First results of a long-term cultivation experiment of different species of Nummulitidae (Foraminifera) from the island of Sesoko (Okinawa, Japan)

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Abstract

Many experimental culture studies have been carried out on larger benthic foraminifera to improve their use in paleoceanography, paleoclimatology or environmental monitoring. However, understanding how to culture larger benthic foraminifera over longer periods of time (several months to years) without deficiencies is limited. Observations of sexual reproduction, in particular, are limited in laboratory setting, let alone the full alteration of generations. In this study, we present the results of a successful long-term cultivation of four species of larger benthic foraminifera, enabling individual recognition (i.e. the traceability of single individuals). *Operculina complanata*, *Palaeonummulites venosus*, *Heterostegina depressa* and *Cycloclypeus carpenteri* were sampled and cultured over a period of 15 months, with the aim of optimizing growing conditions, and approximating changing environmental conditions measured in the field as closely as possible.

Keywords: long-term cultivation, growth strategy, growth rate, larger benthic foraminifera

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Introduction

Larger benthic Foraminifera (LBF) are used in various applications. They are index fossils in stratigraphy (e.g., Cahuzac and Poignant 1997; Serra-Kiel et al. 1998; Molina et al. 2016), as well as proxies for a variety of paleoenvironmental studies, including biological monitoring (Hallock 2012 and references therein). Studies estimate that they contribute ~5% of the total carbonate production in the world's reef and shelf areas, and produce ~0.5–2.5% of the total carbonates in the world's

ocean (Langer 2008, Smith and Mackenzie 2015).

With increasing effects of global warming, ocean acidification, and eutrophication on marine coastal ecosystems, experimental studies of benthic symbiont-bearing foraminifera have become prominent in the scientific literature. Despite the fact, that benthic foraminifera have been cultured since the 19th century (e.g., Gervais 1847; Schultze 1856, 1860; Schaudinn 1894; Röttger 1972; Muller 1974), relatively little information has been added to the understanding of theoretical niches in various species, as opposed to the vast amount of information on the realized niches that has been gathered from distribution and community studies of living benthic foraminifera.

Since the 1980s, a growing number of short-term cultivation experiments have revealed important effects on growth, metabolism and calcification of benthic foraminifera by a number of parameters such as temperature, pH, salinity/conductivity, photosynthetically active radiation, or oxygen content (e.g., Talge and Hallock 2003, Williams and Hallock 2004; Fujita et al. 2011; Schmidt et al. 2011, 2014; Prazeres et al. 2015). An early study by Hallock et al. (1986) reported asymptotic growth responses of *Amphistegina* spp. over time spans of 3–14 months under different artificial light intensities and water motion. However, some experiments have reported shortened live spans, disturbed metabolism, or growth abnormalities. Schmidt et al. (2011) recorded reduced motility in a six-day incubation experiment of *H. depressa* in all but the 32°C group. They also report reduced growth rates for both *A. radiata* and *H. depressa* at all temperatures in comparison to studies using natural light (Uthicke and Altenrath 2010). Haynert et al. (2014), in six month investigation of benthic foraminifera kept in natural sediment at different pCO_2 levels, showed significant decrease of living specimens in the control group, but even a slight increase in population density at 1856 µatm. Even investigations on foraminifera from long-term cultivations, which have successfully reproduced asexually, and thus survived several generations, show significant abnormalities (Röttger 1972; Eder et al. 2016). Furthermore, successful sexual reproduction and the verification of a trimorphic lifecycle has yet to be achieved in the cultivation of benthic foraminifera (Lehmann et al. 2006).

Linshy et al. (2007) presented a detailed list on the history of cultivation experiments, from Meyers (1935) to Grimm et al. (2007). Difficulties in reproducing suitable environments arise from various factors, especially in setups where individual recognition is of importance. When populations are investigated in mesocosms (e.g., Alve and Bernhard 1995; Frontalini and Coccioni 2012) coupled with flow-through systems, they best approximate the natural environment. However, flow-through systems are only feasible in locations with a direct connection to seawater pumps collecting seawater from the organism's place of origin. To retrieve individual foraminifera for observation, they can be kept in small enclosures, for example Petri dishes, which provide them a stable environment.

