

Assessing the effect of calcein incorporation on physiological processes of benthic foraminifera

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Abstract

Rosalina sp. was incubated in seawater spiked with calcein (5 mg/l to 20 mg/l calcein), a dye proposed as efficient marker to tag newly formed calcite, to understand its effect on benthic foraminifera. The experiment was conducted at 25°C and 27°C temperature. The growth of *Rosalina* sp. is not affected by any of the calcein concentrations within the first 4-5 weeks of exposure. Additionally, no distinct difference in abnormality, mortality and reproduction was observed in the control and treatment specimens during the first 3-4 weeks of incubation. The comparable growth, abnormality, mortality and reproduction in both the control and the various treatment suggests that short-term exposure to calcein does not adversely affect benthic foraminifera. However, during the total 15-16 weeks experiment, more than 50% of the specimens died in all the treatment sets, as compared to <40% mortality in control sets, thereby suggesting a slight adverse effect of calcein on benthic foraminifera. Additionally, some of the specimens incubated in calcein spiked media were stunted and developed abnormal shells after 5-6 weeks of incubation. Also the lack of reproduction, the increased mortality and abnormality in specimens subjected to very low calcein concentration (≥ 5 mg/l) for a longer periods of time (5-6 weeks or more), suggests an adverse effect on benthic foraminifera. Although the percentage of abnormal specimens and the percentage of specimens which reproduced was different for the two temperatures (for similar calcein concentrations), no such difference was observed for growth or mortality. The findings support previous studies which proposed that short-term exposure to low calcein concentration can be used as an effective technique to distinguish newly formed chambers in laboratory experiments with foraminifera.

Keywords: Calcein, benthic, foraminifera, size, abnormality, mortality, reproduction

1. Introduction

In paleoclimatic studies, increasing emphasis is being given to quantitative estimation of different climatic parameters including seawater temperature, salinity, pH and ice-volume. Trace metal and stable isotopic ratios of foraminiferal shells are often used to quantitatively estimate past seawater temperature, salinity and pH (Boyle, 1981; Sanyal *et al.*, 1995; Elderfield and Ganssen, 2000; Lear *et al.*, 2003; Barker *et al.*, 2005; Saraswat *et al.*, 2005; 2012; Hintz *et al.*, 2006; Hönisch *et al.*, 2011). A precise knowledge of the effect of individual physico-chemical parameters on the trace metal and stable isotopic composition of the foraminiferal shells is required to estimate these climatic parameters. Culture studies help in assessing the effect of different physico-chemical parameters on the foraminiferal shell chemistry (Spero *et al.*, 1997; Lea *et al.*, 1999; Havach *et al.*, 2001; Lea, 2003; Reichart *et al.*, 2003; Hintz *et al.*, 2006; De Nooijer *et al.*, 2007; Sadekov *et al.*, 2008; Dissard *et al.*, 2010; Raitzsch *et al.*, 2010). In many studies, the living specimens used in the experiments are collected in the field, and have already secreted part of their shell in natural conditions. The early chambers of such specimens will have the trace metal and stable isotopic composition depicting the field conditions, and only the later chambers will reflect the laboratory conditions, imposed after incubation. In such cases, the trace metal and stable isotopic composition analyses of the complete shells of such specimens will not adequately reflect the experimental conditions. With the development of laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS) (Reichart *et al.*, 2003; Eggins *et al.*, 2003; 2004) and secondary ion mass spectrometry (SIMS) techniques, it is now possible to make precise chemical analyses in individual chambers, instead of analyzing the whole shells (Bice *et al.*, 2005; Sadekov *et al.*, 2008; Allison *et al.*, 2010; Glock *et al.*, 2012). However, the strategy to measure trace element composition by ablating the carbonate in a 10-50 μm diameter crater in a single chamber, requires distinguishing between the chambers formed in the field, before incubation, and those secreted after incubation, under controlled laboratory conditions.

Calcein (Bis [N,N-bis (carboxymethyl) aminomethyl]-fluorescein) which binds with calcium in bio-mineralized structures (Hoelzl *et al.*, 1959) such as in the process of biochemical precipitation of bones by fish, was suggested as an effective marker for tagging newly formed biogenic calcite (Brooks *et al.*, 1994; Mohler, 1997, 2003; Logsdon and Pittman, 2012). Carbonate spiked with calcein fluoresces a yellow-green when excited with light with a particular wavelength (~470 nm excitation, 509 nm emission). Detailed work has been done to assess the utility of calcein to tag fish otoliths, fins, and rays (Brooks *et al.*, 1994; Mohler, 1997, 2003; Logsdon and Pittman, 2012). Studies on many other groups of calcium carbonate/phosphate secreting organisms, including corals

(Tambutté *et al.*, 2011; Gagnon *et al.*, 2013), mollusks (Kaehler and McQuaid, 1999; Moran, 2000; Riascos *et al.*, 2007; van der Geest *et al.*, 2011), ascidians (Lambert and Lambert, 1996), echinoderms (Medeirosbergen and Ebert, 1995; Rogers-Bennett *et al.*, 2003), brachiopods (Rowley and MacKinnon, 1995) and cnidarians (Marschal *et al.*, 2004), have confirmed the utility of calcein as a fluorescent marker in skeletons and shells.

Bernhard *et al.* (2004), demonstrated the use of calcein to label foraminiferal tests and reported that short exposure (a couple of weeks) was non-lethal. Since multilocular foraminifera add chambers episodically (Goldstein, 1999), incubation of foraminifera with the fluorescent dye calcein can be used to discriminate between preexisting foraminiferal calcite, and new calcite added under controlled laboratory condition (Bernhard *et al.*, 2004; Hintz *et al.*, 2006; Barras *et al.*, 2010; Dissard *et al.*, 2010; Raitzsch *et al.*, 2010; Diz *et al.*, 2012). Possibly, this technique is also applicable in case of agglutinated specimens in which the calcite cement can be tagged (Bender, 1992). Although calcein tagging has become increasingly popular to understand various processes in foraminifera such as biomineralization (Bentov *et al.*, 2009), trace metal uptake (Hintz *et al.*, 2006; De Nooijer *et al.*, 2007; Dissard *et al.*, 2009; Raitzsch *et al.*, 2010; Allison *et al.*, 2010; Filipsson *et al.*, 2010), ontogenetic variations (Hintz *et al.*, 2006) and even in the impact of ocean acidification (Dissard *et al.*, 2010), the primary assumption made is that the fluorescent dye is not harmful to the microorganism. Earlier, Brooks *et al.* (1994), however, reported mortalities of up to 33% in fish incubated for 6 h in calcein (500 mg/l of water). Subsequently, also Mohler (1997) reported increased mortality in the Atlantic salmon *Salmo salar* treated with 250 mg/l calcein solution for 48 h. The mortality decreased significantly only after drastically reducing the exposure time (to 3.5 min) using an osmotic induction technique (Mohler, 2003). Similarly, significantly increased mortality was reported in elasmobranchs treated with a calcein concentration of 25 mg/kg body weight as compared to those treated with 5-10 mg/kg body weight (Gelsleichter *et al.*, 1997). In all of these studies, the organisms were only briefly (a few minutes to a maximum of a few hours) exposed to different calcein concentrations. Because of the potentially toxic nature of calcein, methods have been suggested to remove it from the waste water (Mohler and Bradley, 2008). However, the susceptibility of organisms to calcein appears to vary among different organisms. No lethal effect was reported for gastropods and bivalves even after exposure to a calcein concentration as high as 150 mg/l (Moran, 2000; Riascos *et al.*, 2007; Mahé *et al.*, 2010).

