6-18-2004

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Publication Info

Published in *Limnology and Oceanography: Methods*, Volume 2, Issue 6, 2004, pages 160-170.

Hintz, C. J., Chandler, G. T., Bernhard, J. M., McCorkle, D. C., Havach, S. M., Blanks, J. K., & Shaw, T. J. (18 June 2004). A physiochemically constrained seawater culturing system for production of benthic foraminifera. *Limnology and Oceanography: Methods*, 2(6), 160-170.
http://dx.doi.org/10.4319/lom.2004.2.160

LIMNOLOGY
and
OCEANOGRAPHY: METHODS

Limnol. Oceanogr.: Methods 2, 2004, 160-170

A physicochemically constrained seawater culturing system for production of benthic foraminifera

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Abstract

We present an apparatus and procedure for culturing deep-sea (i.e., bathyal) benthic foraminifera under physicochemically constrained conditions. A 1600-L recirculating culture system was constructed to contribute negligible trace metal contamination; the system was housed in an environmental room maintained near in situ temperatures but at atmospheric pressure. Peristaltic pumps continuously circulated 3 mL seawater min^{-1} from the reservoir to 9 to 12 culture chambers. A 2-mm-thick layer of high-purity clay-sized silica substrate was used to minimize the impact of sedimentary microhabitats. Physicochemical parameters (salinity, alkalinity, pH, temperature) varied <2% throughout two culture experiments. Trace metal concentrations were initially set near open-ocean values and remained constant (i.e., within our analytical precision) during the experiments. Culture seawater was equilibrated with atmospheric CO_2, thus dissolved inorganic carbon $\delta^{13}C$ varied ~1‰ over the course of each experiment, reflecting the seasonal atmospheric CO_2 isotopic variation. Culture seawater $\delta^{18}O$ varied ~0.2‰. Each culture chamber was inoculated with as many as 100 foraminifers of single or multiple species. Pre-existing foraminiferal calcite was identified by fluorescent labeling prior to specimen introduction into culture. The cultures remained viable for >200 d in each experiment and produced up to 2,800 individuals per culture. The growth and reproduction of benthic foraminifera in a physicochemically constrained culture provide a new method for the experimentation and validation of geochemical proxies.

The chemistry of the calcium carbonate shells (i.e., tests) of certain foraminifera can be used to reconstruct records of past ocean chemistry. This approach is valid for elemental and isotopic proxies that are incorporated into foraminiferal calcite in proportion to their oceanic composition at the time of precipitation (Emiliani 1955; Hester and Boyle 1982; Lea and Boyle 1989). For example, the stable carbon isotope ratio in foraminiferal tests is used as a proxy of past ocean circulation, productivity, and carbon cycling (e.g., Shackleton 1977; Belanger et al. 1981; Curry and Lohmann 1982; Duplessy et al. 1984, 1988; Zahn et al. 1986). The incorporation of the nutrient proxies barium and cadmium into the calcium carbonate of planktic and benthic foraminifera has been used to reconstruct nutrient distributions in past oceans (e.g., Boyle 1988, 1992; Lea 1995; Lea and Boyle 1989; Rosenthal et al. 1997). Field studies have shown reproducible relationships between foraminiferal trace metal incorporation and ambient seawater concentrations, but also suggest that factors other than temperature and bottom-water tracer composition influence geochemical proxy signatures in benthic foraminifera (Boyle 1988; Lea and Boyle 1989; Mackensen et al. 1993; McCorkle et al. 1995; Ohkouchi et al. 1995).

Foraminiferal culturing techniques offer an opportunity to manipulate and control physicochemical variables during calcification to measure the proxy partition coefficient between ambient seawater and calcite tests. Culturing experiments

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Acknowledgments

We thank the captains and crews of the R/V *Cape Henlopen* and R/V *Oceanus*; Dr. Susan Goldstein, Dr. Andrea Habura, Dr. Ken Hintz, Adriana Bejarano, Greg Cane, Tawnya Cary, Christina Durham, Eddie Majzlik, Travis Meador, Natalie Monacci, Ellen Roosen, and Alicia Salizar who aided in sample collection; and Michelle Blickley for her assistance with sample analysis. We thank Dr. Dan Schrag for his measurement of seawater $\delta^{18}O$. We also wish to thank David Thistle and two anonymous reviewers for their suggestions on an earlier version of this manuscript. This work was supported by NSF OCE-9504793 and OCE-9911654.

