Journal of Experimental Marine Biology and Ecology 490 (2017) 64–73

Contents lists available at ScienceDirect

Journal of Experimental Marine Biology and Ecology

journal homepage: www.elsevier.com/locate/jembe

Suitability of three fluorochrome markers for obtaining *in situ* growth rates of coralline algae

Bonnie Lewis [a,*], Guillermo Diaz-Pulido [a,b]

[a] Griffith School of Environment and Australian Rivers Institute – Coast & Estuaries, Nathan Campus, Griffith University, 170 Kessels Road, Brisbane, Nathan, Queensland 4111, Australia
[b] ARC Centre of Excellence for Coral Reef Studies, Queensland, Australia

ARTICLE INFO

Article history:
Received 5 October 2016
Received in revised form 1 February 2017
Accepted 5 February 2017
Available online 27 February 2017

Keywords:
Calcification
Coralline algae
Coral reefs
Fluorochrome
Growth
Growth marker

ABSTRACT

Coralline algae play a central role in reef ecology and have been identified as one of the most sensitive calcifying organisms to ocean acidification, and as such are potential indicators for evaluating the future effects of climate change. Despite their importance, little information is available on their growth and calcification rates *in situ*, highlighting the need for a more effective technique to obtain these measurements. In this study we compared two alternative fluorochrome markers (calcein and calcofluor white - CFW) against the commonly used alizarin red stain in order to establish a more efficient and effective staining protocol for use in the field on both branching (*Lithophyllum pygmaeum*) and encrusting (*Porolithon onkodes*) coralline species. Each marker was tested using three different concentration and immersion times for toxicity, visibility and efficiency. Results from this study found that while alizarin displayed a high visibility frequency it also reduced growth in the branching species. The CFW staining proved unreliable for the encrusting samples with usable marks in only 3 of 9 treatments and also reduced growth in branching treatments. Calcein, however, proved to be an effective and efficient marker for *in situ* coralline studies with a high visibility frequency, no toxic effects on growth and a short immersion time of 3 h or less. This identification of a more efficient and effective stain to use *in situ* on coralline algae can potentially improve demographic studies in both the field and laboratory environments, further facilitating future climate change research.

1. Introduction

Coralline algae play fundamental and ecologically important roles in tropical reef environments. As calcifiers they cement and stabilise the reef framework (Littler and Littler, 1984), provide hard substrate and settlement cues for commercially important invertebrates (Diaz-Pulido et al., 2007; Harrington et al., 2005), provide habitat (Foster, 2001), and are an important food source (Guinotte and Fabry, 2008; Littler and Littler, 1984). Furthermore, rhodolith beds, made up of free living coralline algae (Foster et al., 2013), are one of the world's four largest macrophyte-dominated benthic communities (Amado-Filho et al., 2012; Foster, 2001; Foster et al., 2007) and play a significant, yet largely unquantified, role in calcium carbonate production and carbon sequestration (Amado-Filho et al., 2012; Hill et al., 2015). Due to their importance, and the uncertainty in their carbon storage capacity, empirical studies are urgently needed to quantify the potential of these carbon sinks (van der Heijden and Kamenos, 2015). Coralline algae have also been earmarked among marine calcifying organisms as one of the most sensitive to ocean acidification (Jokiel et al., 2008; Martin and Gattuso, 2009) and as such are potential indicators for assessing the impacts of climate change. Currently, information on *in situ* growth and calcification rates of coralline algae are lacking (Chisholm, 2000; Fisher and Martone, 2014; Morrison et al., 2013; Villas Bôas et al., 2005) due to their slow growth (Blake and Maggs, 2003; Fisher and Martone, 2014; Rivera et al., 2004) (necessitating lengthy studies) and the difficulty in obtaining field measurements (Martone, 2010). With the effects of ocean acidification and warming becoming increasingly evident (Diaz-Pulido et al., 2012; Kuffner et al., 2008; Martin and Gattuso, 2009), a reliable and efficient method of obtaining coralline algal growth and calcification rates in the field is essential to better understand and identify the effects of future climate change.

