Culturing large erect shelf bryozoans: skeletal growth measured using calcein staining in culture

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Bryozoan skeletons are increasingly being exploited as a record of environmental change. This study applied calcein staining to large erect shelf bryozoans in culture with the hope of creating sequential markings for temporal studies. The goals are to improve culture techniques, better understand taxonomic variation in the effectiveness of the method, its utility in illuminating the relative timing of biomineralisation of various parts of bryozoan colonies, and quantifying colony rates. Results indicate that calcein marking works very well in both cheilostome and cyclostome bryozoans. Bryozoan growth rates measured in culture are one to two orders of magnitude less than those measured in the wild. This suggests that we need to improve our techniques for culturing large erect bryozoans. Finally, calcein staining maintains visible fluorescence at least 10 years after exposure.

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GROWTH and longevity are critical parameters in understanding population dynamics and ecological relationships, essential for management of both resources and conservation. Indeed, the capacity to measure growth under various conditions allows experimental investigation of the effects of stressors and changes, including future climates.

While growth and development of terrestrial organisms are often well constrained, determining growth and longevity in marine organisms presents challenges that limit utility, particularly in terms of revisiting the same organism over time for direct measurement of growth. Over the last few decades, a series of effective methods of extracting age and growth data from marine organisms have been developed. Many marine invertebrates present inherent physical growth markers, such as annual growth rings in bivalve molluscs (e.g. Kilada *et al*. 2009) or bands in corals (e.g. Lough & Cooper 2011), which can be used to age the organisms after death. The most common application of this technique is in the ageing of commercial fishes using growth bands in otoliths; Campana (2001) estimates that more than a million fish per year are aged using this technique. Artificial physical markers such as tags and pins (Barnes 1995; Cocito *et al*. 2006) may be used on sessile organisms in mark and recapture studies, but can affect the very growth they are there to measure.

Inherent chemical markers, such as stable isotopes reflecting seasonal or annual environmental changes (Smith & Key 2004; Key *et al*. 2013) or bomb radiocarbon incorporated in the 1950s–1960s (Kilada *et al*. 2009), can allow determination of age and growth patterns, but these methods are destructive. Chemical dyes provide a useful alternative to these methods because they can be readily manipulated, are neither lethal nor destructive, can be used in situ (more than once if required), and can track specific biomineralisation processes (e.g. Szabo *et al*. 2014). Tetracycline (Rowley & MacKinnon 1995), alizarin red (e.g. Schiller 1993), xylenol orange and calcein have all been used, with calcein emerging as most popular, probably due to its low toxicity (van der Geest *et al*. 2011).

Calcein or Fluorexon ($C_{30}H_{26}N_2O_{13}$) is a fluorescent dye that fulfills most of the requirements for a useful skeletal marker. It is commercially available in crystalline form at a low cost. It exerts low toxicity in foraminifera (Bernhard *et al*., 2004), invertebrates (Kaehler & McQuaid 1999), and fishes (Bumguardner & King 1996), especially with short periods of immersion (e.g. Kurtarkar *et al*. 2015), and appears to have little effect on biomineral composition of carbonate (Dissard *et al*. 2009). Calcein binds to calcium and is thus incorporated into $CaCO_3$, where it leaves a bright fluorescent mark that can be easily detected using epifluorescent microscopy under blue light (excitation wavelength of 495 nm) for at least a year after incubation in foraminifera (Bernhard *et al*. 2004), corals (Marschal *et al*. 2004), echinoids (Ebert *et al*. 2008) and molluscs (Linard *et al*. 2011). For these reasons, calcein is commonly used to mark skeletal materials, often as part of growth and ageing studies, but also to investigate how skeletal elements develop, grow and are repaired.