Keeping smaller Petri dishes in a larger tank with stabilised water quality means exposure to reduced water currents, possibly a stressor to certain shallow reef species. Schmidt et al. (2014) used 6-well cell-culturing plates with flow-through lids attached to a pump to mimic natural conditions of wave flow, but reported loosing up to 13% of the specimens. Fujita et al. (2011) used cell strainers (culture cages) attached to transparent vinyl hoses and placed into larger water baths with suction cup and did not report any losses.

Another major problem seems to be adequate lighting and sufficient control over different wavelengths. Stomp et al. (2007) have shown the importance of prevailing wavelengths on annidation of phototrophic organisms. For symbiont-bearing foraminifera coexisting in the same habitat, this factor is presumably of particular importance. Submersible spectroradiometers for UV/VIS offer adequate control, but are costly. Prazeres et al. (2015) and Nobes et al. (2008) suggest that better growth can be achieved by using natural sunlight in combination with shading cloth to reduce light intensities to the desired values. Unfortunately, keeping tanks outside means either significantly reducing the control over the temperature, or turning it into a major cost factor.

To evaluate the quality of a cultivation experiment (i.e., the growth of foraminifera), it must be constantly compared to results retrieved from field observations; concerning growth studies, this can be best achieved by a comparison with the natural laboratory method (Hohenegger et al. 2014).

This work presents a successful experiment of long-term cultivation with daily observation of more than 550 individuals, and their constant comparison with the natural environment obtained by monthly sampling. The main goals of this work are the estimation of the lifespan, growth rate, and reproduction strategies of the larger foraminifera *Heterostegina depressa*, *Palaeonummulites venosus, Operculina complanata* and *Cycloclypeus carpenteri*.

Material and Methods

Sampling

The sampling area for this work is the small island of Sesoko, northwest Okinawa (see Table 1 for exact locations), the main island of the southernmost prefecture of Japan. Over a period of 15 from 23 April 2014 until 14 July 2015, larger foraminifera were cultivated in two tanks (see Fig. 1a). Each

Table 1 Coordinates and sampling dates of all sampling sites

Site	Date	Latitude	Longitude
01A	2014-04-23	26°40.1433'	127°51.6467'
02A	2014-05-02	26°32.2100'	127°52.4050'
02B	2014-05-02	26°37.2000'	127°51.6350'
03A	2014-05-09	26°40.0390'	127°51.3310'
03B	2014-05-09	26°39.7060'	127°52.2930'
04A	2014-05-30	26°40.2200'	127°51.5160'
04B	2014-05-30	26°39.9089'	127°52.1564'
05A	2014-07-18	26°40.4240'	127°51.5324'
05B	2014-07-18	26°39.9362'	127°52.1641'
06A	2014-08-19	26°40.4231'	127°51.4673'
06B	2014-08-19	26°39.9351'	127°52.1659'
07A	2014-09-10	26°40.2410'	127°51.5281'
07B	2014-09-10	26°39.9091'	127°52.1580'
08A	2014-10-03	26°37.4250'	127°52.2624'
08B	2014-10-20	26°39.9080'	127°52.1612'
09A	2014-11-10	26°37.3511'	127°51.4629'
09B	2014-11-10	26°37.4079'	127°51.5399'
10A	2014-12-11	26°40.218'	127°51.517'
10B	2014-12-11	26°39.9008'	127°52.1523'
11A	2015-01-16	26°40.2142'	127°51.5101'
11B	2014-01-16	26°37.4598'	127°51.8458'
12A	2015-02-13	26°40.1711'	127°51.5076'
12B	2015-02-13	26°37.4445	127°51.8420
13A	2015-03-04	26°40.2670'	127°51.4727'
13B	2015-03-04	26°37.4597'	127°51.8360'
14A	2015-04-15	26°40.2362'	127°51.4540'
14B	2015-04-15	26°37.4950'	127°51.8422'
15A	2015-05-18	26°40.2756'	127°51.5099'
15B	2015-05-18	26°39.9471'	127°52.1600'
16A	2015-06-11	26°40.3148'	127°51.6201'
16B	2015-06-11	26°39.9430'	127°52.1642'
17A	2015-07-14	26°40.1600'	127°51.5144'
17B	2015-07-14	26°39.9160'	127°52.1652'

month, two samples were collected: one from 50 m water depth (sample A) and one from 20 m water depth (sample B). The sampling was conducted, whenever possible, depending on weather conditions, at the beginning of every month. In the first month two additional 'trial samples' were taken to optimize and standardize sampling procedures. Whenever possible, samplings were conducted north of the island, where there are generally higher abundances of foraminifera (Hohenegger et al. 1999) and the best possibility to find the rarest of the target species, *C. carpenteri*.