with planktic foraminifera have begun to delineate factors that may be responsible for the variability observed in foraminiferal proxy signatures (e.g., Delaney 1989; Lea and Spero 1992; Spero et al. 1997; Erez 2003). However, the inability to maintain viable benthic foraminiferal cultures in physicochemically stable conditions has limited the success of culturing experiments on benthic species. Three studies have provided preliminary validation of stable isotope and trace metal proxies in benthic foraminifers, but these studies also demonstrated the need for improved culturing methods (Chandler et al. 1996; Wilson-Finelli et al. 1998; Havach et al. 2001). These culturing studies used natural sediment substrates that contributed to microhabitat effects and diagenetic artifacts. Thus, whereas these experiments were successful in their scope, they were not designed to provide the stringently controlled chemical environment used in planktic foraminiferal studies.

The goal of these foraminiferal culture experiments was to maintain a physicochemically constrained culture environment for a period sufficient for bathyal benthic foraminiferal growth and reproduction. As described in *Foraminiferal culturing system*, a large-volume flow-through culturing system was developed to satisfy these requirements. Results from analysis of foraminiferal calcite are not presented here in lieu of later, more developed discussions (Hintz et al. in prep. unref.). Initial results of *Bulimina aculeata* cadmium, barium, magnesium, and strontium distribution coefficients (D_{Cd}, D_{Ba}, D_{Mg}, D_{Sr}) are consistent with previous culturing (Havach et al. 2001) and intermediate-depth field data (Boyle 1988; 1992), especially from the western Atlantic (Rosenthal et al. 1997; Lear et al. 2002).

Materials and procedures

Foraminiferal culturing system—A recirculating-seawater culture system was built from nonmetallic components (high-density polyethylene [HDPE], polypropylene [PP], silicone, and Teflon® [PTFE]) to minimize potential trace element artifacts. All materials were acid-cleaned in 2 M HCl for at least 24 h and rinsed thoroughly with 18 MΩ-cm E-Pure™ water prior to their use in the system. Trace element backgrounds were determined on component materials using HNO_3 and seawater leachates prior to their incorporation into the system. Leachates were analyzed on a Finnigan Element inductively coupled plasma–mass spectrometer (ICP-MS); no significant variance from background was observed.

The entire culture system was housed in an environmental room (~2 × 4 m, Fig. 1). Four 400-L HDPE cylindrical tanks were used as the seawater reservoir. The tanks were connected in series by a PTFE magnetically coupled centrifugal pump, HDPE and PP tubing, and HDPE and PP fittings to allow mixing of the seawater continuously during the experiments.

Initially, the four 400-L tanks were partially filled with deionized water and Instant Ocean®. Filling the 1600-L reservoir, 480 L of Gulf Stream water was added to the system to result in a salinity of 35‰. This seawater was aerated, mixed, and equilibrated for 1 month at 7°C. Large volumes (~10 L min^{-1}) of high-efficiency particulate air–filtered atmospheric (outdoor) air were continuously pumped into the environmental room, through condensation traps, and then equally dispersed into the four seawater tanks via large (200 cm^3) acid-leached glass air stones. Multichannel peristaltic pumps were used to circulate seawater to culture chambers (2 channels per culture chamber, described below), which housed the foraminifera. Silicone rubber tubing (Silastic® 508-007, 0.16 cm inner diameter, 0.24 cm outer diameter) connected the reservoir, peristaltic pump, and culture chambers. Tubing used to draw water from the reservoirs was plumbed from the peristaltic pump through the tank lid and weighted with a PTFE-coated stir bar to submerge the input 0.5 m below the seawater surface. Return flow from the culture chamber was similarly plumbed through the tank lid and was allowed to drip freely. The pump flow rate was set to 3 mL min^{-1}. In this configuration, a maximum of 24 chambers can be maintained simultaneously; in our two experiments, we maintained 9 and 12 culture chambers, respectively.

