Responses to reviews. Author responses in Times New Roman; Reviewer comments in Arial.

Rev 1 (Lennart de Nooijer)

Received and published: 25 August 2015

Dear Editor,

The novel method presented by Bernhard and co-authors as described in their Technical Note may well prove to be a new tool to determine the in-sediment calcification depth of (amongst others) benthic foraminifera. As the authors explain, this is necessary to refine carbonate-based proxies, especially those for redox-conditions, since availability of elements (and hence their incorporation) varies with sediment depth. As noted by the other reviewer too, the relation between calcein concentration (higher closer to the dispenser's port) and intensity of foraminiferal/ bivalve fluorescence was not determined and therefore prevents proving whether bivalves and foraminifera not showing any fluorescence were located too far away from the pump's opening. Since the vertical and lateral extent of the calcein diffusion into the sediment was not directly measured, I wonder how this technique can be applied to determine the (average) living depth of foraminiferal species/ specimens (as suggested in section 3.3). To do this, the diffusion gradient of the released calcein should be sharp enough to label forams (e.g.) in the top cm, but not those living below the top cm, or vice versa. This makes that the calcein-diffusion gradient should be under (some) control by those applying this technique in the future. I think the authors should include in their discussion the necessity to control the diffusion gradient on a scale that is relevant in determining in-sediment living depths.

Sincerely, Lennart de Nooijer

Minor comments: The reference to my 2014-paper (page 9446, line 10), is probably meant to be that in Earth-Sci Rev, and not that in MarMic.

Page 9450, lines 7-10: were the osmotic pumps inspected and checked whether they were (almost) empty? The time to empty for these pumps may be different in the setup employed here than they were originally designed for.

It might be beyond the scope of this paper, but why is not all newly formed shell from the quahogs stained equally brightly? Is the calcein not incorporated in the thin bands that are not fluorescent (fig 2B and D) or is there something present in these layers that 'shades' the fluorescence?

We thank Dr. de Nooijer for his comments and generally positive assessment. We would be pleased to include a short addition to our manuscript discussing how researchers could go about determining their particular diffusion gradients (i.e., "include in the discussion the necessity to control the diffusion gradient on a scale that is relevant…"). If required, we would add a paragraph something like the following, probably between lines 8 and 9 on page 9455 (or we would rewrite and expand the paragraph currently on page 9455 lines 4-8):

The radius of calcein dispersion into sediments will depend on all sediment properties as well as the presence / absence and activities of bioturbators. Diffusion coefficients can be measured directly in sediments (e.g., Jorgensen 1979 *Geomicrobiol J*) or they can be estimated from the sediment's formation resistivity factor, which can be estimated from sediment porosity and other sedimentary characteristics (e.g., Ulmann and Aller 1981 *L&O*).

Responses to Minor Comments:

We corrected the improperly cited 2014 paper (it was indeed intended to be the *Earth-Science Review*).

Regarding page 9450 lines 7-10, we are not entirely clear what Dr. de Nooijer is asking because our point was to remove the osmopump so that the clams would grow additional calcite that was not fluorescent. However, in an attempt to explain, we note that visual inspection of an osmopump does not allow confident assessment of contents. To check if an osmopump continues to dispense calcein, it can be placed overnight, for example, in a clean beaker of seawater. The next day, an aliquot of the water can be analyzed in a spectrophotometer using the appropriate excitation wavelengths. We did such tests early in our investigations to establish accuracy of our calculated estimated dispensation times; results indicated our calculations were adequate (i.e., at our temperature and salinity, the pumps lasted as we expected). We did not include this information in the manuscript, but we could do so if necessary. If a short passage were added, it would likely be inserted on page 9455 between the two sentences on line 10 or, potentially, in the Methods.

Not being bivalve biomineralization experts, we do not know why there are a few thin lines of the shell that do not fluoresce as brightly as adjacent materials in Figs 2B, D. These lines appear to correspond to the thicker ridges. We might surmise that there are more organics in those thick ridges of the shells. The organics may act to mask or "dilute" the fluorescence. We feel this issue is beyond the scope of this short Technical Note.

Rev 2 (anonymous)

Received and published: 22 July 2015

General comments A paper by Bernhard and others introduces a new method to study microhabitats and biomineralization in calcareous microorganisms in situ, using a combination of small osmotic pumps and the fluorescent marker calcein. Osmotic pumps are commercial products and calcein has been widely used for biomineralization of calcareous organisms, but a combination of these two is novel and worth reporting. This method would be particularly useful for deep-sea biology.

However, although I understand this paper is a "technical note", I think supporting data are necessary to assess if this method is really useful or not. I have many questions remained after reading this paper; How much is the actual concentration of calcein? How fast is the diffusion rate in the environment? How far is calcein solution really reached horizontally and vertically?

How long does calcein really disperse? I understand this would be dependent upon substrate types and sediment properties (grain size, porosity, etc.). Authors should present verification tests under several different settings (e.g, mud and fine sand typical for deep sea and shelf setting, respectively). Without such supporting data, this method is so hypothetically based that we cannot assess whether unstained organisms either indicate no calcification during experiments or is due to a low concentration and narrow dispersion range of calcein. Therefore, based on the present version of the paper, I doubt this method is really resolving in situ centimeter scale location and timing of biomineralization, as shown in the title of this paper. I suggest authors take into consideration these comments for the final version of the manuscript.

Specific comments

1. Introduction: this section mainly describes significance in studying biomineralization and microhabitats of calcareous microorganisms as proxy for paleoceanography. However, most of the contents are not directly related to the method shown in this paper, and should be summarized. I would rather like to know more about previous studies of in situ experiments of biomineraliazation and microhabitats if any.