During winter months some samplings had to be conducted south of the island because northerly winds produced unsafe anchoring and diving conditions north of Sesoko. All samples were collected via Scuba diving. At 50 m depth, four subsamples were taken at four separate points (labelled I, II, III, IV) within ~2 m of each other to eliminate effects of patchy distribution. Each subsample consisted of the top 2 cm of sediment (roughly 0.5 kg each) collected manually into a plastic box. At

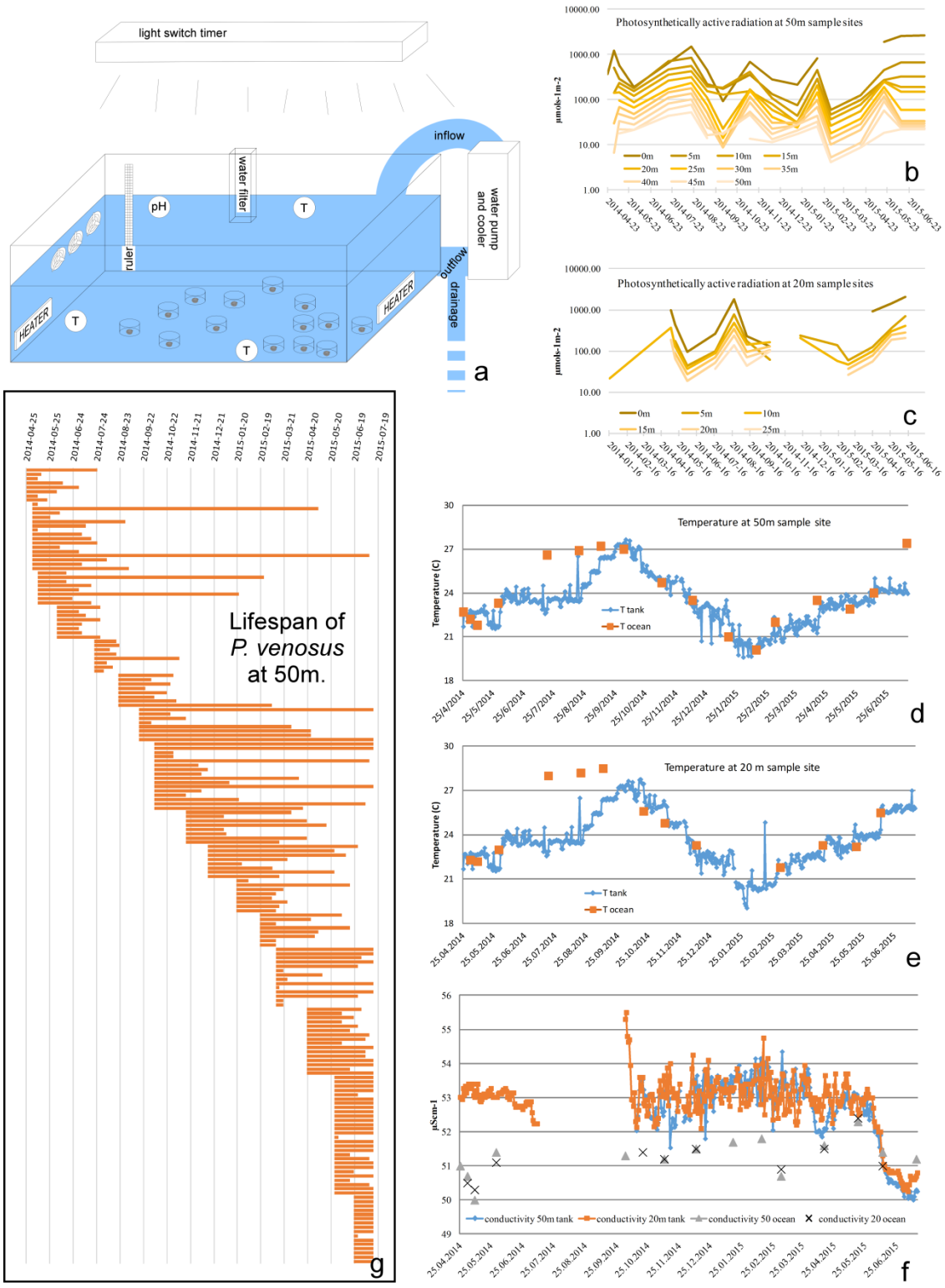

Fig.1 a. Set-up of the cultivation tank, **b**. PAR measurements at 50 m sample sites, **c**. PAR measurements at 20 m sample sites, **d**. Comparison of temperature measurements in tank A and at 50 m sampling site, **e**. Comparison of temperature measurements in tank B and 20 m sampling site, **f**. Comparison of conductivity measurements in tanks A and B and at 50 m and 20 m sampling sites, **g**. Lifespans of cultured specimen of *Palaeonummulites venosus*.

20 m, where sediment is mainly composed of coral rubble, half a bucket of coarse material was collected within a radius of 2 m.

At every sampling, temperature, salinity and photosynthetically active radiation were measured at five meter increments using a WTW Multi350i, combined with a depth conductivity cell TA197-LF and a LI-COR LI-250A light meter with a LI-192 underwater Quantum Sensor. The pH at sample sites was measured for bottom and surface water

Sample preparation

Immediately after collection, the A samples (50 m water depth) were poured into flat sorting trays and carefully washed with seawater, to eliminate the finest, easily suspended clay and silt fraction by decantation. The coral rubble was brushed off using soft toothbrushes and the brushoff was similarly poured into sorting trays. Within two days, all living *H. depressa*, *P. venosus*, *O. complanata* and, if present, *C. carpenteri*, were picked out under an Olympus SZ60 Stereomicroscope. From one tray of the 50 m subsamples, as well as for one tray of the 20 m sample, all living larger foraminifera were picked. The living larger foraminifera from every sample were counted and split into two equal parts. One half was washed in fresh water and dried immediately for later use (not reported here). From the second half, the four largest and four smallest individuals were selected and put into separate glass dishes, labelled, and placed into the aquarium for cultivation. The remainder of the second half sample of each species was put into 'population dishes', and also cultivated in the tanks.

Experimental Set-up