Calcein is either injected directly into the study organism or, more often, added to ambient sea water for a period of time of gradual uptake. In situ studies require the containment of the study specimens and their surrounding water in, for example, a plastic bag (Smith *et al*. 2001) or chamber (van der Geest *et al*. 2011) which is then injected with calcein for a period of time. Study in culture requires the addition of calcein to sea water in tanks. Usually invertebrates are immersed in concentrations of about 50 mg/L over periods of several hours (Smith *et al*. 2001; Lartaud *et al*. 2013; Haag *et al*. 2013). In general, the higher the concentration,

the shorter the time of immersion needed for successful marking, and the brighter the staining. Dissard *et al*. (2009) immersed foraminifera in 5 mg/L for 30 days, whereas Mahé *et al*. (2010) were able to shorten immersion time to 30 min by using a concentration of 150 mg/L. Shorter immersion times allow for a thinner and more precise chrono-mark, whereas longer immersion times increase the mark rate and the chance that all parts of the organism that are actively growing are marked. Both lowered growth rates and mortality have been reported in sea urchins for longer calcein immersion times at high concentrations (see Haag *et al*. 2013).

Like corals, bryozoans are colonial animals, and thus present additional challenges for age determination. In these colonies made of minute modules called zooids, it is not individual zooid growth that is of ecological or sedimentological relevance, but that of the colony. In this case, calcein staining will illuminate only those parts of the colony that are calcifying during the immersion period. Unmarked zooids may be dead, dormant, or temporarily non-functional. Bryozoan colonial morphology, too, presents challenges. Where linear or 'spot' colonies clearly have a defined growing edge (and most growth studies are limited to these simpler forms), more complex colonies can be alive and/or growing in a range of places, making interpretation of incremental growth difficult.

Large, erect-rigid, frame-building bryozoans, which are ecologically and sedimentologically important and potentially endangered by trawling (e.g. Wood *et al*. 2012), usually occur in mid-shelf depths (50–90 m). Many are difficult to reach by diving; it is nearly impossible to photograph the same colony twice, and culturing them in the lab has proved difficult. It is easy to understand why most bryozoan culture/growth experiments have concentrated on small encrusting nearshore taxa such as *Celleporina hyalina* (Hunter & Hughes 1993), *Electra pilosa* (Saderne & Wahl 2013), and *Membranipora membranacea* (Saunders & Metaxas 2009). Nevertheless, we wanted to investigate the potential for culturing and measuring growth in large frame-building bryozoan colonies, because these more robust colonies are often long-lived and may create a potentially useful temporal record of environmental change. We thus developed a method of culturing large shelf bryozoan colonies in the lab for periods on the order of months, and then of marking them with calcein to determine linear extension and allow calculation of equivalent annual growth rates.

METHODS

Sample Collection and Preparation

Live, erect-rigid frame-building bryozoans were collected from the mid-shelf bryozoan 'thickets' (Batson & Probert 2000) on the Otago shelf, using a 1.8 m beam trawl deployed from the *RV Munida* on 18 August 2005. Colonies were chosen from the trawled material on the basis of bright colour and low epifaunal cover, both of which indicate the vitality of the colony. Colonies were placed in round, black PVC tanks with lids and flow-through seawater for transport to the University of Otago's Portobello Marine Laboratory on Otago Harbour. Round tanks reduce the effect of wave slop on samples. Samples were collected from three locations: 45°46.884'S latitude, 170°54.220'E longitude at 75 m water depth; 45°46.468'S, 170°55.186'E at 84 m water depth; and 45°46.536'S, 170°55.604'E at 90 m water depth.

Species collected included the most common shelf-thicket species: *Cellaria immersa, Adeonellopsis* sp.*, Cinctipora elegans, Hornera foliacea, H. robusta, Diaperoecia* spp., and *Celleporina grandis.* There is no way of being certain that samples are genetically different because of fragmentation and the clonal nature of bryozoans (Hageman 2016). Thus, each fragment collected was treated as if it were a separate colony.

In the laboratory, colonies were separated whilst being held in seawater with handling kept to a minimum (i.e. using forceps). Sections of colonies that were clearly dead were removed, and large epifauna were also removed where possible. Live sections of colonies were attached to weighted plastic mesh using double ended clothes pegs which held colonies upright, perpendicular to the tank floor.

Species and replicates from within colonies were randomised so that no species occupied a particular position in the replicate tanks. In each of five identical 20 L tanks, there were two rows of bryozoans; each row contained one colony of each of seven bryozoan species, resulting in ten colonies of each species spread through five tanks which were independent, except for water input.

Feeding occurred once per day, when 1–2 L of each of three phytoplankton species were added to each tank. Water flow and pumps were checked twice to three times daily. Colonies were checked weekly using a long-armed microscope over the aquaria, and notes made of which colonies were observed to be alive. Water temperature was logged every half hour using a small data logger.