3.3. Potential applications: I think the osmotic pumps are useful together with other fluorescent and isotope markers. Authors should consider potential applications of osmotic pumps with other markers.

Technical corrections: This paper is well written and structured. I could not find any typing and grammatical errors in the paper.

We thank the anonymous reviewer for their review and perspective.

Regarding the reviewer's "many questions", we provide the following responses. The actual concentration of calcein loaded in our osmopumps was 100 mg per liter. That concentration can be varied, although considerably higher concentrations have not been tested. The diffusion rate in the environment depends on the temperature, salinity (osmolality), and sediment properties (water content, connectivity, grain size, formation resistivity). The rate of diffusion in the environment depends on the temperature, salinity, etc. The horizontal and vertical distance that the calcein solution dispenses depends on the sediment porosity, grain size, sorting, formation resistivity factor, and connectivity as well as current speeds and the benthic community and its bioturbation rate(s). The length of time that calcein is dispensed depends mostly on the temperature and salinity (osmolality), as well as the configuration (model and size) of the osmopump.

We feel that taking the time to do more calcification experiments with different sediments from different settings is beyond the scope of this Technical Note. As the reviewer admits, the radius of dispersion and timing of dispensation will depend on specific physicochemical conditions (i.e., specific sediment grain sizes, rounding, sorting, temperature, porosity, salinity, etc). We feel we have demonstrated that the method works in two settings: sands for clams and muds for benthic foraminifera. If the paper were hypothetical, then we would not have shown any images. We agree that it will be necessary for people who wish to employ this method to "do their homework", determining the radius of dispersion and the

length of dispensation, depending on exactly what their questions are. For some, however, it may be relevant to merely show that some specimens calcified in a particular setting or how fast specimens grew. It may not be necessary for all researchers to require quantification of the entire population.

A recently finished thesis project at Lund University (MSc student Susanne Landgren) confirms the practical use of the osmopumps using foraminiferal-laden fjord sediments (Landgren S., 2015, Dissertations in Geology at Lund University, report no 431, https://lup.lub.lu.se/student-papers/search/publication/5425178). Further, we presented initial results at a recent meeting (Landgren S., Filipsson, H.L., Charrieau, L., Bernhard., J.M: In situ biomineralisation of benthic foraminifera during hypoxic and normoxic conditions. The Micropalaeontological Society, Foraminifera and Calcareous Nannofossil Groups, Spring Meeting 2015, June 14- 18, 2015, Plymouth University, Plymouth, UK). A manuscript describing those results is in preparation and will be submitted sometime this autumn, but the present Technical Note focuses as an introduction of the method because Bernhard developed the method in her lab. The first true field application was by Filipsson's lab, and thus will be led by her student Landgren. Further, Susanne developed a matlab script to calculate the dispensation time; this will be presented in her publication. Further discussion of diffusion gradients is presented in our response to Reviewer 1, Lennart de Nooijer.

Regarding the title, which begins with the phrase "Towards resolving", it clearly states that this is a method that will help establish location of calcification. We do not claim anywhere that all details are resolved.

Regarding specific comment 1, we feel the Introduction provides context so we do not feel compelled to shorten that aspect of the Introduction. Biomineralization in foraminifera is the focus of considerable research at this time (e.g., de Nooijer et al., 2014 *Earth Science Review*; Bentov et al. 2009 *PNAS*: Nehrke et al., 2013 this journal), however, as far as we are aware none has done *in situ* biomineralization experiments, and this one reason is why this method might prove to be particularly useful to some investigators.

Regarding specific point 3.3 (potential applications), we appreciate the suggestion to include discussion about potential of additional osmopump-dispensed fluorescent or isotopic markers. We prefer to refrain from adding discourse on this topic because we have not tested any additional compounds. We assume that people can develop their own applications, but of course warn that they will have to demonstrate proof of concept. While CellTracker Green could be dispensed from the osmopumps, in most cases, core collection proves adequate for such applications, given the relatively short (6-16 hour) incubations required of that probe. We are not confident that using isotopically labeled nitrate, for example, would work as we do not know if an adequately concentrated solution could be made.

Changes (see tracked changes in pdf document)

1) We fixed a few typographical errors throughout the manuscript (e.g., missing periods, capitalization changes, symbol changes).
2) We added 3 passages that had been discussed in the Responses to Reviewers.
3) We omitted a few superfluous words (e.g., in Abstract).
4) We added a short passage in the Acknowledgements thanking the reviewers.

Technical Note: Towards resolving *in situ*, centimeter-scale location and timing of biomineralization in calcareous meiobenthos— the cCalcein-oOsmotic pump method

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Abstract

Insights into oceanographic environmental conditions such as paleoproductivity, ~~sea-surface temperatures,~~ deep-water temperatures, salinity, ice volumes, ~~circulation patterns,~~ and nutrient cycling have all been obtained from geochemical analyses of biomineralized carbonate of marine organisms. However, we cannot fully understand geochemical proxy incorporation and the fidelity of such in species until we better understand fundamental aspects of their ecology such as where and when these (micro)organisms calcify. Here, we present an innovative method using osmotic pumps and the fluorescent marker calcein to help identify where and when calcareous meiofauna calcify *in situ*. Method development initially involved juvenile quahogs (*Mercenaria mercenaria*); subsequent method refinement involved a neritic benthic foraminiferal community. Future applications of this method will allow

determinations of *in situ* growth rate in calcareous organisms and provide insights about microhabitats where paleoceanographically relevant benthic foraminifera actually calcify.

