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A method for growing a monospecific epilithic cyanobacterial biofilm for use in marine ecological experiments

Sónia Brazão [a,*], Min Chen [b], Richard J. Murphy [c], Stephen J. Simpson [b,d], Ross A. Coleman [a]

[a] *Coastal & Marine Ecosystems, Marine Ecology Laboratories (A11), School of Biological Sciences, The University of Sydney, NSW 2006, Australia*
[b] *School of Biological Sciences (A08), The University of Sydney, NSW 2006, Australia*
[c] *Australian Centre for Field Robotics, The Rose Street Building J04, Department of Aerospace, Mechanical & Mechatronic Engineering, The University of Sydney, NSW 2006, Australia*
[d] *Charles Perkins Centre, The University of Sydney, NSW 2006, Australia*

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ABSTRACT

On most intertidal rocky shores, marine filamentous cyanobacteria Oscillatoriales are important components of natural biofilms. Some studies have already suggested the nutritional importance of benthic cyanobacteria for intertidal grazers; however, due to the complex nature and technical difficulties of observing and manipulating biofilm *in situ*, questions related to the relationship between what is available and what is actually consumed and assimilated by intertidal grazers remain unanswered. An alternative approach could be to use an isolated benthic cyanobacterial strain and grow it on surfaces resembling natural rocky shores under controlled laboratory conditions. Researchers can then test hypotheses about feeding behaviours and nutritional implications of grazers feeding on a monospecific biofilm. In this study a procedure to grow a blue-green cyanobacterial biofilm on experimental sandstone units (ESUs) was developed. Evidence of this growth is demonstrated using two established techniques in the analysis of chlorophyll *a* (Chl *a*) as a proxy for biomass. Destructive (spectrophotometric) and non-invasive (spectrometric) methods were used to measure the photosynthetic pigment in the benthic filamentous blue-green cyanobacterium *Geitlerinema* sp. strain CS-897 in a 26-day experiment. Amounts of Chl *a* were assessed every two days and a continuous and significant growth was observed through time (values ranging from 0.20 μg cm^{-2} on day 0 to a maximum of 1.10 μg cm^{-2} on day 20). A strong linear relationship ($R^2 = 0.87$) was found between the spectrophotometric measurements of Chl *a* and the index NIR/red (R_{750}/R_{672}) obtained from reflectance spectrometry, providing for the first time evidence for the attachment and growth of a monospecific cyanobacterial biofilm on sterilised ESUs under controlled laboratory conditions. The linear regression equation obtained from this relationship can then be used to assess non-destructively the amount of food provided and consumed by key intertidal grazers in future manipulative feeding experiments. These results provide a platform for further studies focusing on the nutritional ecology and feeding behaviour of intertidal grazers. The development of experimental monospecific and/or mixed benthic biofilms on rocky substrata opens up new possibilities for experiments to improve understanding of key ecological processes such as competition, foraging behaviour and the effects that different benthic grazers have on the biofilm.

1. Introduction

Cyanobacteria are significant contributors to primary productivity and are vital to the functional ecology of a diverse range of ecosystems such as microbial mats (Abed et al., 2008; Jodłowska and Latała, 2013), stromatolites (Goh et al., 2009) and lakes (Quesada et al., 1999). They are also an important component of intertidal epilithic biofilms on rocky shores (Brito et al., 2012; Jackson et al., 2013; Nagarkar, 1998), covering even apparently bare surfaces along with assemblages of diatoms, protozoan, fungi, lichens and macroalgal propagules, all embedded in a mucopolysaccharide matrix (Anderson, 1995; Decho, 2000; Hill and Hawkins, 1991). The value of these complex 3-dimensional biofilms as the principal food source (Hill and Hawkins, 1991; Nicotri, 1977; Steneck and Watling, 1982) and the settlement for intertidal grazers is well known (Chiu et al., 2007; Tung and Alfaro, 2011; Zhao and Qian, 2002). There is, however, little current information about the contribution of each component of the biofilm to the diet of grazers or on whether grazers are selecting specific components of the biofilm.