In preparation for the experiments to investigate concentration effects on trace element incorporation into shell calcite, barium [Ba] and cadmium [Cd] were set near typical western Atlantic bottom-water concentrations. The Ba concentration in Instant Ocean is elevated (400 to 500 nmol kg^{-1}) compared to natural seawater (~40 nmol kg^{-1} near the collection site), thus a method was developed to remove Ba from artificial seawater using Mn fiber (after the Ra stripping method described in Moore 1976). An Eheim® aquarium filter partially filled with Mn-coated fiber, crushed coral, and activated carbon was connected in line to the reservoir system tubing. The [Ba] was reduced in the culture-system seawater with ~1 week of continuous flow over this mixed-media filter bed. The experimental concentrations were similar to those observed in shallow porewaters of our collection site (0 to 0.5 cm sediment depth; [Ba] = 120 nmol kg^{-1}) and within the range of glacial, interglacial, and modern values (Chan et al. 1977; Lea 1995; Table 1). Likewise, $CdCl_2$ was added to each tank to create a similarly representative cadmium concentration (Boyle et al. 1976; Table 1). Before Experiment 2, more $CdCl_2$ was added to raise [Cd], while a Gulf Stream seawater exchange (480 L) and further Mn fiber stripping lowered [Ba].

Culture chamber design—Culture chambers to house the foraminifers were custom milled from acrylic stock (Fig. 2). The two halves of each chamber were sealed with a Viton® o-ring and held together by three nylon bolts at the outer edge. The culture chamber volume was 19 cm^3. The chamber held a polystyrene cell culture insert (BD Falcon® 3093, 6-well design) suspended above the culture chamber bottom. Seawater was free to flow in and around the cell culture insert. The BD Falcon® cell culture insert base was made of high diffusivity polycarbonate membrane with an 8-μm pore size and 4.7 cm^2 area. The cell culture insert had solid sides that matched a PTFE bushing sealing the culture to minimize foraminiferal