1 Introduction

Biomineralized carbonate of marine organisms such as foraminifera, coccolithophores, and ostracods has provided an abundance of geochemical data critical to our understanding of modern-day oceanographic conditions and processes as well as critical to reconstructions of paleoceanographic conditions and processes. While geochemical proxies of planktic and benthic foraminiferal tests (shells) have yielded copious insights to past sea-surface temperatures, salinity, ice volumes, deep-water temperatures, oceanic circulation patterns, nutrient cycling and paleoproductivity (Katz et al., 2010;Allen and Hönisch, 2012), in the vast majority of cases, initial proxy calibration was developed from core-top sampling and field calibrations. Culturing studies have also contributed greatly to our understanding of the mechanisms controlling these geochemical processes during biomineralization. While we have gained much knowledge on these topics (reviewed by Katz et al., 2010), there remain some significant issues regarding fundamental and emerging proxies. In brief, a variety of factors complicate proxy interpretations; the most common ones in this context include “microhabitat preferences”, “vital effects”, and rapid changes in carbonate chemistry occurring in the uppermost sediment column. Microhabitats refer to the micron- or millimeter-scale distribution of foraminifera with respect to the sediment-water interface, some other physical structure (e.g., worm tube), or chemocline. Vital effects, which can include ontogenetic differences (Filipsson et al., 2010;McCorkle et al., 2008), are physiological processes that impact test geochemistry, although some researchers include environmental processes in the definition of "vital effects" (de Nooijer et al., 2014).

In particular, changes in environmental parameters occurring in the uppermost part of the sediment might affect proxy reconstruction and it is crucial to obtain an increased understanding of where in the sediment biomineralization occurs. For example, one of the most often used temperature proxies, foraminiferal Mg/Ca, relies on temperature, but is also influenced by carbonate ion concentration (Elderfield et al., 2006;Healey et al., 2008;Raitzsch et al., 2008;Rosenthal et al., 2006) and pH (e.g., Russell et al., 2004), which vary significantly with sediment depth. Stable isotopes of oxygen and carbon ($\delta^{18}O$ and $\delta^{13}C$) also are impacted by the carbonate ion effect (Spero et al., 1997;McCorkle et al., 2008;Lea et al., 1999;Bijma et

al., 1999). With increasing sediment depth, pore water becomes increasingly depleted in $\delta^{13}C$ due to a steep gradient in pore-water dissolved inorganic carbon (DIC) because of organic carbon remineralization. This same process is simultaneously lowering the carbonate ion concentration. Thus, for benthic foraminifera, determining where within (or on) the sediments they calcify is very important for determining the fidelity of their test chemistry and the resulting proxy relationships, as well for improving the precision of proxy reconstructions.

Although we know much about where many benthic foraminifera live in sediments (e.g., infaunal vs. epifaunal (Jorissen et al., 1995;Corliss, 1985)) on the centimeter scale, the true depth habitats and calcification microhabitats of benthic foraminifera that are used in paleoceanographic reconstructions are not known. Indeed, as discussed by McCorkle et al. (1990), abundance peaks of Rose Bengal stained foraminifera are typically several centimeters thick yet these authors showed species' $\delta^{13}C$ ranges of 1-2‰, which may suggest calcification in a narrower depth horizon. While it is possible that foraminiferal species calcify at the sediment-water interface or a particular sediment horizon, thereby incorporating only the DIC from bottom waters or the horizon's pore waters, subsequent migration into more oxygen-depleted zones characterized by extremely low $\delta^{13}C$ values hypothetically results in an apparent disequilibrium between ambient conditions and foraminiferal calcitic tests (McCorkle et al., 1990;Stott et al., 2002). Such activity would explain at least in part the disequilibrium observed in down core studies (e.g., McCorkle et al., 1990). In reality, while benthic foraminiferal calcification horizons are inferred from distribution studies (e.g., Stott et al., 2002), the actual depth of calcification or the related geochemistry is not known, especially for paleoceanographically relevant species, such as, for example, *Cibicides* spp., *Uvigerina* spp., and *Oridorsalis umbonatus*. Furthermore, distribution patterns may not be reliable given that the classically employed method to distinguish live from dead foraminifera, Rose Bengal stain, has been shown to undoubtedly also stain foraminiferal carcasses (i.e., dead foraminifera (Bernhard et al., 2010)). The requirements for monospecific (single species') analyses as well analyzing specimens within a well-defined size range to avoid biases caused by vital effects or microhabitat effects can minimize geochemical proxy uncertainty (Ravelo and Hillaire-Marcel, 2007;Katz et al., 2010), but at this time it is not established that all conspecifics calcify in the same microhabitats and/ or depth horizons or that vital and ontogenetic effects are consistent among an entire population of a given species.

To resolve some of these unknowns, we developed a method that will assist in documenting the timing and location of calcification in sediments for calcareous benthic meiofauna. The method employs commercially available osmotic pumps to deliver calcein, which is a fluorescent compound that binds to calcium in biomineralized structures as it is precipitated (e.g., Medeiros-Bergen and Ebert, 1995;Monaghan, 1993;Moran, 2000;Collin and Voltzow, 1998;Hernaman et al., 2000). Using full immersion incubations, calcein has been used to mark bivalves (e.g., Kaehler and McQuaid, 1999;Moran and Marko, 2005;van der Geest et al., 2011) and also has been used in laboratory studies regarding foraminiferal calcification (Bernhard et al., 2004;Denoyelle et al., 2012;Dissard et al., 2009;Filipsson et al., 2010;Kurtarkar et al., 2015;Nardelli et al., 2014). In this contribution, we describe a novel point-source calcein dispensation method and show proof-of-concept for quahog (hard clam) bivalves and benthic foraminifera.