In many of the studies in which newly formed foraminiferal calcite was tagged, the specimens were exposed to calcein concentrations varying from 5 mg/l to 20 mg/l, for 3-4 weeks, before the start of the experiment (pre-staining phase), so that the incorporation of calcein did not

interfere with uptake of minor and trace elements (Bernhard *et al.*, 2004; Dissard *et al.*, 2010; Allison *et al.*, 2010; Raitzsch *et al.*, 2010; Wit *et al.*, 2012). However, calcein incorporation and exposure time may vary from species to species, for instance due to different chamber arrangement and test wall structure (thickness and composition) (Bernhard *et al.*, 2004). Surprisingly, the effect of calcein media on the growth rate and reproduction in foraminifera, has never been studied. This poses the question of the utility of a calcification marker which might alter the growth process of the organism and hinder reproduction. To assure the validity of results obtained in experimental studies, it is necessary to ascertain that foraminifera grew under conditions close to optimum, at which the species can incorporate calcein into its tests and at the same time retain normal growth rates and other physiological processes. In view of this, we have conducted a laboratory culture experiment to determine the exposure times and calcein concentrations for which the shallow water benthic foraminifera *Rosalina* sp. does not show a negative response.

2. Materials and Methods

2.1 Collection and Separation of Live Specimens

To obtain living specimens of *Rosalina* sp., surface sediment samples and sea-grass were collected from shallow waters at the Dias Beach, Goa, India (latitude 15°27'12" N and longitude 73°48'05" E). The seawater temperature at the time of sampling was 27°C. The material collected from the sampling location was sieved through a 63 µm size sieve and >63 µm material along with seawater was brought to the laboratory. Additional sea water was also collected in 20 l LDPE plastic cans at the time of sampling. In the laboratory, the >63 µm material was observed under the stereo-zoom microscope (Olympus SZX-16). Living specimens were picked using a micropipette or a very fine brush. These living specimens were then transferred to sterilized polystyrene multi-well (6-wells) tissue culture plates (Axygen TM). The live status of the specimens was further confirmed by observing activities like movement, collection of food and pseudopodial extension, under an inverted microscope (Nikon ECLIPSE TE2000-U). The living specimens so obtained were divided into several batches and kept at different conditions (salinity varying from 20 to 40 salinity units (psu) and temperature varying from 20°C to 30°C) considered optimal for reproduction. The optimum growth and reproduction for *Rosalina* sp. was reported at 25-27°C. In order to avoid any possible temperature related bias, we only used juveniles of specimens maintained at 27°C. The juveniles so produced, which had 4-6 chambers, were used for the experiment. We tried to pick juveniles of a size range as narrow as possible, for all experimental sets. The average size of the juveniles was 113±16 µm and the average number of chambers in these juveniles was 5±2. The advantage of using juveniles is that they provide ample experimentation time, allowing us to vary the conditions of the

medium to invoke a response of these specimens before they became mature and reproduced. In this way, foraminifera can be studied over the full range of sizes/ ages and it is thus possible to investigate any ontogenetic effects.

2.2 Experimental Procedure

A total of 200 juveniles of *Rosalina* sp. were used. Special care was taken to ensure that the specimens had only 4-6 chambers before incubation in different calcein spiked media. The media were prepared beforehand by dissolving the desired weight of freshly powdered calcein (5 mg, 10 mg, 15 mg, 20 mg) in 1 liter of 0.22 μ m filtered sea water (35 salinity). The range of calcein concentrations (5-20 mg/l) was chosen based on previous studies involving incubation of foraminifera in calcein (Bernhard *et al.*, 2004; Allison *et al.*, 2010). Simultaneously, a control was taken, i.e. calcein-free seawater, to determine 'background' levels of survival, growth, abnormality and reproduction. The pH of the control was noted and all the other stock solutions were adjusted to this pH by means of addition of 1N NaOH (20-50 μ l) following Allison *et al.* (2010). The pH was measured with a ThermoScientific Orion Star A329 multi-parameter meter with a precision of ± 0.01 pH units. The specimens were cultured in 6 well tissue culture plates (trays). Each well had a diameter of 35 mm and a height of 20 mm. The wells were filled with about 10 ml of the media. 10 specimens were exposed to the same calcein concentration at a given time. Two working temperatures (25°C and 27°C) were chosen, for two reasons: 1) during pre-experimental incubation, specimens responded well at both of these temperatures; 2) in an earlier study conducted on a species of the same genus (*Rosalina globularis*), large differences were observed in growth and especially in reproduction between specimens incubated at 25°C and 27°C (Saraswat *et al.*, 2011). Growth at 25°C temperature was lower than at 27°C, and also the highest percentage of reproduction was reported for the specimens subjected to 27°C temperature (Saraswat *et al.*, 2011). The incubation temperature was kept constant throughout the experiment by maintaining the culture trays in cooling incubators (Sanyo make, Model MIR-154, temperature fluctuation $\pm 0.2^\circ\text{C}$, temperature uniformity $\pm 0.5^\circ\text{C}$) with 12 hour light and dark cycles (Chandler *et al.*, 1996; Wilson-Finelli *et al.*, 1998; Havach *et al.*, 2001). The study was carried out in replicate, but because of the low number of surviving individuals after more prolonged incubation periods, the replicates were merged for the study of test growth. For the study of mortality, test abnormalities and reproduction rate, the replicates were kept separately.. Food in the form of live diatoms *Navicula* sp. (50 μ l/well) was added twice a week. *Navicula* species have been reported from the area where living specimens were collected. Although the diatoms were alive when fed to the foraminifera and the foraminifera were kept at a light/dark cycle, the difference in pH between treatments was negligible. The *Navicula*

stock was maintained by a batch subculture method. The diatoms were grown in F2 medium. The F2 medium was prepared by following the method described by Guillard and Ryther (1962).

Media were changed every week with care being taken so as to keep the pH of the stock solutions constant. Foraminifera were examined with a Nikon ECLIPSE TE2000-U inverted microscope with epi-fluorescence attachment and photographs of the living specimens were taken with an Olympus DP21 camera, while changing the media. The maximum diameter of all 200 specimens was measured by using Cell Sens Standard™ prior to incubation and once a week thereafter (a total of 14 times during the experiment) until either the specimens reproduced, died or stopped responding (no growth, desticking of food material or no signs of reproduction) (SOM Table 1). In order to minimize the stress and to assess the degree of calcein incorporation in the tests, the calcein spiked media in the wells were carefully replaced momentarily (15-20 minutes, once a week while changing the media) with calcein free media (with all other parameters the same as the calcein spiked media), which was replaced again with calcein spiked media after documenting the growth and calcein incorporation. As it was impossible to follow individual specimens throughout the experiment, growth was estimated as the average size increase of all living specimens at the time of measurement. The error associated with the measurement of the maximum diameter by using Cell Sens Standard™ is smaller than 2 μm . The error is based on measurements of the maximum diameter of 25 specimens, 50 times each.