Culturing of Bryozoa

Prior to sample collection, five insulated, gravity-fed 20 L tanks were each fitted with a 600 L/h (30mA) submersible pump connected to a submersed spray bar which recirculated water in a horizontal current, thus holding food particles in suspension. Sand-filtered seawater was held in a 200 L insulated header tank maintained by a ballcock, so that temperature could be monitored using an aquarium thermometer and heated automatically as necessary. Water in the header tank was heated using two 300 W aquarium heaters governed by a thermostat and circulated by a submerged pump.

Water temperature of 10ºC is the norm at mid-shelf depth on the Otago shelf in winter (Jillett 1969; Sutton 2003), and we attempted to cool the warmer Otago Harbour water to close to this value. After an initial adjustment period, water temperature in August (late austral winter) was maintained by altering water flow rates. By mid-September (austral spring), water temperatures in the tanks reached 12ºC. On 16 September, a refrigeration unit with titanium heat exchanger was fitted, enabling water cooling to 11ºC ± 0.1ºC. Because of limitations in cooling capacity, the aquarium flow-through rates were reduced, which also kept food in the tanks for a longer period after feeding occurred. By the end of the experiment in November, the cooling system was running near capacity. To counteract daily temperature variations, tanks were insulated with 20 mm polystyrene.

Bryozoans were fed using a variety of phytoplankton. We do not know what food bryozoans on the Otago shelf eat. Previous studies (e.g. Jebram 1977; Hunter & Hughes 1993; Amui-Vedel *et al*. 2007) have fed bryozoans a range of phytoplankton species, covering a range of sizes. Dissolved organic material and bacteria were still in the water when it reached the tanks, and they could be a food

source for some species (Johnson & Wendt 2007). In addition, phytoplankton were cultured in 50 L bag cultures using standard Guilliards Enrichment F2 media (Guillard & Ryther 1962) at 20ºC. Water for culture was filtered at 10, 5, 1 and 0.35 µm then UV sterilised. Bag cultures take a minimum of two weeks to reach a good cell density, depending on the size of the initial inoculation. Two species that have been successfully fed to bryozoans in our lab in the past, *Isochrysis galbana* (3–5 µm) and *Dunaliella tertiolecta* (10–12 µm), were used along with a third, mid-sized species, *Tetraselmis chui* (5–10 µm).

Food source can affect growth, vitality, and the attainment of maturity. Jebram (1977) noted that each time food source species were changed, active polypides were reduced and replaced by new ones, the old ones being unable and/or disinterested in feeding on the new food source. Thus, all three food species were made available throughout the culture trials. Duration of food availability ranged from three to six hours per day, depending on circulation rates.

Marking with Calcein and Measurement of Growth

On 7–8 September 2005, bryozoan colonies were immersed for a 24 h period in buffered calcein mixed with sea water at a concentration of 30 mg/L. For this, seawater was drained off and 10 L (enough to cover the colonies) of calcein solution was added. Aerators were used and the tanks held in a constant temperature room set at 10ºC. Food was added in case it was necessary for calcein uptake. Unfortunately, the constant temperature room lost power and ambient room temp increased to 13ºC, which raised the water temperature for a brief period (Fig. 1). After 24 hours, the dyed water was removed and the tanks returned to the tank room and normal flow conditions, temperatures and feeding regime.

Specimens were collected and killed on 11 November 2005, 65 days after marking. They were lightly bleached and dried, then examined and photographed under fluorescent light in March 2007 using an Olympus Provis AX70 microscope with excitation wavelength set at 495 nm. Photos were taken at 4× and 10×, often with blue filters to enhance fluorescence. Some specimens were examined and photographed again in July 2015.

The amount of growth was measured as the linear distance from the most distal fluorescence to the growing tip at the time of harvesting. Measurements were made on the digital photographs to the nearest 0.01 mm using Image-Pro Express v. 5.0.1.26 software by MediaCybernetics with a mean measurement error of 0.02 mm based on repeatability experiments. Growth rate was calculated as the amount of growth divided by the time between calcein marking and harvesting. As the colonies may have died before harvesting, these calculated growth rates are minimum growth rates.