2 Materials and Methods

2.1 Osmotic pumps and calcein

The means used to dispense the calcein are ALZET®™ osmotic pumps (Fig. 1A; DURECT Corporation, Cupertino, CA, USA). Osmotic pumps are devices designed to deliver pharmaceuticals to animals; as originally intended, they are installed under the skin of an animal. Different osmotic pump models allow different delivery rates and durations. We used model 2ML2 or 2ML4, each with a reservoir of 2 ml. The 2ML2 was designed to dispense (in mammals) at a rate of 5 µl h^{-1} for 14 days; the 2ML4 at a rate of 2.5 µl h^{-1} for 28 days. Dispensation rate depends on the model, as noted, but also on osmolality and temperature of the environment. A calculator to determine flow rate under specific relevant conditions conveniently exists on the ALZET web page: (http://www.alzet.com/products/guide_to_use/pump_selection.html). In our quahog incubations, we expected each 2ML2 to dispense for about 2 months and, in the foraminifera incubations, for about 4 months. Because incubations were performed in seawater, to avoid corrosion, we replaced the stainless steel tubing that is standard in the ALZET osmotic pumps with PEEK (PolyEtherEtherKetone) tubing.

The osmotic pumps were filled with a concentrated solution of calcein (100 mg L^{-1}; Fig. 1A). A thin wooden rod was secured to each osmotic pump via elastic bands to allow emplacement of the pump into the sediments as desired. The dispensation hole, heretofore referred to as the

"port", could be placed facing downward or upward, depending on the research objective. To avoid clogging of the port, for downward pointing osmotic pumps, a thin plastic film (conventional kitchen plastic wrap) was loosely wrapped over the dispensation end during pump emplacement into sediments. After the pump was located within sediments as desired, the thin film was removed by gently pulling one edge vertically so as to minimize disturbance to the sediments. Upward facing osmotic pumps did not require such protection during emplacement.

Visual inspection of an osmopump does not allow confident assessment of contents. To check if an osmopump continues to dispense calcein, it can be placed overnight, for example, in a clean beaker of seawater. The next day, an aliquot of the seawater can be analyzed with a spectrophotometer. We did such tests early in our investigations to establish accuracy of our calculated estimated dispensation times; results indicated our calculations were adequate (i.e., at our temperature and salinity, the pumps lasted as expected).

2.2 Bivalve incubations

Our initial incubations employed juvenile bivalves (quahogs and surf clams; initially ~5mm and 1 cm in length, respectively), starting in December 2012. Intertidal sediments that were collected from a local salt pond were divided into four containers so that each had a sediment layer of ~10 cm of sediment. Bivalves were seeded into the sediments at a density of about 1 bivalve per square centimeter. Both species (*Mercenaria mercenaria*; *Spisula solidissima similus*) are surface dwelling or shallow-infaunal taxa. One calcein-filled osmotic pump was placed into each container so that the port was located in the container center, just below the sediment-water interface so that the calcein would emanate near the sediment-water interface.

These containers were initially maintained at 7°C. During that time, containers were installed in a recirculating seawater system containing ~10 L. Two containers were installed in each circuit. Salinity was monitored weekly with a refractometer and adjusted to 35 as needed. After the first ~3 weeks, in order to increase bivalve calcification rate, the containers were thereafter maintained at room temperature (21°C). Due to logistic reasons, circulating the containers at 21°C was not possible so each container was aerated with an aquarium bubbler. During this time, salinity was monitored and new seawater was added approximately every 2 weeks.

Throughout the incubations, living algal food (*Dunaliella tertiolecta*; *Isochrysis galbana*) was provided to each container every week (Hintz et al., 2004). The algae were concentrated via gentle centrifugation and ~40-50 ml was introduced into each container, so as not to disturb the sediment-water interface, each week.

Every 2-3 weeks whole specimens (live) were removed from containers noting their location with respect to the osmotic pump. Specimens were typically burrowed into the top cm. Each bivalve was examined with epifluorescence microscopy (see below) to determine if they had incorporated the fluorescent marker calcein. After examination, each individual was placed back in the sediment near its original location. After ~2 months (i.e., the approximate end of calcein dispensation), the osmotic pumps were removed from the sediments and the quahogs allowed to grow for another ~2 weeks. Then, the quahogs were removed from the sediments and preserved in 70% ethanol.

2.3 Foraminiferan incubations

Sediment cores containing benthic foraminifera were collected in May 2013 on a 3-day RV *Endeavor* cruise. Material was collected from a site south of Martha's Vineyard. This site, the Mud Patch, is on the broad continental shelf (40°30'N, 70°45'W), at a depth of approximately 75 m (Bothner et al., 1981). The site is well known as being a sediment-focus area, causing sediments to be muddy. Sediments were collected with an Ocean Instruments MC800 multicorer. The top 14-18 cm of designated multicores was extruded into 40-cm long core liners of identical internal diameter (i.e., 10-cm). Bottom waters from the collection site, which were collected in Niskin bottles attached to a CTD-rosette sampler, were carefully introduced into each core liner to produce a seawater header of ~20 cm. These introductions did not visibly disturb the sediment-water interface of any core. Cores were maintained near *in situ* temperature (8-10°C) and brought back to our shore-based cold room (7°C).

As with the bivalve incubations, one calcein-filled osmotic pump was placed into each core so that the dispensation port was located in the core center. In most cases, the port was placed just below the sediment-water interface. In other cases, the port was placed deep (4 cm) below the sediment-water interface. The cores were maintained at 7°C. Due to logistic reasons, circulating the cores with seawater was not possible so each core was aerated with an aquarium bubbler. Salinity was monitored with a refractometer weekly and adjusted to 35 as

required. Throughout the foraminiferan incubations, living algal food (*Dunaliella tertiolecta*; *Isochrysis galbana*) was provided to each core every week, as noted above for bivalves.