The specimens developed a protective cyst prior to reproduction which helped in identifying the specimens which were about to undergo reproduction. Such specimens, that developed a cyst, were monitored daily. After reproduction, juveniles remained either attached to or in the close vicinity of the parent for several days, what helped to count the number of juveniles per reproduction. The individual specimens were far apart to facilitate distinction between specimens. The parent died during reproduction when the juveniles came out of the parent test when it broke open. The death of the specimens was confirmed by a definite lack of movement and pseudopodia extension. This was followed by a gradual disintegration of the feeding cyst adhering to the test as well as by a change in colour of the specimens from brown to opaque, due to decay of protoplasm. The process of reproduction, from the development of a protective cyst to the separation of all juveniles from the parent test took 2-4 days. The specimens which underwent reproduction were not included in the calculations of mortality which are exclusively based on specimens which died without undergoing reproduction. In fact, some specimens neither died nor reproduced during the experiment. The experiment was terminated after 112 days. t-tests were performed to assess the significance of differences ($p \leq 0.05$) in growth rate, reproduction, mortality and abnormality of

Rosalina sp., in groups of specimens subjected to different calcein concentrations at both 25°C and 27°C temperature. Specimens raised in experimental conditions were compared with the control specimens, by using 'Statistica6' software. The results of the t-tests have been included as Excel tables in the supplementary online material (SOM).

3 Results

Increase in the size of *Rosalina* sp. specimens was observed in all experimental conditions (Figure 1, Table 1, SOM). The increase in the size of the specimens only concerns the portion of the total population that was alive at any time. Although exact measurements of the fluorescence intensity were not taken, from a preliminary analysis of the photographs, it appears that calcein incorporation into the newly formed chambers occurred already during the first week. After 7 days a very faint fluorescence was noted in specimens incubated at 5 mg/l and 10 mg/l calcein at both 25°C and 27°C temperatures, while those incubated at higher calcein concentrations had a prominent fluorescence. In specimens incubated in weaker concentrations, until the end of the experiment, it was difficult to distinguish between chambers formed prior to and after the incubation in calcein (Figure 2). No distinct visual difference in calcein incorporation was noted between specimens subjected to similar conditions at different temperatures. Apparently, calcein incorporation increased with increasing calcein concentration, as suggested by the increasing visual fluorescence intensity.

3.1 Size

The average size at any given day is the average of the size of all living specimens taken together, calculated separately for each calcein concentration and temperature. The average final size of the specimens is calculated from the size of all living specimens on the last day of measurement. The reported variability is the mean of the standard deviation of the average size of all living specimens taken together. The final size ($492 \pm 60 \mu\text{m}$) of the control specimens subjected to 25°C temperature was larger than those subjected to 27°C ($368 \pm 55 \mu\text{m}$) (Figure 1, Table 1). The average final size of the specimens incubated at all different calcein concentrations (5 mg/l - $320 \pm 74 \mu\text{m}$; 10 mg/l - $342 \pm 29 \mu\text{m}$; 15 mg/l - $348 \pm 62 \mu\text{m}$; 20 mg/l - $270 \pm 85 \mu\text{m}$) at 25°C temperature was smaller than the average size of the control specimens ($492 \pm 60 \mu\text{m}$) at the same temperature (Figure 1). However, at 27°C, the final size of the control ($368 \pm 55 \mu\text{m}$) was not significantly different from specimens incubated in 5 mg/l ($370 \pm 60 \mu\text{m}$) and 10 mg/l ($394 \pm 34 \mu\text{m}$) calcein (Figure 1). The final size attained by the specimens incubated at 15 mg/l ($248 \pm 6 \mu\text{m}$) and 20 mg/l ($287 \pm 16 \mu\text{m}$) at 27°C, however, was significantly ($p < 0.05$) smaller than the rest of the specimens. The pseudopodial activity also reduced in wells of 15 mg/l and 20 mg/l calcein concentration. Interestingly, at 25°C, a continuous size

increase was observed until about 85 days for all concentrations, whereas at 27°C size leveled off after 68 days (Figure 1). There was hardly any increase in size after ~85 days of incubation of specimens in both controls as well as in different calcein media.

3.3 Test Abnormality

All specimens in both the control and treatment sets and temperatures, were morphologically normal during the initial 3-4 weeks of incubation. However, deformation began after 4-5 weeks of incubation of *Rosalina* sp. specimens into calcein media, and this led to a progressive increase in the percentage of deformed specimens (Figure 3, Table 2, SOM Data Table). The type of abnormalities included chambers with a much larger or smaller size than the preceding chambers or newly added chambers being oriented in a plane different than the normal plane of chamber addition (Figure 4). At 25°C temperature, at the end of the experiment, about half of the specimens were abnormal at both 15 mg/l (55±15%) and 20 mg/l (50±40%) calcein concentration. At the end of the experiment all the specimens of the control sets at both 25°C and 27°C temperature were normal.

3.4 Mortality

The mortality increased with increasing calcein concentration at both 25°C and 27°C (Figure 5, Table 3). In all calcein treatment sets, more than 50% specimens died ultimately, although the timing was variable and was shorter at higher calcein concentration. The mortality was significantly higher at 15 mg/l (80±0%, 25°C; 90±10%, 27°C) and 20 mg/l (95±5%, 25°C; 89±1%, 27°C) calcein concentration as compared to that at 5 mg/l (70±10%, 25°C; 70±0%, 27°C) and 10 mg/l (65±5%, 25°C; 60±0%, 27°C) calcein concentration. Mortality was not significantly different for both control sets (25°C, 40±10% and 27°C, 20±10%), and was significantly lower than mortalities in all treatments with calcein added to the media (Figure 6).

3.5 Reproduction

In the control conditions, significantly more specimens reproduced at 27°C temperature (55±5%) as compared to 25°C (25±5%) (Figure 7, Table 4). At 25°C, reproduction was also noted at 5 mg/l (15±5%), 10 mg/l (10±10%) and 15 mg/l (2 specimens) calcein concentration, though it occurred much less as compared to the control specimens (55±5% at 27°C and 25±5% at 25°C). At 27°C, except the control specimens, none of the specimens subjected to different calcein concentrations, reproduced. None of the specimens incubated in 20 mg/l calcein media at both 25°C and 27°C temperatures reproduced.

4 Discussion

Although the calcein tagging method is suggested to allow easy identification of chambers formed before and after calcein incubation, it presents the problem to determine the time frame and concentration at which this incubation is to be carried out. As different species show different responses to calcein tagging (*Quinqueloculina* sp. fluoresced very brightly as compared to perforate low-Mg species; Bernhard *et al.*, 2004) suggesting different mechanisms of bio-mineralization (Hemleben *et al.*, 1986; Erez *et al.*, 1994; Erez, 2003; Zeebe and Sanyal, 2002; De Nooijer *et al.*, 2009), it is necessary to ascertain the optimum time period and concentration to carry out this incubation such that the specimen growth is unaffected. However, the effect of calcein on foraminifera may not exclusively relate to calcite production, but can also affect the metabolism (not directly related to calcification) of different species by different magnitudes.

4.1 Morphological changes as a response to calcein

Due to the small number of surviving specimens towards the end of experiment, , all living specimens belonging to the two replicate sets were taken together to calculate average size. The large difference (>100 μm) in size of the control specimens for the two different temperatures suggests a large effect of incubation temperature on growth.