Ideally, the study of relationships between patterns of food supply and effects on grazers should be done by manipulating the spatial abundance of different foods (i.e. specific components of the biofilm assemblage) in natural habitats. This step, however, implies *a priori* knowledge of which foods (amount, composition and nutritional consequences for grazers) are available in natural habitats. Such information

* Corresponding author.
E-mail address: sonia.estevesbrazao@sydney.edu.au (S. Brazão).

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is largely unavailable for rocky intertidal systems. Several studies focusing on the distribution of intertidal epilithic biofilms on natural and artificial rocky shores have revealed their spatial and temporal variability even at very small scales (e.g. Anderson, 1995; Chapman and Underwood, 1998; Thompson et al., 2005). Bottom-up (e.g. wave exposure, topography features) *versus* top-down (e.g. grazing) forces can strongly influence the distribution and composition of biofilms on rocky shores, making their study and manipulation *in situ* extremely difficult and logistically challenging. Nonetheless, tropical rocky shores have been found usually to be dominated by cyanobacteria-based biofilms (Kaehler and Williams, 1996; Nagarkar, 1998; Nagarkar and Williams, 1999), while diatoms tend to prevail on temperate rocky shores (Hill and Hawkins, 1991).

The complex and patchy nature of intertidal epilithic biofilms together with the difficulties in observing, identifying and then manipulating their different components *in situ* have contributed to the lack of studies on the nutritional consequences for intertidal grazers of ingesting a particular component of the biofilm assemblage, for example cyanobacteria. An alternative approach would be to develop monobiofilms of typical 'foods' (cyanobacterial and/or diatom, for example) under controlled laboratory conditions on sterilized substrata to avoid contamination with other biota. Laboratory feeding experiments could then be used to test the influence of particular biofilm compositions on the behavioural and physiological responses of key intertidal grazers. Although the potential value of benthic diatoms as food has already been tested for marine organisms such as sea urchins (Xing et al., 2007) and abalones (Carbajal-Miranda et al., 2005; Courtois de Viçose et al., 2012; Takami et al., 1997), the dietary value of benthic cyanobacteria is still poorly understood. To understand the nutritional consequences of grazing on a cyanobacterial biofilm it is first necessary to develop methods to grow a monospecific biofilm on experimental units.

One exception to the paucity of studies on the dietary value of cyanobacteria is the work of Nagarkar et al. (2004) who explored the nutritional quality of 13 species of cyanobacteria isolated from intertidal epilithic biofilms from rocky shores in Hong Kong, and suggested that biofilms dominated by cyanobacteria were a high value food source for intertidal grazers. This hypothesis is, however, yet to be tested experimentally. An important first step to doing so is ensuring a constant food supply for grazers during manipulative feeding experiments. To this end, the benthic blue-green marine cyanobacterium *Geitlerinema* sp. strain CS-897 (Class: Cyanophyceae, order Oscillatoriales) was used as a model in this study due to their highly adhesive properties and potential to be used as a possible food source for intertidal grazers. This genus belongs to one of the most dominant orders covering rocky shores all over the world. Cyanophyceae are also one of the first groups to colonize bare rocky surfaces (MacLulich, 1986). The aim was to develop a technique for growing a monospecific biofilm. This was tested in respect of whether *Geitlerinema* sp. would attach and how long it would take for a biofilm to form on sterilised sandstone units (as an analogue for natural rocky shores) under controlled laboratory conditions.