After ~4 months, each osmotic pump was gently removed and each core was subsampled as follows. All overlying water was carefully removed. A plastic ring identical in diameter to the core barrel was placed atop the barrel and the core barrel was gently lowered 1 cm so that 1-cm of the core extended into the ring. A thin stainless steel plate was then passed between the core barrel and ring to isolate the surface cm. Our goal was to obtain samples at horizontally and vertically discreet distances from the osmotic pump port. Because surface sediments had high water content, the first core sectioned was subsampled by taking 8-mm-diameter syringe cores along four radii in the surface centimeter (Fig. 1B). The remaining 0-1 cm sediments were retained separately.

All subsequent subsampling of 1-cm intervals was configured in concentric rings (Fig. 1B, C). Thus, the next 1-cm interval was extruded into the large diameter ring, three thin-walled plastic rings were concentrically placed into the core ~1 cm, the thin stainless steel plate was used to slice the core horizontally while the concentric rings were held in place, and the sediments delimited between concentric rings were placed into plastic bottles, and properly labeled (as center, inner, outer or rim) along with depth interval below the sediment-water interface. The 1-2-cm, 2-3-cm, 3-4-cm, and 4-5-cm intervals of each core were subsampled using this concentric ring approach. All sediment samples were preserved in 70% ethanol. Each sediment sample was sieved with artificial seawater over a 63-µm screen and >63-µm fraction microscopically examined.

2.4 Microscopy

Epifluorescence microscopy (480-nm excitation; long pass 518-nm emission) was used to assess calcein incorporation. Preserved materials were examined with a Leica FLIII stereomicroscope equipped with epifluorescence capabilities and an Olympus DMP70 digital camera. Whole quahogs ~~were examined.~~and ~~W~~whole foraminifera, obtained from the >63-µm fraction of sieved sediment aliquots, were ~~also~~ examined.

Once imaged at low magnification, select quahog shells were cut with an Isomet slow-speed rock saw (0.4-mm-thick blade) to obtain valve cross sections. These valve cross sections had to be polished with fine grit wet-dry sandpaper to obtain a smooth surface. To remove organics, shells were exposed to 3% sodium hypochlorite for 20 minutes. After rinsing and drying, these were examined and imaged with the epifluorescence-equipped Leica FLIII

stereo-dissecting microscope and DMP digital camera and/or with an Olympus Fluoview Confocal Laser Scanning Microscope (CLSM).

3 Results and Discussion

3.1 Proof of concept

The surf clams only survived 1-3 weeks in the cores, but the quahogs remained active and grew, evidenced by the observations that many (~25%) of the quahogs had portions of their shell that were fluorescent (Fig. 2). The observation that the umbo and other oldest parts of each quahog valve were not fluorescent is consistent with the fact that we seeded the cores with juveniles that were ~0.5 cm in length. Keeping in mind that bivalves add material to their valves in increasingly larger concentric annuli, somewhat akin to tree rings, it was noted that the fluorescence appeared as bands, where some portions fluoresced and others did not. Incorporation of calcein into newly precipitated carbonate was confirmed by CLSM of bleached valves (Fig. 2G).

Because the system used during bivalve incubations was recirculating or lacking flow, it is important to consider the maximum concentration of calcein possible if all contents of the osmotic pump were dispensed into the seawater. We calculate that, at most, the recirculating seawater would have had 0.02 mg l^{-1} calcein. For mollusc studies, typically a calcein concentration equaling or exceeding 80 mg l^{-1} is used to label calcite (e.g., Kaehler and McQuaid, 1999;Klunzinger et al., 2014). Thus, the maximum possible calcein concentration in recirculating seawater during the first 3 weeks of bivalve incubations was far below the level required to fluorescently label calcite. Of course if the bivalves were near the concentrated source of calcein (i.e., pump port, 100 mg l^{-1} calcein), then we predict the new calcite would be fluorescent, as observed.

In the static (but aerated) setup, the maximum concentration of calcein possible if all contents of the osmotic pump were dispensed into the seawater was 0.2 mg l^{-1}, which again is far below the minimum mollusc threshold of 80 mg l^{-1}. There are two more lines of evidence that further support the inference that the lack of constant water replacement did not cause artifactual calcein labeling in the bivalves: (1) The seawater overlying the cores did not have strong fluorescence when viewed with the appropriate optics using the stereo dissecting microscope (i.e., there was no significant background signal). (2) Not all bivalves were

labeled with calcein. Some quahogs did not fluoresce in any part of their valves, indicating they were sufficiently removed from the point source throughout the incubation.

Some of the calcareous benthic foraminifera in the cores exhibited bright fluorescence while others did not (Fig. 3A,B). As established for the calcein labeling method, the non-fluorescent calcareous specimens either did not calcify during the incubation (Bernhard et al., 2004) or were too far from the osmotic pump port to incorporate calcein. Some of the calcareous foraminiferal tests fully fluoresced (Fig. 3A-D), while others had only one or two brightly fluorescent chambers (Fig. 3E,F). It is possible that the fully fluorescent specimens were the result of reproduction during the experiment (Filipsson et al., 2010;Hintz et al., 2004). In contrast to brightly fluorescent rotalids, entire tests of miliolid (porcelaneous) calcareous foraminifera fluoresced dimly; no agglutinated foraminifera fluoresced (not shown). It is known that at least some miliolids fluoresce after incubation in calcein, even without calcification (Bernhard et al., 2004). It is also established that when rotalid (hyaline) calcareous foraminifera add new chambers, a thin veneer of calcite is precipitated over existing chambers (Erez, 2003;Nehrke et al., 2013). Such a growth habit explains the differential fluorescence patterns in some foraminiferal specimens, where 1-2 chambers are brightly fluorescent and remainder of the test has less intense fluorescence (Fig. 3F).