The species specific response of benthic foraminifera to temperature variation has been studied by many workers (Bandy, 1963, Theyer, 1971, Murray, 2006, Nigam *et al.*, 2008; Saraswat *et al.*, 2011). During the first 3-4 weeks, there is no significant difference between the average size of all control and treatment set specimens for both temperatures. The similar average size attained by the control specimens and those subjected even up to 20 mg/l calcein concentration during the initial 3-4 weeks of incubation, suggests no perceptible adverse effect on growth of benthic foraminifera, of short-term incubation even in as high as 20 mg/l calcein concentration. Earlier Bernhard *et al.* (2004) also reported that exposing the foraminifera to 10 mg/l calcein for a couple of weeks does not result in any adverse effect. Similarly, Dissard *et al.* (2009) also observed no adverse effect after exposing the foraminifera to 5 mg/l calcein for four weeks. A difference in average size of the specimens incubated in all calcein concentrations at 25°C and those incubated at 15 and 20 mg/l calcein at 27°C temperature, as compared to the control specimens, starts to be apparent after ~50 days. This difference in the final size between the control specimens and specimens incubated in all calcein concentrations, as low as 5 mg/l, after a prolonged exposure, leads us to propose that there is some process that acts as a barrier to the normal growth in the presence of calcein. This is the first report of an adverse effect of such a low calcein concentration on the growth of calcite secreting marine

organisms. A similar decrease in size leading to stunted specimens has previously been reported from anthropogenically polluted (See Nigam *et al.*, 2006 for review; Romano *et al.*, 2008; Frontalini and Coccioni, 2011) and naturally ecologically stressed sites as well as in laboratory culture studies (Alve, 1995, Martínez- Colón and Hallock, 2010; Dolven *et al.*, 2013). The large drop in average size of specimens subjected to 20 mg/l calcein, at 25°C after ~80 days of incubation, is due to the mortality of all large specimens; only small specimens remained alive..

The presence of predominantly normal specimens in both the control and treatment sets till 3-4 weeks of incubation at both temperatures, suggests that short-term exposure to calcein with concentrations as high as 20 mg/l does not induce any abnormality in benthic foraminifera. However, the continued absence of abnormal specimens in the control sets contrasting with a large percentage of abnormal specimens in treatment sets after 4-5 weeks of incubation at both 25°C and 27°C temperature, suggests that the prolonged exposure to calcein (even as low as 5 mg/l), can be detrimental to foraminifera. In fact, although the size of specimens incubated in 5 mg/l and 10 mg/l calcein concentration was not statistically different at the two different temperatures, the percentage of abnormality was higher in the specimens incubated at 27°C temperature. The lower proportion of abnormal specimens at 15 mg/l and 20 mg/l calcein concentration at 27°C temperature, may be due to increased mortality. Instead of adding new (anaomalous) chambers, many of the specimens died.. The type of abnormalities, which included chambers of size much larger or smaller than the preceding chambers or newly added chambers being oriented in a plane different than the normal plane of addition of previous chambers, are the same as those noticed in benthic foraminifera from both polluted (See Nigam *et al.*, 2006 for review; Romano *et al.*, 2008; Frontalini and Coccioni, 2011) as well as naturally ecologically stressed environments (range of extreme salinity, temperature, pH) (Boltovskoy *et al.*, 1991; Alve, 1995, Stouff *et al.*, 1999; Geslin *et al.*, 2002; Martínez-Colón and Hallock, 2010; Dolven *et al.*, 2013). Laboratory experiments with pollutants also showed a development of similar abnormal specimens (Saraswat *et al.*, 2004; Le Cadre and Debenay, 2006; Nigam *et al.*, 2009). A few other papers report, however, that presence of pollutants may not always lead to abnormalities in foraminifera (Alve and Olsgard, 1999; Ernst *et al.*, 2006; De Nooijer *et al.*, 2007). We infer that foraminifera can survive under calcein concentrations up to 10 mg/l (as evident from growth comparable with that of control specimens) but they are probably stressed (as indicated by the development of a relatively large proportion of abnormal chambers), so that long exposure to calcein will result in the development of many abnormal tests.

4.2 Effect of calcein on mortality in benthic foraminifera

The absence of significant differences in mortality between the control and treatment sets until 6-7 weeks of incubation for both the temperatures suggests that benthic foraminifera can tolerate short-term exposure to calcein. However, a two to three times higher mortality after 12 weeks of incubation in all the calcein treatment sets as compared with control sets incubated at both 25°C and 27°C temperatures, suggests that prolonged exposure of foraminifera to calcein is detrimental. Some specimens (at 5-10 mg/l calcein) developed abnormalities before dying while others, especially at 15-20 mg/l calcein, died without becoming abnormal. This suggests that low calcein in the ambient environment affected a few physiological processes in such a way that benthic foraminifera responded by not being able to add chambers in a normal manner, while the prolonged exposure to high calcein concentrations was fatal for many individuals. Normal growth and reproduction was however reported in specimens of *Rosalina vilardeboana* incubated in 10 mg/l calcein for 24 days (Bernhard *et al.*, 2004), suggesting a species specific response to calcein. In view of this, we feel that the susceptibility to calcein may vary from species to species.

4.3 Effect of calcein on reproduction in benthic foraminifera

Although benthic foraminifera can grow in a wide variety of conditions, they reproduce only within a narrow range of ecological conditions (Boltovskoy and Wright, 1976). Even a minor temperature change, of a few degrees Celsius, can cause a drastic change in reproduction frequency as well as the number of juveniles produced by each individual (Saraswat *et al.*, 2011). The large difference in reproduction in the control sets at the two different temperatures further supports the above findings. Therefore, it is possible that a part of the change in percentage of specimens reproducing in the calcein treatments at the two different temperatures reflects the effect of temperature. The strongly reduced reproduction at ≥ 15 mg/l calcein treatment sets at both 25°C and 27°C temperatures, however, suggests that persistent exposure to high calcein concentrations affects reproduction and thus the physiology of the benthic foraminifera. We would however like to mention that the findings are based on a limited number of specimens and therefore should be viewed with a caution.

A few of the findings of this experiment support previous studies while others are in contrast with previous studies. Earlier, Bernhard *et al.* (2004) directly investigated the effects of calcein on some aspects of foraminiferal performance. They incubated foraminifera in 10 mg/l calcein for 3 weeks and found no test abnormalities in chambers deposited in calcein or in foraminifera moved to calcein free seawater. Survival rates were similar in control and calcein treatments and specimens treated with calcein reproduced during calcein exposure or afterwards. We also report the absence of

significant differences in size, abnormality, mortality and reproduction during the initial few weeks of incubation in different calcein media. Our findings also support the work by Hintz *et al.* (2006), who used the same procedure to stain *Bulimina aculeata* and *Rosalina vilardeboana* for 2-3 weeks; both species went on to reproduce prolifically in the culture system. Similarly, Allison *et al.* (2010) stained *Elphidium williamsoni* with 20 mg/l for 3-4 weeks and noticed no difference in survival. Some test abnormalities (usually confined to a single chamber) can be seen in the SEM micrographs presented by Allison *et al.* (2011), but it is not clear if these abnormalities reflect the effect of calcein or if they reflect the mechanical handling of the specimens when the foraminifera were transported into and out of the calcein stain. In view of these results, it seems most likely that the only relevant difference between this experiment and previous work is the duration of the experiment.

A visual examination of specimens incubated at different calcein concentrations revealed increasing fluorescence of the tests at higher calcein concentrations. This was also reported for cockles (Mahé *et al.*, 2010). We found that the sutures incorporated relatively more calcein and fluoresced brighter as compared to the rest of the chambers. Bernhard *et al.* (2004) also reported increased fluorescence near sutures of *Bulimina aculeata*. The absence of a distinct change in fluorescence intensity of the organisms incubated at the same calcein concentration but at different temperatures, suggests that though the adverse effect of calcein at different temperatures was different, the amount of calcein incorporated in shells does not vary with temperature.