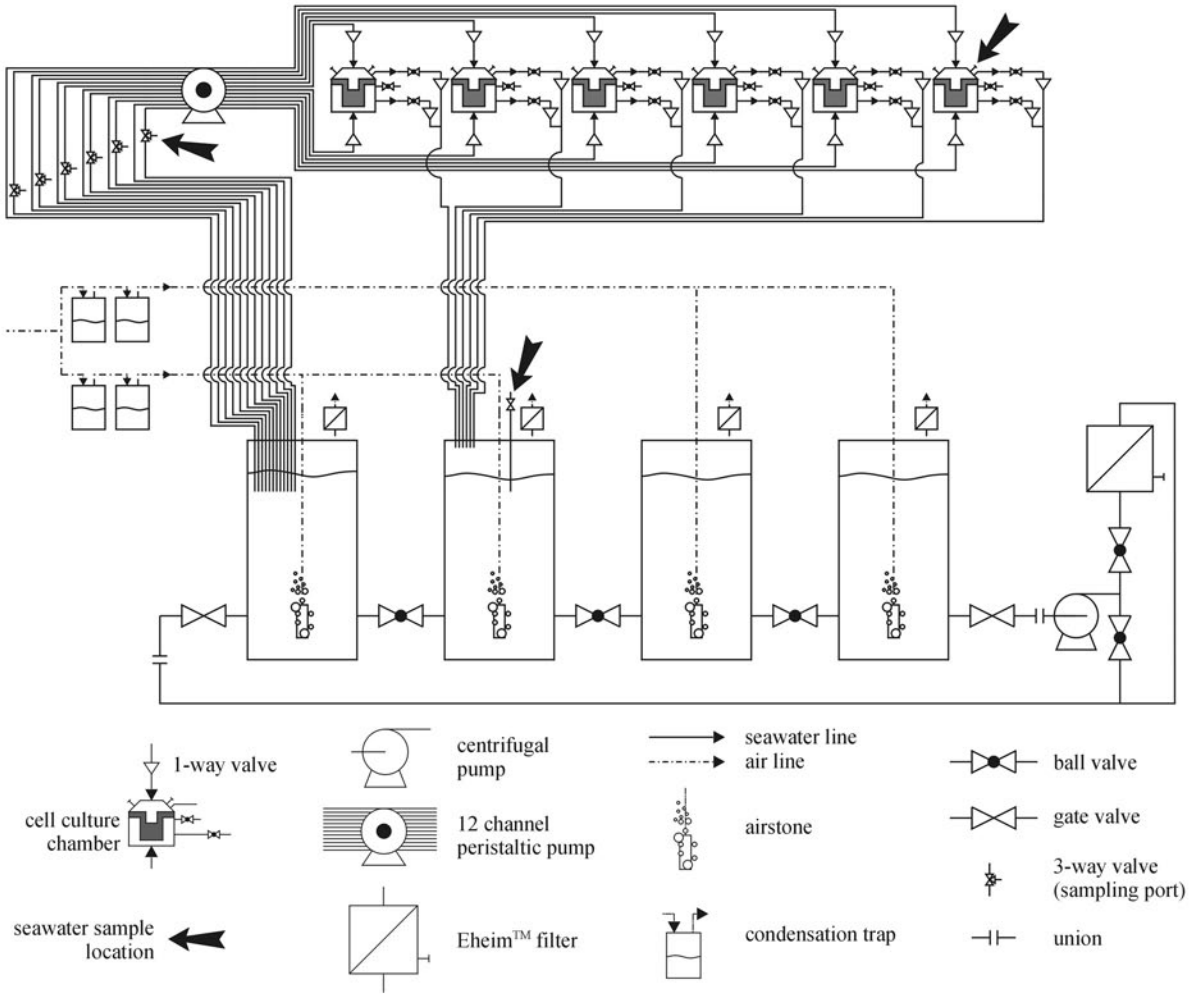

Fig. 1. Benthic foraminiferal culturing system. Only 6 culture chambers are shown for clarity; an additional 3 (Experiment 1) or 6 (Experiment 2) culture chambers were connected to Tanks 3 and 4.

migration out of the culture insert. Polycarbonate Luer-slip stopcocks were positioned vertically at the top and bottom of each chamber to serve as seawater inputs. Two additional stopcocks, which were angled upward to promote entrained air export from the culture chamber, served as seawater outputs. They were capped with female Luer-hose barb adaptors slipped into the silicone tubing connected to the peristaltic pump. Two additional stopcocks were inserted into the top half; these were used to inject food, take water samples, or insert chemical microprobes. These auxiliary stopcocks were closed when not in use. With a flow rate of 3 mL min^{-1}, the seawater residence time was 37 s inside the cell culture insert and 6 min below the cell culture insert.

Selection of culture substrate—Initial experiments demonstrated the need for an alternative culture substrate to natural sediments (Wilson-Finelli et al. 1998; Havach et al. 2001). Culture experiments with various size fractions and types of silica (amorphous silicon dioxide) indicated that clay-sized (~3 µm) 99% silica (Sigma, catalog nr S5631) was suitable for foraminiferal growth and reproduction. When mixed with seawater, the silica formed a fluffy layer similar to natural sediment that was easily separated from foraminiferal adults and juveniles at experiment's end. Silica gels were tested but abandoned due to a tendency to form large aggregates instead of the desired fluffy substrate.

Before the silica was used, it was cleaned extensively and analyzed for release of metals over time. A 1:1 solution of silica suspension in 2 M HCl was agitated for 3 d, centrifuged at 2,000 rpm for 3 min, decanted and resuspended in E-Pure™ water, washed, and centrifuged again. The substrate was similarly agitated in E-Pure™ for 3 d (twice) and in culture system seawater for 7 d (twice), with centrifugation between each solution change.