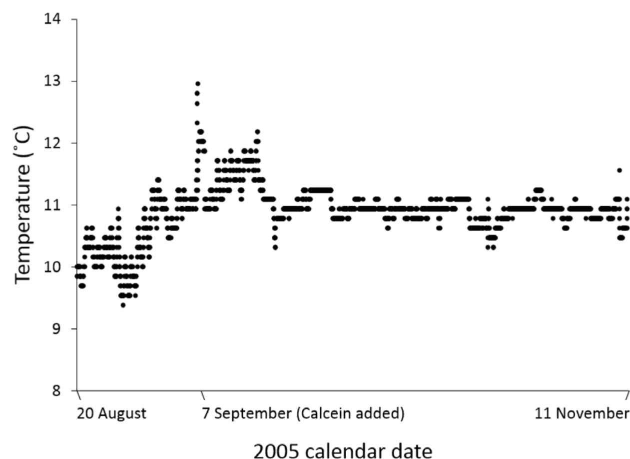

Figure 1. Plot of water temperature versus time for the duration of the experiment, showing when calcein was added.

RESULTS

Bryozoans entered culture on 20 August 2005 and were marked on 7 September 2005. After 84 days, most bryozoans were still alive, with protruding lophophores. By 11 November 2005 few-to-no bryozoans were still alive so the experiment was terminated and the colonies harvested. Thus there were at most 65 days of growth after marking.

Throughout the experiment tank temperatures averaged 10.9ºC (Fig. 1; range: 9.4–13.0ºC; standard deviation: 0.4ºC). Before the calcein was added while the system was equilibrating, the mean temperature was 10.5ºC with a relative standard deviation of 5.0%. After the system equilibrated and the calcein was added, the mean temperature was 11.0ºC, and the relative standard deviation dropped by half to 2.6%, showing that water temperature had become more consistent.

Bryozoan colonial skeletons showed bright fluorescent marks from calcein. They were easy to see in specimens that had been marked two, ten, and 17 years previously. Marking with calcein was successful in both cheilostomes and cyclostomes. The marked colonies included our intentionally-collected seven large, erect and rigid frame-building bryozoan species (*Cellaria immersa, Adeonellopsis* sp.*, Cinctipora elegans, Hornera foliacea, H. robusta, Diaperoecia* spp., and *Celleporina grandis*), also associated (accidentally included) small erect-flexible or delicate-rigid colonies (*Cellaria, Annectocyma, Diaperoecia, Mecynoecia, Odontionella*) and encrusting colonies (e.g. *Celleporina*, *Filaguria*) using larger bryozoans as a substratum. We calculated growth rate only for those species where we had more than two colonies and several measurements (Table 1).

Three of the cyclostome species showed essentially no post-marking growth (Table 1, Fig. 2), suggesting those species did not grow after the introduction of calcein. Those species that did grow, did so at a surprisingly consistent rate: they added about 0.2 mm in the experimental period, equivalent to about 1 mm/y. On average the six species grew 0.60 mm/y with the two cheilostome species growing four times faster (1.19 mm/y) than the four cyclostome species (0.30 mm/y) (Table 1, Figs 2 & 3).

DISCUSSION

Calcein marking works in bryozoans

Many bryozoans have now been successfully marked with calcein (Fig. 3). Rowley & MacKinnon (1995) first demonstrated that calcein could be incorporated into bryozoan skeletons. Subsequently, Smith *et al*. (2001) elucidated growth rate in the large erect-rigid cheilostome bryozoan *Adeonellopsis* sp. using calcein marking in situ (in diveable water depths of 12–15 m). Saderne and Wahl (2013) used calcein in aquaria to mark thinly encrusting bryozoans and spirorbid worms encrusting kelp fronds.

Table 1. Measured growth in bryozoans from Otago shelf in up to 65 days in culture. The equivalent mean annual growth rate (assuming the bryozoan was alive when the experiment terminated) is calculated.