5 Conclusions

Based on the laboratory culturing of benthic foraminifera in different calcein concentrations, we infer that short-term (5-6 weeks) exposure to calcein concentrations as high as 20 mg/l is non-lethal and does not cause any perceptible adverse effect. However, exposure for longer periods of time, even to concentrations as low as 5 mg/l, causes increased mortality, test abnormalities and hampers reproduction. Therefore we recommend short-term exposure of benthic foraminifera to calcein concentrations up to 20 mg/l. Conversely, long-term exposure, even to low calcein concentrations, should be avoided. These findings have a strong bearing on the application of calcein spiked media in laboratory culture studies. On the basis of this culture experiment, we suggest that it is much better to use previously calcein tagged foraminifera in experiments (incubation of foraminifera in the calcein for a couple of weeks to obtain tagged specimens, and conducting the experiment in seawater without calcein) than incubating foraminifera in calcein spiked sea water throughout the experiment.

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Legend to Figures and Tables:

- Figure 1: Increase in test size through time, at different calcein concentrations, for 25°C and 27°C temperature. Due to a small number of surviving specimens towards the end of the experiment, the size represents the average of all specimens, taken together. Only the living specimens were considered to calculate the average size. The number of specimens living at any given time, which is used to calculate the average size is given in the supplementary online material table. The drop in average size in the later stages of the experiment is due to reproduction or death of the large specimens. Specimens that either reproduced or died were not considered to estimate average size.
- Figure 2: Fluorescence in typical specimens after incubation without calcein (A- 25°C and F- 27°C) and in different calcein media. B- 5 mg/l, 25°C; C- 10 mg/l, 25°C; D- 15 mg/l, 25°C, E- 20 mg/l, 25°C; G- 5 mg/l, 27°C; H- 10 mg/l, 27°C; I- 15 mg/l, 27°C; J- 20 mg/l, 27°C. The specimens subjected to 20 mg/l calcein concentration were distinctly abnormal. All the photographs were taken after incubating the specimens for 62 days, except A which was taken after 44 days of incubation. All specimens have added a minimum of 5-6 chambers, as the juveniles had only 3-4 chambers when subjected to experimental conditions. The white scale bar is 100 µm.
- Figure 3: The percentage of abnormal specimens in control and different calcein concentration for both 25°C and 27°C temperature. The total number of abnormal specimens (both living as well as dead) at each concentration, during the course of entire experiment, was used to calculate percentage of abnormal specimens. The error bar indicates the standard deviation calculated from the percentage of abnormal specimens in replicate sets.
- Figure 4: Abnormal specimens at different calcein concentrations. Specimens A, B, C and E were photographed after 98 days, while D and F after 62 days. The abnormalities included chambers bigger than the normal size or newly added chambers being oriented in a plane, other than the normal horizontal plane of chamber addition.
- Figure 5: The percentage of specimens that died over the course of the experiment. Only specimens that died without reproducing, for each concentration, were included in the mortality percentage. Specimens that died as a result of reproduction were not included. The error bar (multiplied by a factor of 0.5 for clarity) indicates the standard deviation calculated from the percentage of dead specimens in the two replicate sets.

- Figure 6: The total percentage of specimens that died during the experiment in both control as well as treatment sets at both 25°C and 27°C temperature. Specimens that died as a result of reproduction, were not included. The error bar indicates the standard deviation calculated from the percentage of dead specimens in the two replicate sets.
- Figure 7: The percentage of specimens in both control as well as treatment sets at both 25°C and 27°C temperature, that reproduced during the experiment. The total number of specimens that reproduced at each concentration, during the course of entire experiment, was used to calculate reproduction. The error bar indicates the standard deviation calculated for the two replicate sets. The absence of bar indicates no reproduction.
- Table 1: Weekly observations of average size (μm) and standard deviation (SD) for the control and treatment sets (both replicate sets taken together) at 25°C and 27°C.
- Table 2: Percentage of abnormal specimens with its SD in control and treatment sets at 25°C and 27°C.
- Table 3: Weekly mortality (%) and its SD throughout the experiment in all sets at 25°C and 27°C.
- Table 4: Average reproduction (%) with its SD in control and treatment sets at 25°C and 27°C.
- Supplementary Table: Weekly measurements of diameter, state of individuals (living or dead) and details of t-Tests conducted to assess the significance of differences.