Table 1. Physicochemical data from the culture experiments*

	Temp. (°C)	Salinity (‰)	Total alkalinity† (meq kg^{-1})		DIC (mmol kg^{-1})	pH	Ba (nmol kg^{-1})	Cd (pmol kg^{-1})	Ca (mmol kg^{-1})	$\delta^{13}C$ (‰ PDB‡)	$\delta^{18}O$ (‰ SMOW§)
Experiment 1	*n* = 38	*n* = 64	*n* = 39	*n* = 35	*n* = 35	*n* = 41	*n* = 21	*n* = 7	*n* = 8	*n* = 8	*n* = 19
Range	6.7-8.5	34.3-35.7	3.18-3.28	3.172-3.187	2.870-2.885	8.062-8.214	104-119	185-380	9.9-10.8	–0.04-0.29	–4.52- –4.27
SD	0.3	0.3	0.02	0.005	0.006	0.027	7	80	0.3	0.08	0.11
Experiment 2	*n* = 34	*n* = 60	*n* = 91	*n* = 17	*n* = 17	*n* = 88	*n* = 68	*n* = 23	*n* = 12	*n* = 39	*n* = 42
Range	6.6-8.4	33.6-36.0	2.99-3.08	2.949-2.964	2.669-2.683	8.079-8.239	94-111	224-370	8.8-10.1	–0.22-0.76	–3.48- –3.27
SD	0.5	0.2	0.02	0.003	0.003	0.011	4	50	0.5	0.05	0.02

*Reported are the range of values measured during each experiment with the number of samples and method precision (as same-day replicate pooled standard deviation [SD]).
†Total alkalinity values reported include small volume (4 mL, left column) and high-precision (100 mL, right column) analyses.
‡PDB = Pee Dee Belemnite.
§SMOW = standard mean ocean water.

The silica was then stored in culture-system seawater until use. ICP-MS measurements on the slurry water indicated that the silica contributed negligibly to Ba and Cd backgrounds.

Field sampling—Three sample sites were visited on two oceanographic cruises to collect foraminifera for culture inoculation. One site, the Charleston Bump (32°N 79°W), which is 120 km southeast of Charleston, South Carolina, has been previously visited to collect benthic foraminifera for culture (Wilson-Finelli 1998; Havach et al. 2001). This site is relatively shallow (220 m) with a bottom-water temperature ~8.9°C. Two deeper sites are located off the North Carolina coast. One site with a water depth of 740 m and bottom-water temperature of ~4.8°C is 65 km east of Cape Hatteras (35°N 75°W). The other site has a water depth of 1,020 m, bottom-water temperature of ~4.4°C, and is located 190 km southeast of Cape Fear (33°N 76°W). Each site was sampled 8 to 10 times (by Soutar box core, see description in next paragraph) for bulk foraminifer collection. Three and seven multi-core deployments at the Charleston Bump (2001) and Cape Fear (2002) sites, respectively, collected undisturbed shallow sediments for porewater and live foraminifer samples, as determined with the vital probe Cell Tracker Green™ CMFDA (5-chloromethylfluorescein diacetate; Molecular Probes).

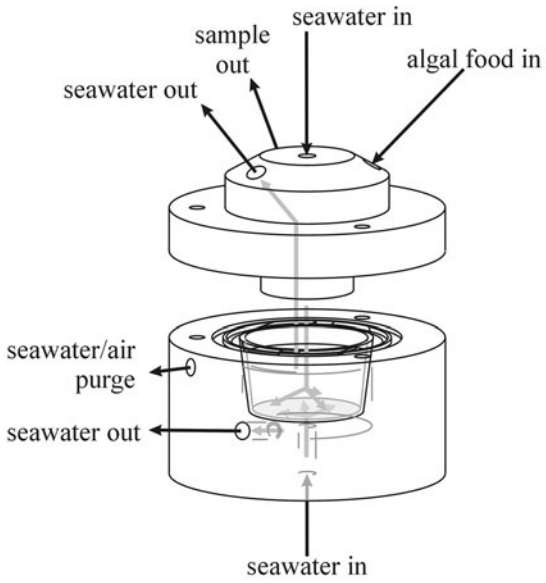

Fig. 2. Schematic of culture chamber and flow pattern. The PTFE bushing is omitted for clarity.

Within minutes of recovery, the surface ~3 cm of sediment from a Soutar box core was siphoned onto nested stainless steel sieves (500 µm, 125 µm, 75 µm). These sediments were washed with chilled bottom water that was previously collected from the same site. In general, the 125 to 500 µm fraction was kept, but occasionally the >500 µm and 75 to 125 µm fractions were also kept. Each size fraction was transferred to a Tupperware® container (~25 × 12 × 3 cm) and maintained at ~5°C for the remainder of the cruise and during transport to the laboratory. After returning to the laboratory, these sieved-sediment cultures were maintained following methods described by Chandler and Green (1996) and Chandler et al. (1996). These stock cultures provided foraminifers for experiments using the trace-metal–controlled recirculating culture system. Stock cultures were maintained for ~1 to 3 months prior to experimental initiation.