Species	Order	Number of colonies measured	Measured Growth (mm)			Calculated mean annual growth rate (mm/y)
			Range	Mean	Std Dev	
Adeonellopsis sp. (Fig. 3C)	Cheilostomata	9	0 – 0.41	0.18	0.14	1.00
Figularia sp. (Fig. 3A)	Cheilostomata	4	0 – 0.48	0.25	0.17	1.38
Cinctipora elegans (Fig. 3B)	Cyclostomata	3	0 – 0.36	0.21	0.15	1.15
Diaperoecia spp.	Cyclostomata	5	0	0	0	0
Hornera spp.	Cyclostomata	8	0 – 0.01	0	0	0.05
Tubuliporid (Fig. 3D)	Cyclostomata	5	0	0	0	0

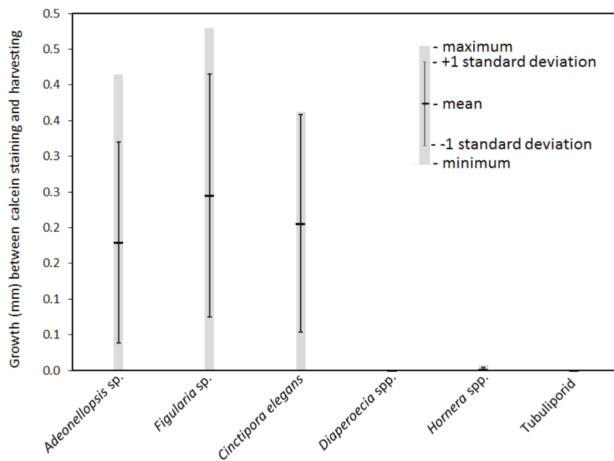

Figure 2. Growth in mm during the 65-day period in culture for six bryozoan species with more than one marked colony.

The present study shows that large, erect, frame-building bryozoans, small bushy colonies and tiny spot encrusters, in both cheilostome and cyclostome taxa, can be successfully marked with calcein, with hour-to-day immersion times and using concentrations that are non-lethal.

At any given time, parts of a bryozoan colony are actively growing, other parts are dead, and some may be temporarily inactive. Calcein marks only the skeleton that was actively precipitated on the day (or perhaps for a day or two later as the calcein worked its way out of the zooids' systems). We found colonies where only peristomes were growing at that time (Fig. 3D). In other colonies, some zooids were making avicularia, while others were precipitating wall material (Fig. 3E). Lack of marking does not necessarily mean the zooid is dead or even dormant, just that it did not make carbonate during the time calcein was in the system. For growth studies, where a good bright line is wanted across the width of the colony, longer immersion times might be useful (e.g., Saderne & Wahl 2013 used 5 days).

We do not know how long calcein remains in the system of a zooid. While no bright fluorescent carbonate can be precipitated prior to treatment with calcein, we cannot be sure when precipitation of marked carbonate ceases. There is no particular reason to think that marked skeleton reflects only the 24 hours of immersion in calcein — there may well be some lag time. Figure 3A shows several zooecia with brightly fluorescing carbonate — either that particular *Filaguria* colony was actively precipitating skeleton on that day, or perhaps it reflects several days of calcification and/or movement of calcium ions through the colony.

Calcein has been characterised as 'long-lasting' by various authors; it is not always clear whether these authors mean that the chemical has a long shelf life (as noted by Rowley & MacKinnon 1995) or that the marks remain visible in carbonate for a long time (as described by Mahé *et al*. 2010). There is some disagreement on this last point: whereas calcein marks in invertebrate skeletal calcite lasted at least one year in several studies (Marschal *et al*. 2004; Ebert *et al*. 2008; Linard *et al*. 2011), one study found that marks in scallop calcite faded after 10 months, possibly owing to the low concentration used (Lucas *et al*. 2008). Fish reared in natural light have been shown to lose

