhodolith	Noumea (New Caledonia)	0.6 ± 0.2	ARS, radiocarbon dating, Mg/Ca, major banding	Adey and Vassar (1975)
					Payri (1997)
					Darrenougue et al. (2013)

6. Conclusions

Band widths were approximately the same in the pre- and post-ARS growths suggesting that the artificial microcosm environment did not cause the algae to produce unnatural banding nor exert a control over the width of a band. Instead, the microcosm setup may have influenced the total amount of growth, e.g. the number of bands produced. The number of bands counted in the post-ARS growth ranged from 0 to 20 with up to a 12 band difference in a single rhodolith and a 7 band difference in a single axis. These observations indicate that bands are not always annual and that the bands would not be a universally reliable measure of time. Secondary lines of evidence, such as radiometric dating, are advisable to gain confidence in the timing of rhodolith growth if the dating method is accurate. Radiometric dates, particularly when derived from near modern samples, need to be interpreted in light of potential sources of error such as poorly constrained reservoir effects influencing absolute radiocarbon ages and open system behavior associated with U-series dating – both are the most commonly used dating methods in coralline algal studies (Linge et al. 2008, Halfar et al. 2013).

The most likely cause of the post-ARS growth variation was the axial position of the rhodolith branches to the light sources. This was evidenced by preferential growth in axis A compared to axes B and C that had similar amounts of growth. Bands grew [irregularly] even though seawater conditions (e.g. temperature, salinity, pH, nutrients) were stable and non-oscillatory. In particular, temperature did not appear to trigger changes in growth such as band formation or number of cell rows grown based on the observation that the temperature records during the experiment did not correlate with the frequency of bands, supporting Kamenos and Law's (2010) observations that temperature does not have a consistent relationship to band width in corallines. This is also the case for encrusting CA *Clathromorphum nereostratum* (Halfar et al., 2011a) and *C. compactum* (Halfar et al., 2013) where light exerts a dominant control. In contrast, Halfar et al. (2011b) use band width as a temperature proxy using *C. compactum* in the Atlantic showing that in some species of CA and locations there is connection between temperature and growth. This highlights the inconsistency of rhodolith proxy sources and that each one needs to have

its growth patterns assessed before paleoenvironmental interpretations can be made. Lastly, the distance the rhodoliths were placed from the light sources (direct versus indirect) in this experiment was shown to be the most significant explanation for variance in overall growth rate.

Further study is needed to compare *in situ* and experimental growth. Experiments can introduce anomalous conditions that inhibit results from being directly transferable to organisms grown in their natural habitats. Close environmental monitoring of wild rhodolith populations would likely yield complementary data that will clarify a broader range of causes and periodicity of growth. In particular, more natural lighting and better control on spatial light variation would help constrain the influence of light. Additionally, the observations that the rhodoliths grow sub-annual bands may be further elucidated through geochemical analyses, such as the measurement of Mg and Ca in order to use Mg/Ca ratios for enhanced band identification (Halfar et al., 2000).

Even considering these limitations of an experimental approach, our results suggest caution should be taken when performing paleo-reconstruction aimed projects using Panamanian GOP rhodoliths, and perhaps other rhodoliths of a similar genus or morphology. Here we have demonstrated that within the same rhodolith, let alone from one alga to the next, there can be a large difference in the number of bands produced and overall extension rates. This has significance for researchers attempting to build environmental time-series proxies using rhodoliths and indicates caution when selecting only a few branches as the basis for a study. Branch selection maybe biased toward long branches, perhaps in axis A, that have an accelerated growth that does not truly match environmental records. Yet our results suggest that even in these branches with rapid growth, there is likely variation in rate, and perhaps periods of growth cessation, that may be difficult to detect. One solution to this may be to select a large (>50), random number of samples to improve statistical significance and representation of the population. Then, through a process of time series averaging, model a more reliable growth signal.

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