As noted for the non-recirculating bivalve incubations, we do not believe the calcein concentration of the overlying seawater would exceed the minimum labeling threshold in the foraminiferal incubations even if the entire osmotic pump contents were released. For foraminiferal calcite labeling, a calcein concentration of 10 mg l^{-1} has been typically used previously (Bernhard et al., 2004;Denoyelle et al., 2012;Nardelli et al., 2014;Filipsson et al., 2010) although concentrations as low as 5 mg l^{-1} reportedly also labeled foraminiferal calcite (Dissard et al., 2009;Kurtarkar et al., 2015). The maximum possible calcein concentration in overlying waters of our foraminiferal incubations was less than 1 mg l^{-1}. As for bivalves, if growing calcareous foraminifera were near the concentrated source of calcein (i.e., pump port, 100 mg l^{-1} calcein), then we predict new calcite would be fluorescent, as observed.

Unfortunately, the calcein-labeled foraminiferal densities were insufficient to determine the vertical and horizontal extent of calcein diffusion into our muddy sediments. Specifically, calcein labeled foraminifera were absent from all small-volume radial subsamples of the 0-1 cm interval of one core. Calcein-labeled foraminifera were found, however, in the remaining bulk 0-1 cm interval of the first multicore. Time and resource limitations prohibited full

processing of additional multicores; spot checks in those samples did not yield convincing fluorescent foraminiferal calcite. A recently finished Master's thesis project confirms the practical use of the osmotic pumps using foraminiferal-laden fjord sediments (Landgren, 2015).

3.2 Notes and caveats

In the course of our method development, a number of lessons were learned. To assist future users of the method, these topics are discussed. Orienting the osmotic pump port downwards is problematic in fine-grained and/or low water-content sediments because conditions likely impede calcein dispensation or the diffusion is too localized to expose many specimens to calcein. It is expected that sediments with high water content (e.g., sediments with "fluff" layers) would not impede dispensation as much as more compacted or consolidated sediments. Attempts to test the osmotic pump port at 4 cm depth did not result in any fluorescent specimens, but we do not know if that was due to lack of calcification or spatially limited calcein diffusion.

To document specifics regarding infaunal calcification horizons, it will be critical to determine the extent of calcein diffusion into sediments. The radius of calcein dispersion and d~~D~~iffusion will vary with sediment grain size, sorting, water content, compaction, hydrodynamics, and community composition (e.g., presence or absence and activity of bioturbators). Diffusion coefficients can be measured directly in sediments (e.g., Krom and Berner, 1980) or they can be estimated from the sediment's formation resistivity factor, which can be estimated from sediment porosity and other sedimentary characteristics (e.g., Ullman and Aller, 1982). Initial verification tests should be considered prior to initiating a lengthy or complicated experiment.

Calculations based on expected dispensation rate, temperature, and salinity can provide estimated duration of calcein efflux. Osmotic pumps are single use; they will not dispense if refilled. Per manufacturer's instructions, osmotic pumps will not perform well if handled without clean gloves.

The cytoplasm of at least one benthic foraminiferal species autofluoresces using excitation and emission wavelengths similar to those for calcein (Apotheloz-Perret-Gentil et al., 2013). The foraminiferal species known to autofluoresce lacks a carbonate test, so it cannot be confused with our calcein-labeling approach. If there are calcareous foraminifera with

similarly autofluorescent cytoplasm, distinguishing between cytoplasmic fluorescence (from viability indicators reliant on similar excitation and emission wavelengths) and carbonate fluorescence is not difficult if one considers the patterns and shapes of the signal (Nardelli et al., 2014).

3.3 Potential applications

The calcein-osmotic pump method can be used without modification to assess growth rates and calcification locations of juvenile and meiofaunal metazoans with calcareous hard parts (e.g., gastropods, echinoids, ostracods). These units can be deployed in shallow marine waters near shellfish fisheries and in reef areas with sediment pockets. Determining rates of calcification and locations where individuals grow are important to benthic ecology and ocean acidification studies.

As noted, our method will help to better understand foraminiferal microhabitats. Such knowledge will help to minimize uncertainty and increase precision in records of paleoceanographic proxies preserved in foraminiferal tests. For instance, recently the difference in the $\delta^{13}C$ for epifaunal *Cibicides wuellerstorfi* and for deep infaunal *Globobulimina* spp. was used to reconstruct bottom-water oxygen concentration (Hoogakker et al., 2015), an approach that could be further improved by this method. Foraminiferal–based ecology studies under shifting environmental conditions, such as varying water oxygen concentration (Nardelli et al., 2014) or pH conditions would also benefit from our calcein-osmotic pump method. Of course, our method should be considered a first step given that most paleoceanographically-relevant foraminifera presumably are able to migrate within the sediment column. It is unknown, however, if benthic foraminifera actually migrate vertically in situ and, if they do, where they calcify. Regardless, additional refinements to the approach will be required to prevent or minimize foraminiferal migration during and/or after calcification.

Our osmotic pump method can be further modified to deploy these units in deep-sea sediments using a Remotely Operated Vehicle (e.g., *Jason*) or a Human Occupied Vehicle (e.g., *Alvin*). Further, calcein-filled osmotic pumps can be installed in habitats that are spatially restricted, such as hydrocarbon seeps or brine pools, where we have little growth data for any sediment-dwelling species.

4 Conclusions

While calcein has been used in growth studies for a variety of organisms, to our knowledge, calcein has not been used as a point source to determine calcification in the environment. Most studies using calcein to determine growth rates immerse entire specimens in the laboratory and then release them into nature for later recapture. Our new calcein-osmotic pump approach can help pinpoint where and when meiofaunal organisms calcify in nature. This information is important because, for example, in situ rates of shell growth are not well known.