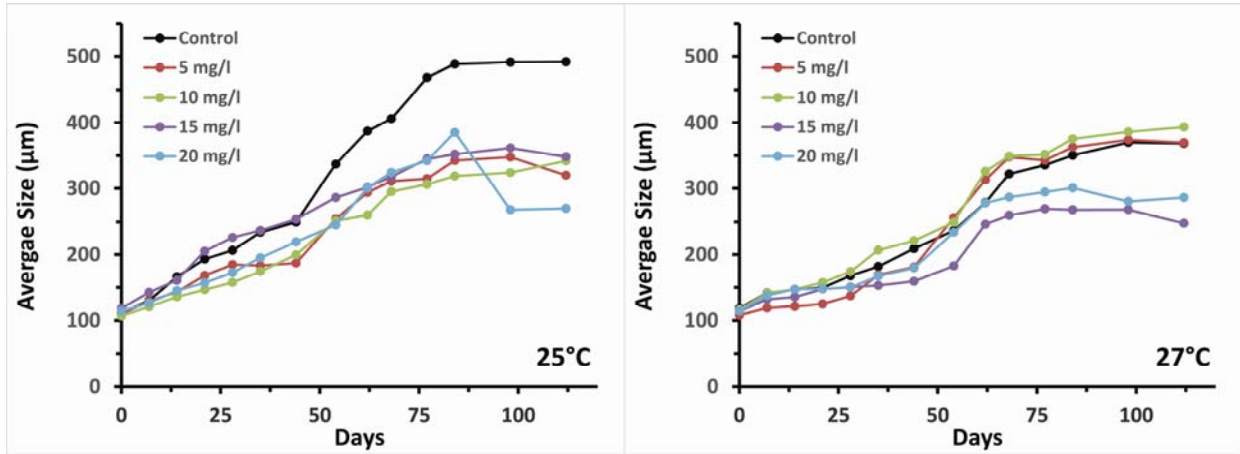


Figure 1

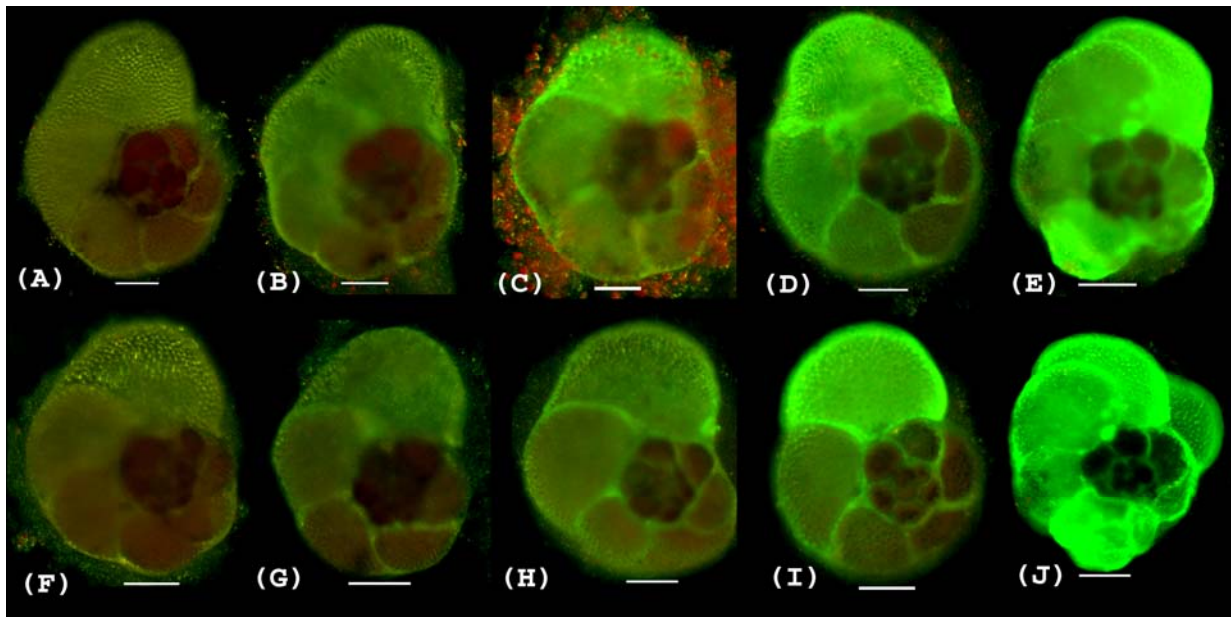


Figure 2

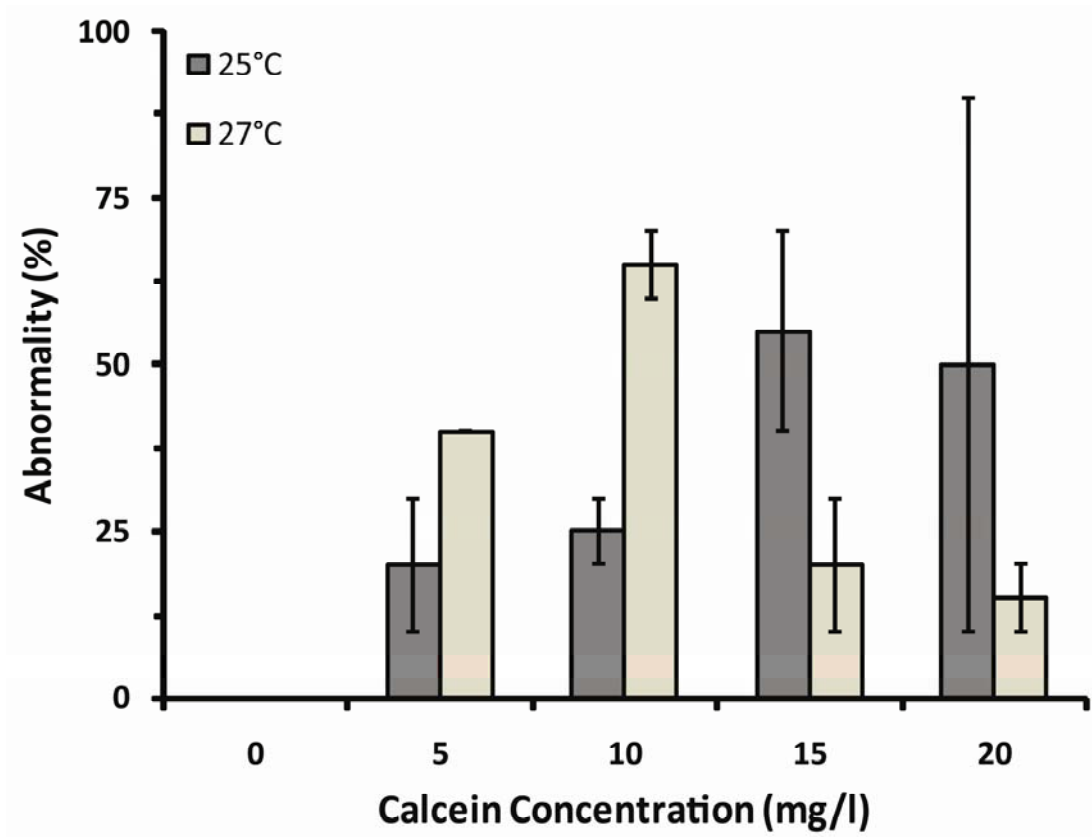


Figure 3

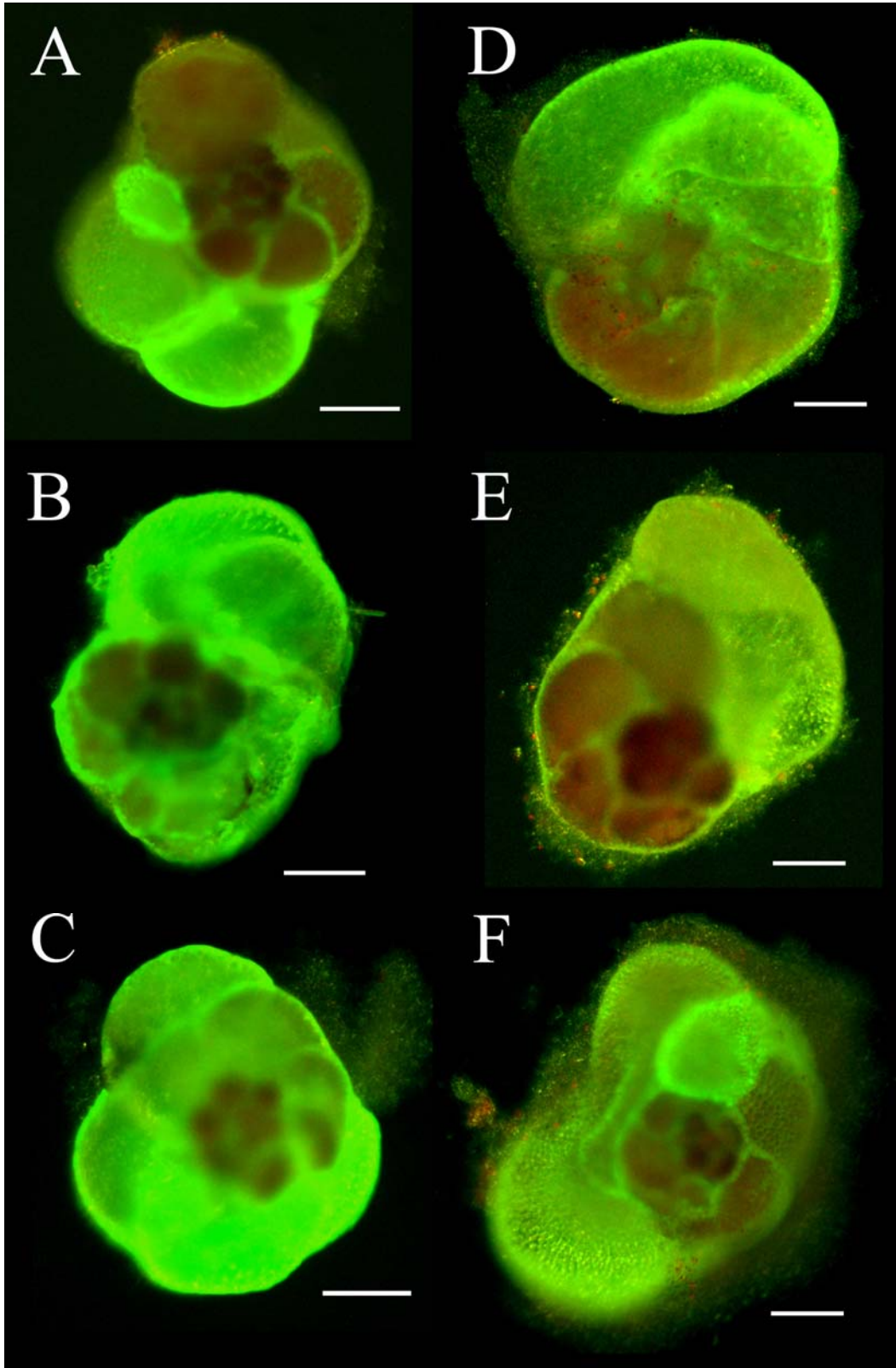


Figure 4

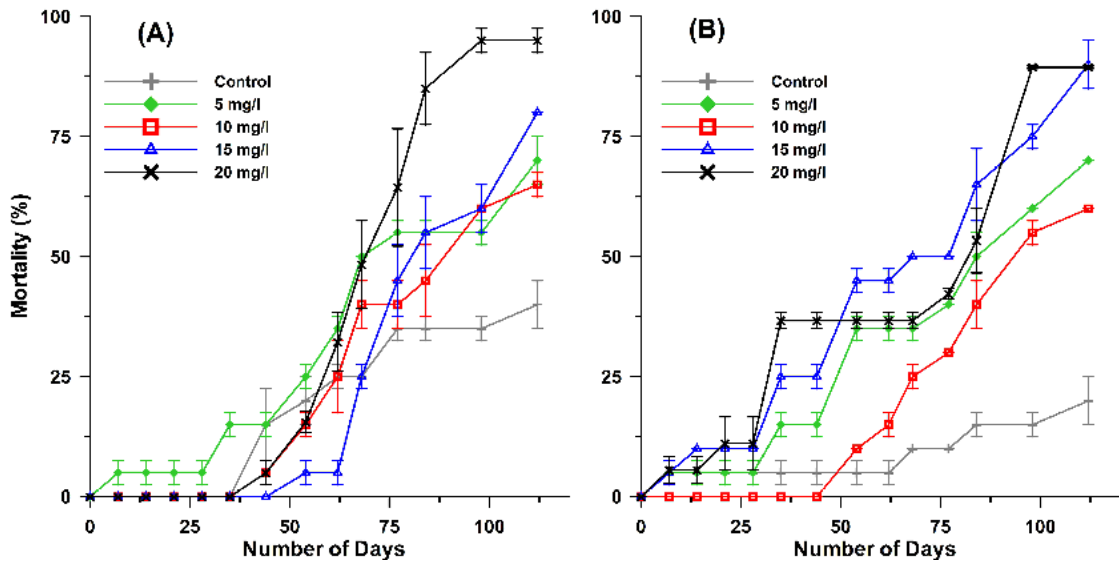


Figure 5

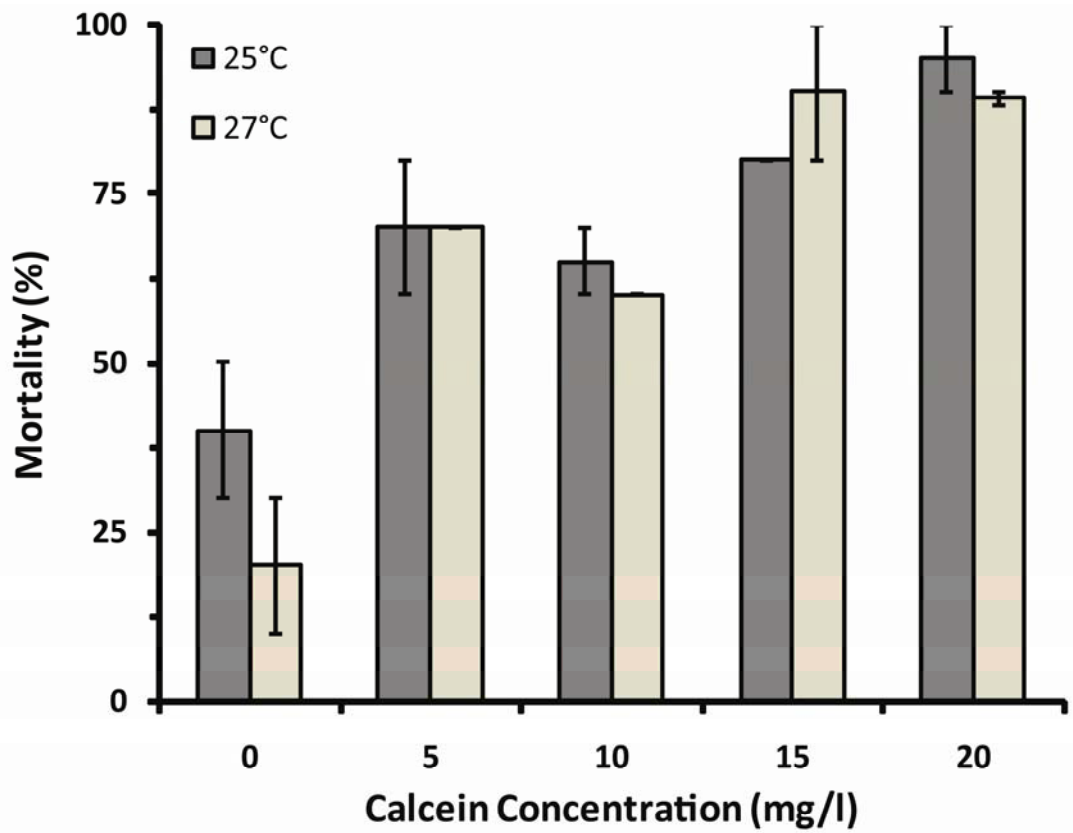


Figure 6

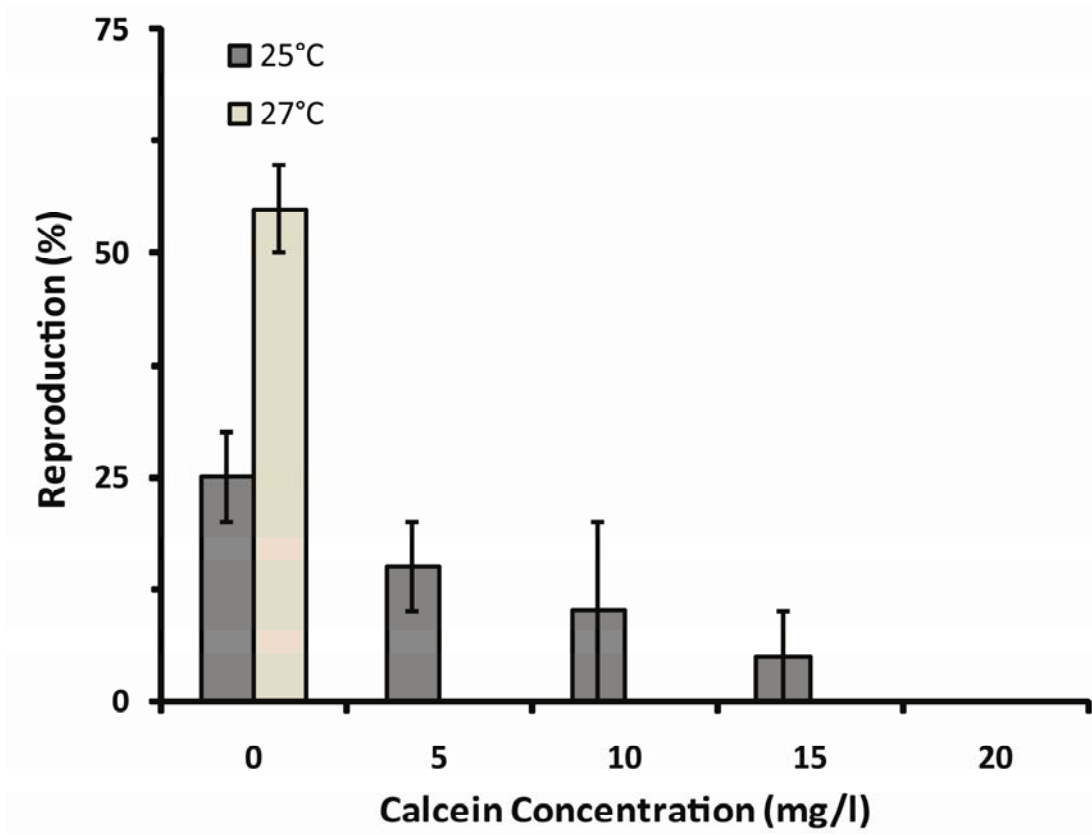


Figure 7

Table 1

Temperature 25°C										
Day	Average Size (µm)					Standard Deviation (µm)				
	Control	5 mg/l	10 mg/l	15 mg/l	20 mg/l	Control	5 mg/l	10 mg/l	15 mg/l	20 mg/l
0	111.2	109.5	106.9	117.8	113.9	15.3	19.6	17.1	12.2	14.6
7	129.7	128.6	120.6	142.3	125.8	23.8	24.3	27.8	23.2	22.3
14	165.8	143.2	135.4	160.6	145.0	38.4	34.0	37.0	32.0	33.7
21	193.2	167.9	146.5	205.2	156.8	49.8	46.2	37.0	44.2	38.4
28	206.4	184.6	157.5	226.1	172.3	60.4	52.0	40.9	44.4	43.7
35	233.8	183.1	174.0	236.8	195.2	65.2	53.1	42.2	48.3	45.6
44	249.8	187.1	199.7	254.1	219.3	73.1	52.6	49.1	46.2	42.6
54	336.9	254.5	251.8	286.4	245.2	99.2	49.9	57.3	59.7	65.8
62	387.3	294.1	260.1	301.6	300.3	109.8	50.6	60.0	68.3	104.8
68	406.1	310.6	295.0	317.3	323.9	110.3	78.3	50.8	68.6	112.8
77	468.1	314.3	305.9	344.7	342.1	65.7	88.6	43.0	63.4	85.1
84	488.6	342.5	318.4	351.8	385.3	55.0	76.5	44.0	67.3	84.9
98	491.1	347.7	323.7	361.3	268.0	56.1	75.2	43.5	69.3	na