The culturing set-up is shown in Fig. 1a. Two of these set-ups were built for cultivating the foraminifera from different depths. The size of the 50 m tank (for A samples) was 90 x 135 x 30 cm while the tank for the 20 m samples was 60 x 90 x 30cm.

The lighting tubes used in the deep set-up were 2–4 Sudo Carribean Blue S-3420 (20W) and in the shallow set-up Sudo Ocean Clear S-3520 (20W). The tubes were connected to timers set to 10/14 (winter) and 13/11 (summer) light/dark cycles. In each tank, the light intensity was constantly adjusted to the intensities measured in the field every month. They were kept at 30–40 µmol m^{-2} s^{-1} during the first five summer months, but subsequently reduced to 15-20 µmol m^{-2} s^{-1} (Figs. 1b-c). This light reduction occurred after field observations revealed that foraminifera were mainly found 'hiding' under live and dead corals branches, inside rubble cracks and crevices (mainly *H. depressa*), or underneath some grains of sand (mainly *O. complanata* and *P. venosus*).

To control temperature in both tanks, two separate water coolers (GEX GXC-201X 240W) in combination with three (50m)/2 (20m) Nisso IC Auto Neo Type 180 Heaters were used. Temperatures were adjusted to those measured in the field (Fig 1d-e).

Water-characterization

The Tropical Biosphere Research Station Sesoko, where the cultivation experiment took place, has seawater readily available, using a pipeline from a depth of ~10 m in front of the station on Sesoko Island. In addition to unfiltered seawater, filtered seawater, used for this experiment, is also readily available, produced by the stations' filtering system. This filter system consists of two main parts: a piston to crush larger particles sucked into the piping system and a rapid pressure sand filter. The filtered seawater from the tap was further filtered using an ADVANTEC 10517P52 micro pore filter (5µm and 1µm cartridges). Moreover, commercially available aquarium mini filters using customary filter pads containing active charcoal were inserted to improve water circulation within the tanks, as well as to reduce the amount of dissolved organic compounds.

A flow-through system, where water was continuously added and drained from the tanks, optimizing water quality, had to be replaced by frequent slow water changes, to enable better temperature control. Water levels where kept at 15 cm in both tanks and checked three times daily to estimate water evaporation/condensation. Temperature, salinity and pH where checked three times per day (Fig. 1f), and the daily water changes adjusted to ensure the most stable conditions possible. On average, 1/3 of the total water volume was changed daily.