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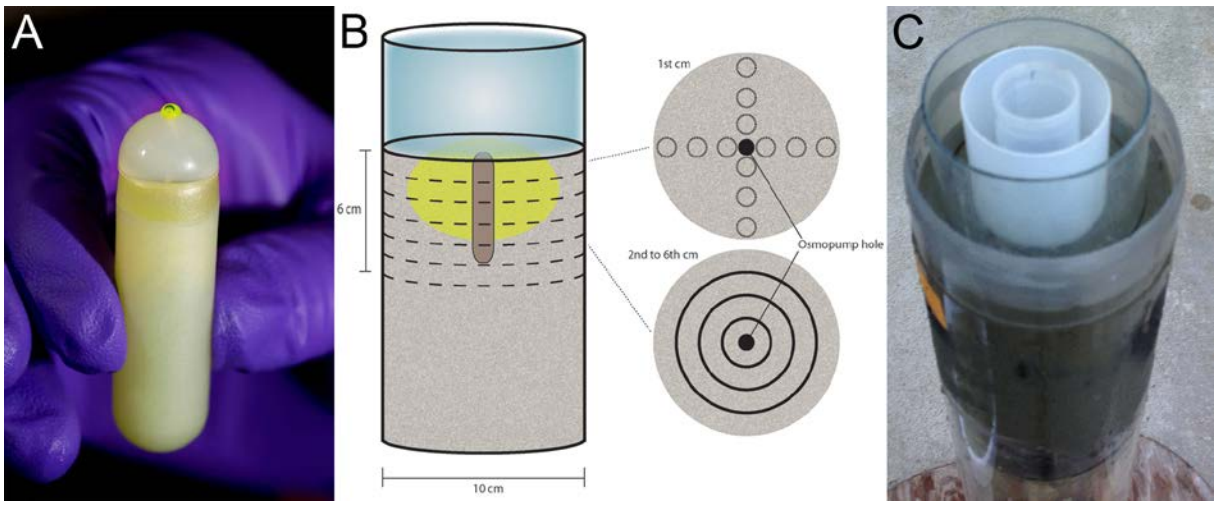

Figure 1. A. ALZET® 2ML4 osmotic pump filled with concentrated calcein. The visible calcein at the top is emanating from the pump's port. B. Schematic of sediment core and overlying water with osmotic pump in place (left) and of two sediment collection approaches: one for top 1-cm layer, using 3-ml syringe cores (right, top); the other for underlying layers, using concentric rings (right, bottom). Yellow ovoid is theoretical dispensation of calcein from osmotic pump emplaced port up in core. C. Image showing concentric rings in place prior to section slicing. Core is 10 cm in diameter.