To grow a biofilm containing this genus of cyanobacteria on experimental sandstone units (ESUs) required achieving five key objectives. Previous studies have suggested that the mineralogical or elemental nature of a substratum may play a significant role in the intertidal biofilm formation process (Bavestrello et al., 2000; Cattaneo-Vietti et al., 2002; Faimali et al., 2004). Consequently, the first objective was to determine the elemental composition of ESUs before growing any type of biofilm on their surfaces. Future comparisons can then be made with studies using sandstones of known and similar elemental signature. The second objective was to establish whether ESUs could be used as a surface for attachment of cyanobacteria which resemble natural rocky shores. This was done by testing the hypothesis that procedures such as sterilising (i.e. to assure that there is no previous biofilm attached) or adding algae-enriched media (i.e. to provide cyanobacteria with all major nutrients for optimal algal growth) would not change the spectral features of the ESUs. The third objective was therefore to describe the conditions under which *Geitlerinema* sp. could be grown to and develop a protocol for growing them on the top surface of sandstone units. Consequently, the fourth objective was to provide evidence for growth and to quantify it using destructive (spectrophotometric) vs. non-invasive (spectrometric) techniques for estimating chlorophyll *a* (Chl *a*) as a proxy for biomass abundance. These data tested the hypothesis that if *Geitlerinema* sp. attaches and forms a biofilm on the ESUs then the amount of Chl *a* will increase over time. The fifth and final objective was to establish the nature and strength of the relationship between the above two techniques using linear regression. Similar linear regressions were established in previous studies to estimate non-destructively the amount of intertidal epilithic biofilm available (Murphy et al., 2006; Murphy et al., 2011) and/or consumed by intertidal grazers (Iveša et al., 2010; Jackson et al., 2009; Murphy and Underwood, 2006) on rocky shores and/or in the laboratory. None of those equations could, however be applied in this study because the growth of *Geitlerinema* sp. on ESUs involves the use of algal enriched media. Consequently a new equation had to be created in order to check if the R_{750}/R_{672} was, in the presence of residual growth medium, an adequate indicator of the biofilm growth. The derived regression coefficients will then be used to assess, non-destructively, the amount of food provided and/or consumed by key intertidal grazers in future manipulative feeding experiments using algal growth media.

2. Materials and methods

2.1. Elemental composition of the experimental sandstone units

Experimental sandstone units obtained from a quarry were used in this study as a standardised substratum for growing a cyanobacterial biofilm, as a surrogate for a natural intertidal substratum. Sandstone is one of the commonest types of sedimentary rock found on artificial (e.g. intertidal seawalls, breakwaters, pilings, jetties) and natural intertidal platforms (Bulleri, 2005; Bulleri et al., 2004; Chapman and Underwood, 1998). Such habitats support a wide range of epilithic grazers (Bulleri et al., 2005; Chapman, 2006). It is mostly composed of sand-sized grains of minerals compacted into rock but also contains cements such as calcite, silica or iron oxides which act to bind the sand grains together and so dictate their strength and mineral composition (Bowen et al., 2007; Hunt, 2008; Morton and Hallsworth, 1999). Quarried sandstone blocks, panels and/or discs have been widely used on rocky shore platforms and/or artificial seawalls (Bulleri, 2005; Murphy et al., 2011; Murphy et al., 2005a) to grow epilithic biofilm and as settlement plates for invertebrates.

All ESUs (27 × 27 mm, 20 mm deep) were cut from locally sourced sandstone panels of uniform colour, texture and hardness. X-Ray Fluorescence Spectrometry (XRF) was used to determine which elements were present in the sandstone and their relative abundances. Note that this was done to determine the basic elemental composition of the sandstone, not to determine if there were any significant differences among individual ESUs. Three individual ESUs were randomly selected and were dried and pulverised in a vibrating disc pulveriser. Powders were then cast using a 12:22 flux to form a glass bead. Carbonate content was estimated indirectly by loss-on-ignition (LOI-1000) using a robotic Thermo-Gravimetric-Analyser (TGA). All results were reported on a dry sample basis (% dry weight).

2.2. Pilot study on the use of experimental sandstone units as a surface for attachment

Growing a monospecific biofilm on an artificial surface implies that there is no previous biofilm developed on it. All ESUs were therefore autoclaved (sterilised) at 121 °C for 1 h. It was also necessary to provide cyanobacteria with all the major nutrients, trace metals and vitamins