Various techniques have been previously used to determine coralline growth and calcification rates in the field, these include growth banding (Darrenougue et al., 2013; Freiwald and Henrich, 1994; Halfar et al., 2007; Kamenos and Law, 2010; Kuffner et al., 2008), Mg/Ca ratio cycles (Darrenougue et al., 2013; Halfar et al., 2000; Hetzinger et al., 2009), buoyant weight (Johnson et al., 2014; Jokiel et al., 2008; Payri, 1997; Potin, 1990; Steller et al., 2007), and the alkalinity anomaly (Chisholm and Gattuso, 1991; Egilsdottir et al., 2012; Payri, 1997;

Abbreviations: CFW, Calcofluor white; GBR, Great Barrier Reef; HIRS, Heron Island Research Station.

* Corresponding author.
E-mail addresses: bonnie.lewis@griffithuni.edu.au (B. Lewis), g.diaz-pulido@griffith.edu.au (G. Diaz-Pulido).

http://dx.doi.org/10.1016/j.jembe.2017.02.004
0022-0981/

Steller et al., 2007), each with their own specific limitations and levels of accuracy (Carricart-Ganivet, 2011; Morrison et al., 2013; Steller et al., 2007). For example, some species show clearer annual banding than sub-annual banding patterns (Halfar et al., 2008; Kamenos et al., 2008) limiting their use in the analyses of short-term responses to environmental changes. Meanwhile, a study by Steller et al. (2007) found the buoyant weight method gave two-fold greater rates of calcification than the alkalinity anomaly method in the same experiment, highlighting the possible inaccuracies between methods.

Staining the coralline algae with a chemical marker such as alizarin is another growth and calcification measurement technique that has been widely utilised (Agegian, 1981; Andrake and Johansen, 1980; Blake and Maggs, 2003; Rivera et al., 2004; Steller et al., 2007). Fluorochromes such as alizarin work by binding to the calcium in newly deposited skeleton, providing a reference point that can then be used to measure growth from the time of exposure. This enables identification of skeleton that has been deposited during a specific time-frame, allowing analysis of short-term responses to changes in environmental conditions such as seasonal changes in light and temperature. Advantages of this staining technique for use in the field include its simple implementation (Holcomb et al., 2013), quick incorporation into the skeleton (Lartaud et al., 2013), and ease of detection (Holcomb et al., 2013; Lartaud et al., 2013). Importantly, staining methods (unlike the buoyant weight or the alkalinity anomaly) provide a direct measurement of the calcium carbonate deposited by the thallus (skeletal extension), excluding secondary calcification processes occurring deeper in the skeleton that may confound measurements of primary calcification.

Alizarin applied with a 24 h immersion time is currently the most utilised method of staining coralline algae (Agegian, 1981; Blake and Maggs, 2003; Payri, 1997; Ragazzola et al., 2012; Rivera et al., 2004; Steller et al., 2007) and, while highly successful, this long immersion time is less suited to *in situ* staining. A study carried out by Andrake and Johansen (1980) found an immersion time of just 5 h to be successful in marking the articulated (geniculated) *Corallina offincinalis* with alizarin in rock pools. The success of this shorter immersion time, however, has yet to be tested on other coralline species, including encrusting (non-geniculate) forms. Shorter immersion times in other marine calcifying organisms such as gastropods have resulted in faint imprecise banding (Riascos et al., 2007) or, in corals, a complete absence of marks (Harriott, 1999). Studies by Blake and Maggs (2003) and Ragazzola et al. (2012) have also found alizarin to be species specific in corallines with the percentage of visible marks falling from 80 to 30% between species and from 30% to non-distinguishable for the two studies, respectively. The use of alternative markers for growth studies, such as the fluorochromes calcein and calcofluor white (CFW) has gained momentum in recent years. Calcein has been successfully used to indicate skeletal growth in a wide variety of marine animals including corals (Tambutte et al., 2012), gastropods (Moran, 2000), and molluscs (Linard et al., 2011), and on early stage calcification of corallines in a laboratory setting (Bradassi et al., 2013). With a short immersion time and no detectable or sub lethal effects on these organisms, calcein displays the characteristics needed in an efficient and effective marker for use on corallines in the field. The CFW marker is a fluorochrome that has been successfully used to stain articulate corallines in the field by Martone (2010) and Fisher and Martone (2014). These studies found CFW to be an effective marker on the three temperate coralline species and suitable for use in the field due to its short immersion times (5 min) and high marking success rate. The effectiveness of CFW on encrusting corallines however is yet to be determined.