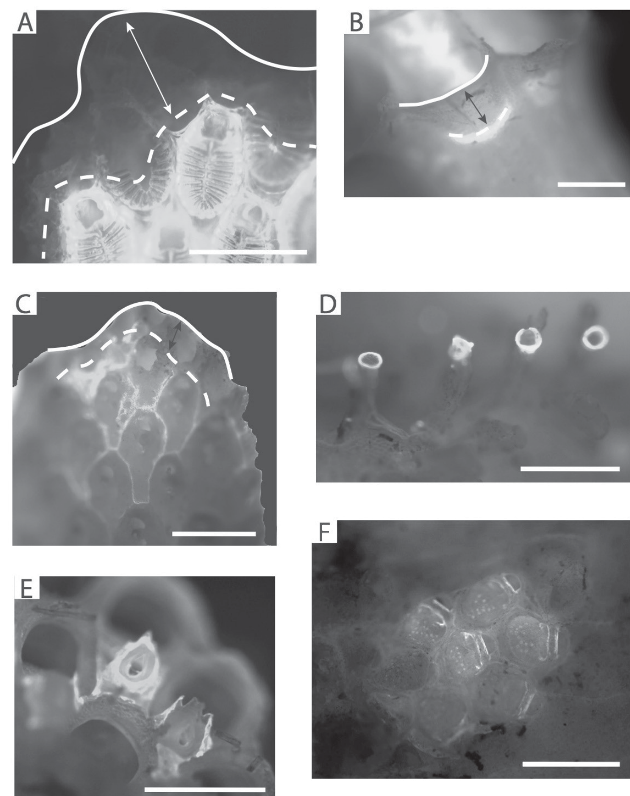

Figure 3. Fluorescent photographs showing calcein marks and growth in some of the colonies in this study. **A**, *Filaguria* sp. growing margin shows maximum growth of 0.48 mm (arrow). **B**, In *Cinctipora elegans* the skeketal shield shows growth of 0.36 mm (arrow). **C**, *Adeonellopsis* sp. showing zooecial growth of 0.20 mm near growing tip. **D**, Idmidroneiform/tubuliporine cyclostome peristome rims marked. **E**, *Odontionella cyclops* shows marking of avicularia. **F**, Early colony development in *Micropora* sp. Scale bars all 0.5 mm. Dashed white lines in A–C represent end of skeletal fluorescence presumably 7 September. Solid white lines represent end of growth presumably 11 November when colonies were harvested.

marking in only a few months (e.g. Elle *et al*. 2010), though those in shaded or indoor tanks retained marks for up to three years (e.g. Crook *et al*. 2009). How relevant this is to invertebrates is hard to say, but it is not unreasonable to suggest that storage of calcein-marked material is best in darkness (e.g. Honeyfield *et al*. 2008).

We examined marked bryozoan material under the microscope after two years of storage, and marks were both bright and common. In 2015, there was no apparent change in the markings of these bryozoans, nor of some which were marked in 1998 (see Smith *et al*. 2001) (Fig. 4). We could see no difference between calcein marking in aragonite vs calcite. It appears that in bryozoan skeletons stored in dry and dark conditions, calcein marking can last at least 17 years.

The complexity of calcein staining in bryozoans has a surprising benefit: it can illuminate the relative timing of biomineralisation of various parts of bryozoan colonies. For example, in *Filaguria* sp., the frontal wall of the previous zooecium was completed before the basal and vertical walls of the next distal zooecium were finished (Fig. 3A). In *Odontionella cyclops*, we can see the development of adventitious avicularia on the frontal wall of the underlying zooecium (Fig. 3E), as described by Smith & Girvan (2010). And in Figure 3F, an encrusting *Micropora* sp. colony shows that six zooids are adding skeletal material to the frontal wall and apertural bar at the same time while the underlying cheilostome colony of a different species was not. Finally, an ancestrula was not adding skeletal material during immersion, but the first two generations of zooids were (Fig. 3F).

In a taxon where biocalcification mechanisms are largely unknown, the simple use of calcein dye may allow useful elucidation of inter-specific differences in skeletogenesis. For example, photos of *Hornera robusta* show all zooids adding material simultaneously to peristomes, whereas *Diaperoecia* spp. shows calcification only at the tip of the branch. In general, cyclostomes were observed growing at the tips of structures (e.g. Fig. 3D); whereas cheilostomes were more extensive and complex (e.g. Fig. 3A). We anticipate that in a similar way, skeletal marking could be used to show rejuvenation, repair and fresh growth after breakage (e.g. as Szabo *et al*. 2014 did in serpulid worms). In an ecological setting, skeletal marking of an encrusting community could give insights into the timing of overgrowth, fouling and competition (Fig. 3F).

When colonies are well-marked, post-mark material must have been precipitated after the addition of calcein (Figs 3A–C). Measuring of post-mark material offers the potential to consider calcification and growth on a variety of time scales. While it would be possible to examine calcification over days or weeks, in our case the experiment lasted more than two months (65 days), which allowed for measurable growth in at least some species.