112	491.7	319.7	341.8	348.0	270.0	59.7	74.1	28.9	62.0	na

Temperature 27°C										
Day	Average Size (µm)					Standard Deviation (µm)				
	Control	5 mg/l	10 mg/l	15 mg/l	20 mg/l	Control	5 mg/l	10 mg/l	15 mg/l	20 mg/l
0	117.7	108.0	115.8	113.6	114.3	12.4	16.3	17.0	14.8	9.8
7	141.4	118.7	141.3	131.5	137.5	23.8	17.5	20.2	20.1	20.2
14	146.6	121.2	146.9	135.1	147.2	26.7	17.4	24.4	18.3	28.5
21	149.3	124.5	157.5	147.2	147.7	26.9	19.3	30.8	23.5	32.4
28	167.6	136.9	173.8	150.6	149.8	40.0	32.2	36.0	23.4	37.0
35	181.8	168.8	206.9	152.7	167.1	31.8	39.6	28.3	19.7	40.6
44	209.1	180.8	221.1	159.1	179.3	54.5	44.5	32.8	19.2	43.5
54	236.6	256.0	249.3	182.5	233.9	74.5	58.7	41.2	27.1	40.0
62	278.8	312.5	325.7	246.4	278.3	70.4	74.3	37.5	46.2	37.0
68	321.6	347.5	348.8	259.9	286.8	60.6	71.1	39.4	53.8	34.7
77	335.4	342.7	351.4	269.4	294.6	59.8	72.1	42.0	54.3	34.7
84	350.3	362.6	375.3	268.0	300.6	54.8	63.0	32.9	53.9	32.6
98	369.7	373.8	386.0	268.2	280.5	51.3	66.2	11.6	39.2	12.5
112	368.4	369.5	393.5	248.0	286.5	55.4	60.5	33.8	6.0	15.5

na = The SD could not be calculated as only single individual was living.

Table 2

Calcein Conc. (mg/l)	Abnormal Specimens at 25°C (%)	Std. Dev.	Abnormal Specimens at 27°C (%)	Std. Dev.
0	0	0	0	0
5	20	10	40	0
10	25	5	65	5