Here we present a study on the most suitable staining technique to measure *in situ* on both encrusting (*Porolithon onkodes* (Heydrich) Foslie) and branching (*Lithophyllum pygmaeum* (Heydrich) Heydrich) non-geniculate tropical coralline algae. The aim was to test three fluorochromes and identify the marker that was non-toxic, reliable and highly visible, and efficient for use on these two abundant coralline species.

2. Materials and methods

2.1. Sample collection & staining

In this study we compared the suitability of calcein and CFW against the commonly used alizarin for *in situ* staining on two abundant species of coralline algae; the encrusting *Porolithon onkodes* and the branching *Lithophyllum pygmaeum*. Both species were selected for the study due to their high abundance (Ringeltaube and Harvey, 2000) and importance as reef-builders (*P. onkodes*) and habitat providers (*L. pygmaeum*). Using these two species also allowed a comparison of the three different markers effectiveness between the two (encrusting and branching) morphologies. We initially conducted an experiment over the austral spring season (Experiment 1) comparing alizarin (Chem Supply C.I 58005), calcein (Sigma-Aldrich, CAS 1461-15-0) and CFW (18909 Fluka). No visible marking resulted from the CFW (18909 Fluka) treatment, however, so a more concentrated version of CFW (Fluorescent Brightener 28, Sigma-Aldrich CAS 4404-43-7) was used and compared to alizarin in a second experiment conducted over the summer season (Experiment 2). Due to logistic constrains, we were unable to compare all three markers simultaneously in the same climatic season.

The two consecutively run experiments were based on Heron Island Research Station (HIRS), southern Great Barrier Reef (GBR), Australia. Specimens were carefully collected using hammer and chisel from the nearby reef crest and upper reef slope (4–6 m) of Heron Island (Tenements 1; 23°26′00.4 S, 151°55′41.3 E) and transported back to the outdoor flow-through tank facilities on HIRS where fresh seawater was supplied directly from the reef lagoon. While in the flow-through tanks samples were gently cleaned by hand to remove epiphytes, invertebrates and loose material and cut to size (*P. onkodes*: 3 × 3 cm chips, *L. pygmaeum*: 2 cm high fragments of approx. 5 branches) as shown in Fig. 1.

Each of the three markers (Alizarin, Calcein and CFW) were tested under three different concentrations for three different immersion times (as shown in Table 1) to determine best staining protocol. Concentration and time treatment levels were based on previous use on marine calcifying organisms found in the literature (Blake and Maggs, 2003; Linard et al., 2011; Marschal et al., 2004; Martone et al., 2010; Riascos et al., 2007; Rivera et al., 2004; Steller et al., 2007). For each treatment combination, five individuals of both *P. onkodes* and *L. pygmaeum* were randomly selected to be stained together in 5 L of fresh seawater. Each treatment container had a small pump for circulation and was placed in a temperature controlled room to maintain the current *in situ* water temperature (22 °C for Experiment 1, 26 °C for Experiment 2) and prevent overheating. Alizarin was dissolved in a small beaker of fresh seawater and then added to the treatment, while calcein and CFW were first dissolved in de-ionised water (<100 mL), with the addition of small amounts of sodium bicarbonate ($NaHCO_3$) to enhance solubility (Wilson et al., 1987) and then added to the fresh seawater treatment. After staining, individuals were rinsed under fresh seawater to remove any residual stain, set in a PVC ring (40 mm diameter) with epoxy (Selleys Aqua Knead It) to secure the sample and prevent dissolution of exposed skeleton (Fig. 1), and attached to the reef slope (4–6 m depth, approximately in the same area where samples were collected) on galvanised racks for a period of 3 months (Fig. 2)

2.2. Detection of marker using fluorescence microscopy and vertical growth measurements

Following the 3 month period all samples were collected from the reef slope, brought to the HIRS labs and oven-dried at 60 °C for 24 h.