Measurement of growth in bryozoans

Colonial growth rates that we measured were small, and many specimens that were marked showed no additional skeletal precipitation after marking. We have no evidence as to whether or not calcein or something else killed these bryozoans or if they were just not able to produce more skeletal material under laboratory conditions. In almost all species that did not grow after marking, it was true for every specimen. Those species that did grow were less consistent:

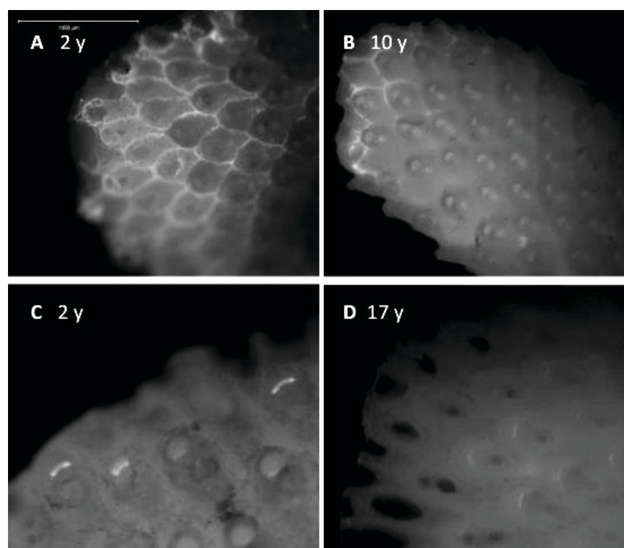

Figure 4. Calcein mark intensity and clarity in marine bryozoan *Adeonellopsis* sp. does not fade over time. **A**, marked in 2005, photographed in 2007. **B**, marked in 2005, photographed in 2015. **C**, marked in 1998, photographed in 2000. **D**, marked in 1998, photographed in 2015.

some specimens grew almost 0.5 mm in 65 days, whereas others added no carbonate. The mean growth and standard deviation of all three species that did grow are, however, surprisingly consistent: each added on average 0.2 mm in the experimental period, equivalent to 1 mm/y.

Our culture methods achieved little mortality (for some months) in large erect-rigid frame-building bryozoan colonies, but also little or no growth. We can speculate that factors such as temperature variation, especially the sudden 2ºC warming when calcein was added, and diet (composition or concentration) may have influenced metabolic rate and hence calcification.

A recent review of growth rates in bryozoans (Smith 2014) allows our growth data to be placed in context. Including our work here, we now have some measured linear extension rates (mm/y) for 40 species of bryozoans (Table 2; Smith 2014, supplementary data table 1). Growth rates in bryozoans have been determined mostly by two methods: either artificial settling panels have been left in the sea for a period, after which growth has been measured (16 species), or skeletal material produced between naturally occurring annual growth checks has been measured (16 species). Growth rate has also been determined by the use of stable oxygen and carbon isotope proxies (four species), and by long-term observation in aquarium culture (1 species). This study adds the new method of mark-and-recapture in culture (four species), and there are two studies that used mark-and-recapture in situ (Smith *et al*. 2001 used calcein; Cocito & Ferdeghini 1998 used alizarin red). In general, most of these labour-intensive studies have few or no replicates (either within experiments or across seasons), which limits their general applicability.

Saunders & Metaxas (2009) measured growth in *Membranipora membranacea* using a variety of techniques in the field and in the lab. They found, as might be expected, that growth measured in tanks/aquaria was much less than in the field. Figure 5 shows that, on average, bryozoan

Method of measuring growth rate (linear extension in mm)	Number of spp. measured this way	Mean growth rate (mm/y)			
		Min.	Max.	Mean	Std Dev.
Naturally occurring growth checks	16	2.9	12.5	5.7	2.4
Isotope proxy for seasons	4	8.0	40.0	20.8	11.9
Mark and recapture in situ	2	6.9	36.0	21.5	14.6
Mark and recapture in culture (this study)	4	0.1	1.4	0.9	0.5
Observation in culture	1			0.06	
Settlement panels	16	1.3	77.1	7.8	17.9
Total	**43**	**0.1**	**77.1**	**8.3**	**13.2**

Table 2. Bryozoan growth rates as determined by a variety of methods.

growth rates measured in culture are significantly less than those measured in the wild. In fact, it could be argued that there is no point in measuring growth rates in culture until we find better ways to keep, feed and rear bryozoans in the lab.