Handling of specimens and analysis

Two individuals of the same species were kept in one Petri dish to reduce the number of dishes in the aquarium while ensuring identification. After 6 months, sieved and sterilized fine sand and coral fragments were added to each Petri dish containing *O. complanata* and *H. depressa*, to enable individuals to hide beneath the sand or the coral to better adjust light absorption: a significant improvement of lifespans and growth rates was registered from this point.

Operculina complanata and, to some extent, *H. depressa* at the beginning of the experiment, tended to be very active within the Petri dishes and eventually escaped (maximum distances of 0.4 m in 6 h for *O. complanata*). Motility was reduced significantly after the introduction of sand and coral fragments into the culturing dishes.

Foraminifera, climbing the walls of Petri dishes, were set back gently using fine brushes. Every four days, pictures of each specimen were taken to document growth/chamber building. An interval of four days was chosen to avoid disturbing the individuals too often, hence disrupting the chamber building process. When possible, pictures were taken during the dark cycle of the aquarium because

the protoplasm was most retracted during the absence of light, noticeable by a clearly paler and patchier colouration during this time (Fig. 2).

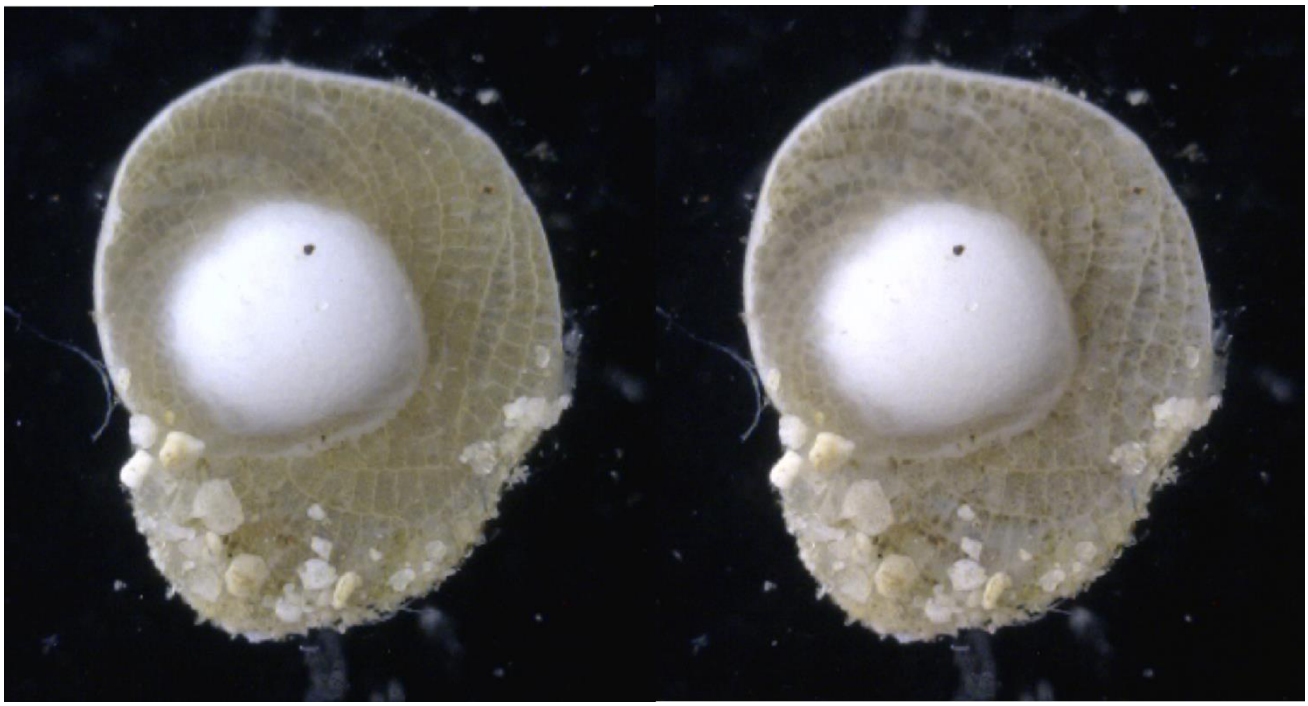

Fig.2 Picture of retracted protoplasm during dark cycle (left) vs. expanded during day cycle (right).