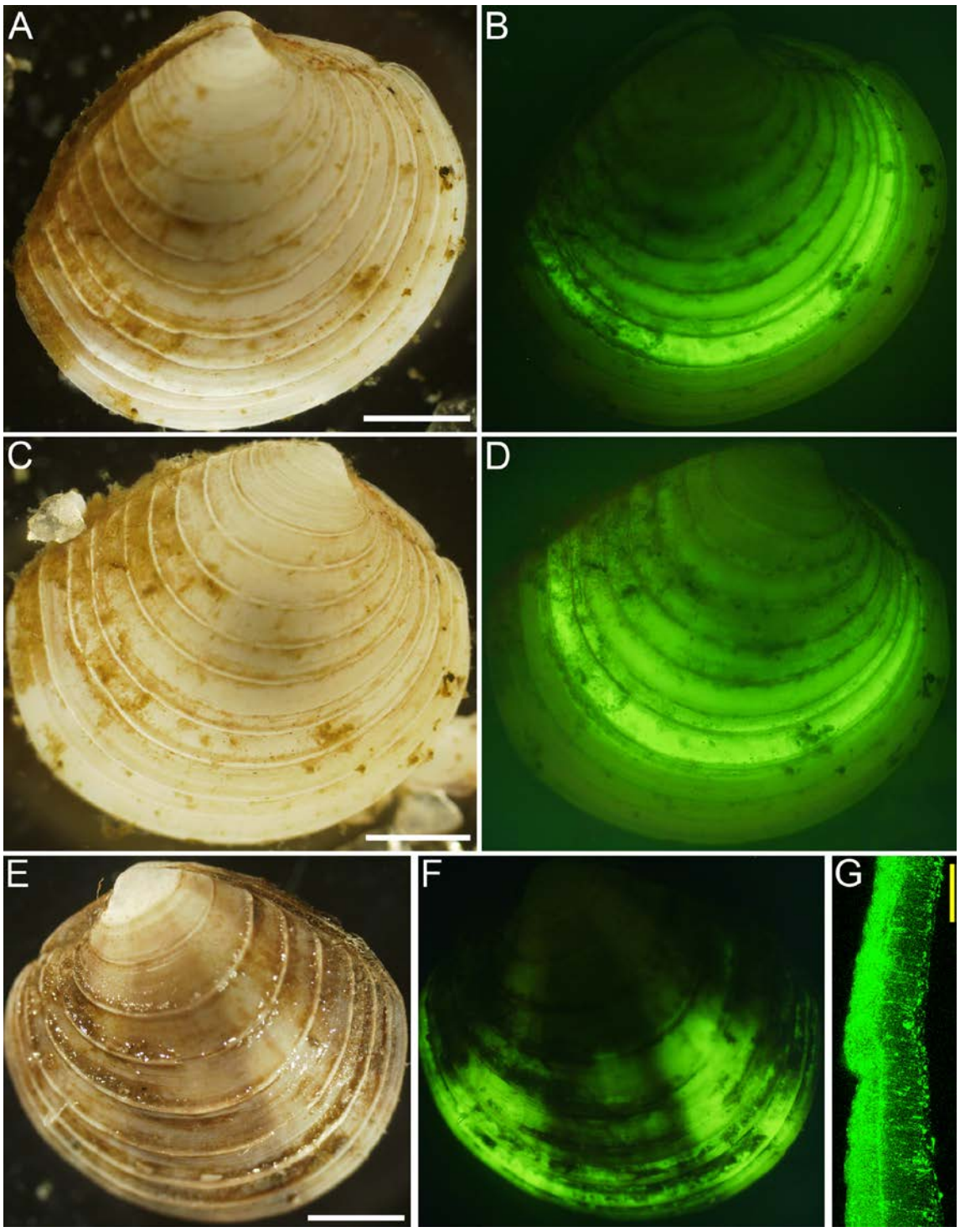

Figure 2. Paired micrographs of quahogs after calcein osmotic pump incubations. Reflected (A, C, E) and epifluorescence (B, D, F) images of quahog after short bleach and air drying (E was not completely dry). Note calcein banding in valves. In F, calcein extends to edge of valve because this specimen died during experiment. G. CLSM micrograph showing cross section of specimen E,F valve after thorough bleaching. Scales: A, C, E= 2 mm; G = 200 µm.

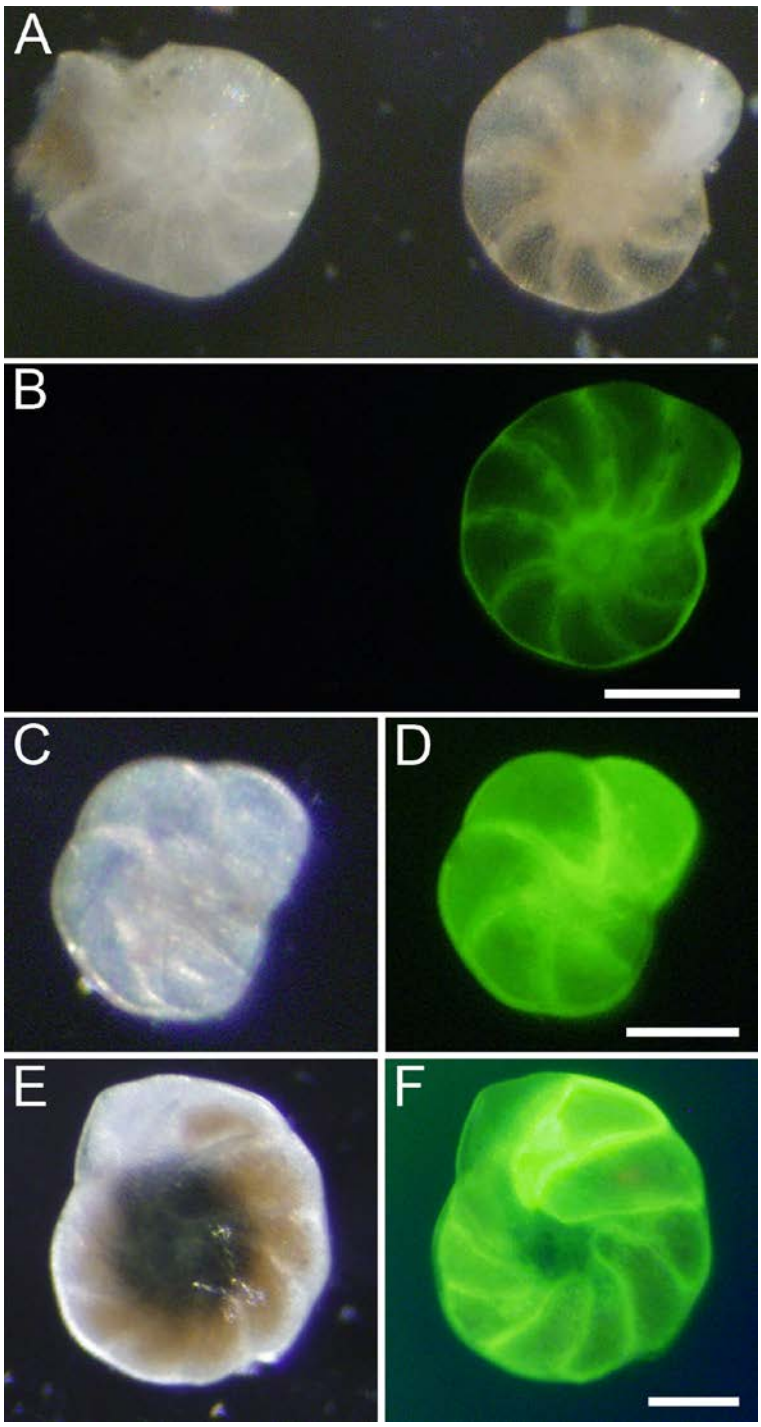

Figure 3. Paired micrographs of foraminifera after calcein osmotic pump incubations. Reflected (A) and epifluorescence (B) images of two *Cribroelphidium* sp. Note the specimen on the right fluoresces strongly in B (vs. left specimen shows no fluorescence). Thus, the specimen on the right grew during calcein incubation and the other did not grow or was too far removed from the osmotic pump. Reflected (C) and epifluorescence (D) images of fully fluorescent small calcareous foraminifera, too small to speciate. Reflected (E) and epifluorescence (F) images of fully fluorescent *Cassidulina* sp. Note that the penultimate chamber fluoresces brightest, indicating its calcification during calcein incubation. The youngest chamber was likely being precipitated at the time of sampling. Scales: B, F = 100 µm; D = 50 µm.