15	55	15	20	10
20	50	40	15	5

Table 3

Temperature 25°C										
Day	Mortality (%)					Standard Deviation (%)				
	Control	5 mg/l	10 mg/l	15 mg/l	20 mg/l	Control	5 mg/l	10 mg/l	15 mg/l	20 mg/l
0	0	0	0	0	0	0	0	0	0	0
7	0	5	0	0	0	0	5	0	0	0
14	0	5	0	0	0	0	5	0	0	0
21	0	5	0	0	0	0	5	0	0	0
28	0	5	0	0	0	0	5	0	0	0
35	0	15	0	0	0	0	5	0	0	0
44	15	15	5	0	5	15	5	5	0	5
54	20	25	15	5	16	10	5	5	5	4
62	25	35	25	5	32	5	5	15	5	12
68	25	50	40	25	48	5	0	10	5	18
77	35	55	40	45	64	5	5	10	15	24
84	35	55	45	55	85	5	5	15	15	15
98	35	55	60	60	95	5	5	10	10	5
112	40	70	65	80	95	10	10	5	0	5

Temperature 27°C										
Day	Mortality (%)					Standard Deviation (%)				
	Control	5 mg/l	10 mg/l	15 mg/l	20 mg/l	Control	5 mg/l	10 mg/l	15 mg/l	20 mg/l
0	0	0	0	0	0	0	0	0	0	0
7	5	5	0	5	6	5	5	0	5	6
14	5	5	0	10	6	5	5	0	0	6
21	5	5	0	10	11	5	5	0	0	11
28	5	5	0	10	11	5	5	0	0	11
35	5	15	0	25	37	5	5	0	5	3
44	5	15	0	25	37	5	5	0	5	3
54	5	35	10	45	37	5	5	0	5	3
62	5	35	15	45	37	5	5	5	5	3
68	10	35	25	50	37	0	5	5	0	3
77	10	40	30	50	42	0	0	0	0	2
84	15	50	40	65	53	5	10	10	15	13
98	15	60	55	75	89	5	0	5	5	1
112	20	70	60	90	89	10	0	0	10	1

Table 4

Calcein Conc. (mg/l)	Average Reproduction at 25°C (%)	Std. Dev.	Average Reproduction at 27°C (%)	Std. Dev.
0	25	5	55	5
5	15	5	0	0
10	10	10	0	0
15	5	5	0	0
20	0	0	0	0