Growth checks are generally found only in bryozoans living in places where seasonality is severe. Colonies slow or even stop growing, which leaves a visible mark. It is no coincidence that the majority of bryozoan species for which growth checks have been used to elucidate growth are from polar regions; 14 of the 16 species were collected from Antarctica (e.g. Winston 1983; Barnes 1995). The strong cold-climate bias may account for the low average growth rate relative to isotope and in situ measurements. Limitation to cold-water taxa means that numerous temperate bryozoans cannot be studied with this technique.

Growth-check ageing is, however, the technique with most reliable applicability to the fossil record. Smith's (2014) data included Tasmanian fossils that seemed to have annual growth checks. It is, of course, nearly impossible to validate those marks. Similarly, few (no?) studies have so far validated the annual nature of growth checks in Antarctic bryozoans.

Settlement panels measure growth immediately after settlement, and these studies usually only document the growth of encrusting 'spot' or 'sheet' colonies. At least some species of this kind of opportunist bryozoan grow faster when they are larger (Saunders & Metaxas 2009), so the moderate growth rate found on panels might not reflect growth of more mature colonies. In any case this technique is inappropriate for large long-lived erect-rigid bryozoans living below diveable depths.

It appears that the most commonly used and logistically straightforward ways of determining growth rate in bryozoans could be systematically underestimating real growth rates and/or cannot be applicable to temperate erect species. Marking wild colonies in the field with calcein or alizarin red dye, then revisiting after a period of time to collect and measure growth since marking offers a good alternative for species in water depths less than 30 m. It allows for measurement of hard-to-culture erect species, for measurement of mature colonies, and for growth to occur in a natural environment, avoiding effects of temperature and food composition or concentration in the lab. Marking with tags or pins is another in situ marking method, but it can result in damage to colonies, especially in environments with strong currents or waves.

So how can we study large erect bryozoans that live too deep for divers to reach them? We must improve our culture techniques, or develop a submersible-based dyeing technique, or perhaps consider a modified mark-and-recapture technique to better reflect real growth rates, in order to determine growth and calcification rates for these important but hard-to-study bryozoans.

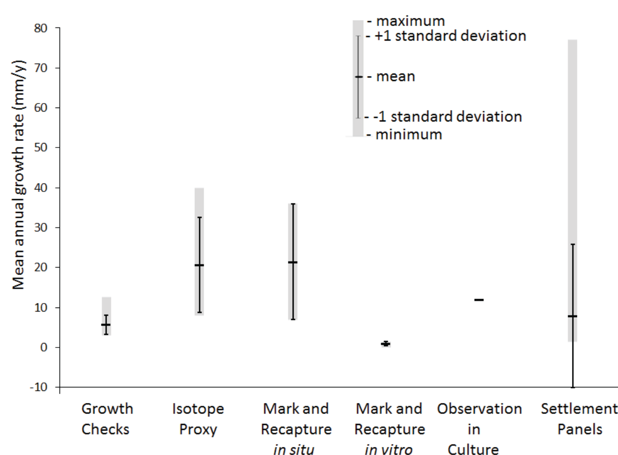

Figure 5. Methods used for determining growth rates in bryozoans produce significantly different results.

SUMMARY AND CONCLUSIONS

Calcein marks large erect bryozoans well, and can be used to determine minimum growth rate. Calcein is an excellent chemical marker for skeletal growth and biomineralisation sequences in bryozoans because it is easily obtained and applied, effective and efficient, and the marks last for at least 17 years after marking. We note that there is no standard protocol for archiving and storage of marked material, but suggest, given the literature, that material is stored dry and in the dark. Chemical marking and recapture, however, works better in the wild than in tanks, unless culture techniques are good enough to support normal, vigorous growth. While our culture methods resulted in some growth in some species of large erect-rigid bryozoans for the first time, there is no reason to think that our measured growth rates have any particular relevance to wild populations. Examination of growth-rate measurements in bryozoans shows that different methods give significantly different results, and that all available methods have limitations.

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