Results and Discussion

Success was mixed with respect to collections and survival in culture. Only 26 specimen of *C. carpenteri* were found in 15-month sampling campaign, suggesting that, in the sampling area, their depth distribution lies well below 50 m (Fujita and Kanda 2015). None of the 26 specimens lived longer than 3 months. *Heterostegina depressa* could usually be found hiding (or partially hiding) beneath coral fragments, while O. *complanata* buried itself in the fine sand. For *P. venosus*, no difference in motility or colouration was noted with sand or coral rubble. Fig. 1g shows the life span of all cultivated and studied specimens of *P. venosus* in the tank of specimens from 50 m; similar data were obtained for the other investigated taxa. The average life time in cultures of *O. complanata* was 77 days; 13 individuals lived more than 200 days, three reproduced asexually and one individual produced gametes. Moreover, 14% of 186 individuals were lost by manipulation, while 22% did not survive for more than one month. For *H. depressa* the average survival in culture was 99 days. Nine individuals reproduced asexually, while 12% were lost. *Palaeonummulites venosus* on average survived 117 days in culture, 5 individuals reproduced asexually, and 10% of specimens were lost. *Palaeonummulites venosus* not only had the longest lifespans, but also survived best in culture, while *O. complanata* seemed most vulnerable and bleached the fastest. While no effort was made to

semiquanitatively record stages of bleaching, we observed that some specimens showed a continuous decrease in coloration intensity. This bleaching was most pronounced in *C. carpenteri*, followed by *O. complanata*, but hardly occurred in *P. venosus* and only rarely in *H. depressa*, and varied greatly among individuals.

Remarkable change in individuals usually occurred 1-2 weeks after being placed in the tank. During these first weeks of culturing even larger individuals of all species except *C. carpenteri* could frequently be found to produce an ectoplasmic sheaths, as described by Röttger (1973); this process seemed impaired later. Since the recognisable cause of the death of specimens seemed to be the overgrowth with mostly red or, rarely, green cyanobacteria, mortality due to impaired protection can be assumed. This would also explain why *Palaeonummulites*, with an extremely smooth test surface, was best protected against the settling of cyanobacteria.

Arnold (1954) described the problems that algae can cause for benthic foraminifera. He and later authors (e.g., Röttger 1972, Krüger et al. 1996, Holzmann et al. 2001) tried to overcome this obstacle by cleaning the foraminifera with fine brushes. In this study, individuals also occasionally had to be cleaned to take pictures and free them from overgrowth. Whenever possible, this was done during the evening hours, as the cleaning procedure can easily destroy very young and not fully calcified chambers, which are usually initiated during the morning hours (Röttger 1972b).

All asexual reproductions observed during the winter period occurred after a reduction of light intensity (i.e., either the removal of a neon tube or the reduction of light hours), while summer reproductions occurred regardless of any environmental variation. This leads to hypothesis, that, in the investigated species, duration of light and dark cycles, as well as temperature, may be important triggers in the coordination of reproduction.

The mean pH in both tanks was 8.26, with standard deviations of 0.06 (50 m) and 0.054 (20 m) that can be assumed constant and consistent with measurements in the field.

Since filtered water has been used for this work, an excess of dissolved organic nutrients in the tank must be assumed due to the excretory products of organisms including large serpulids and bivalves settling within the station's filter system. To avoid clogging due to settling fauna, the system was cleaned once a year. The clean-out revealed the remarkable sizes (some bivalves >20cm) reached in one year due to the constant influx of nutrient-rich water. While flow-through experiments with unfiltered seawater tend to be more costly due to difficulties in temperature regulation, they should always be preferred. Filtered water is recommended for those cases where there is no flow-through, as it avoids algal blooms within the tank system. This inevitably implies changing the chemical and biological composition of the water depending on pore size used, possibly preventing successful long-term cultivation.

While this experiment has given valuable clues and insights about the cultivation of larger benthic foraminifera, further experiments are needed to express the full alternation of generations within an experimental setup. Only then will experimental studies be able to determine boundaries of theoretical niches for the different species. This, in turn, is crucial for a better understanding of the role of competition among species as well as assessing the impacts of rapid changes in many marine environments of coastal areas.

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