Measurement of physicochemical parameters—The physical and chemical seawater parameters (temperature, salinity, pH, total alkalinity [small volume and high-precision methods], dissolved inorganic carbon, $\delta^{13}C$, $\delta^{18}O$, [Ba], and [Cd]) were measured in each of the four tanks prior to and during each experiment. Sample collection ports were connected to tubing directly exiting from each tank. Tanks 2 and 4 had sample ports located in line with the inlet tubing to the multi-channel peristaltic pumps. Tanks 1 and 3 had sample ports located just outside the tank lid with no other connection to the culturing system. A small set of seawater samples from each experiment (~1% of total) was collected directly from sample ports on

each culture chamber to verify that in-culture conditions were not significantly different from the bulk seawater conditions.

System salinity was measured weekly with a Wescor 5100C vapor pressure osmometer. An Orion 520A pH meter and Ross combination electrode standardized with NIST buffers were used to measure the pH of each tank several times weekly. Small-volume total alkalinity (SVTA, 4 mL) was measured weekly by Gran titration (Gran 1952; Gieskes et al. 1991) standardized against gravimetric sodium bicarbonate standards.

Seawater $\delta^{13}C$ samples, collected biweekly, were kept on ice and immediately sealed in prepoisoned glass ampules under ultra-high purity nitrogen. $\delta^{18}O$ samples, also collected biweekly, were sealed in glass scintillation vials. High-precision dissolved inorganic carbon (DIC) and total alkalinity samples (250 mL) were collected monthly by siphoning directly from seawater tanks with tubing through the temporarily opened tank lids. The sample was allowed to rinse the borosilicate sample bottle with a 3-volume overflow and was immediately poisoned with 50 µL of saturated $HgCl_2$.

High-precision total alkalinity (HPTA) and DIC concentrations were determined on 100 mL samples using an automated closed-vessel titration system, with equivalence points located using a nonlinear curve fitting approach (Bradshaw et al. 1981; Brewer et al. 1986; Department of Energy 1994). Titrations were standardized using certified reference materials obtained from Dr. A. Dickson (Scripps Inst. of Oceanography); the standard deviation of replicate analyses was 5 µeq kg^{-1} for alkalinity and 3 µmol kg^{-1} for DIC. DIC in small (3 to 5 mL) samples sealed in glass ampules was determined by acidification and vacuum extraction, using an automated version of our standard extraction system and methods (McCorkle et al. 1990; McCorkle and Klinkhammer 1991). The carbon isotopic composition of the extracted CO_2 was then determined on the VG-PRISM mass spectrometer in the National Ocean Sciences Accelerator Mass Spectrometer laboratory at Woods Hole Oceanographic Institution. The standard deviation of replicate $\delta^{13}C$ extractions and analyses was 0.04‰. The oxygen isotopic composition of water ($\delta^{18}O$) was determined in the laboratory of Dr. D. Schrag (Harvard University) using a VG Optima mass spectrometer with a VG Isoprep 18 automated shaker/equilibrator (Schrag et al. 2002). The standard deviation of replicate $\delta^{18}O$ analyses was 0.09‰.

Seawater samples for trace metal concentration measurement were collected weekly, immediately filtered with a 0.22-µm polyether sulfone syringe filter, and acidified with triple distilled concentrated HNO_3 to a pH < 2. At least 1 mL of sample seawater was passed through the syringe filter before the remainder was collected into acid-washed 4-mL polystyrene sample vials or 2-mL screw cap HDPE microcentrifuge tubes.

Cadmium (^{111}Cd spike, ^{114}Cd analyte) and barium (^{135}Ba spike, ^{138}Ba analyte) seawater measurements were by isotope dilution ICP-MS. Each filtered, acidified, and ^{111}Cd-spiked sample (1.5 mL) was preconcentrated by $Mg(OH)_2$ coprecipitation (after Wu and Boyle 1997). Ba was measured on a 1:20 dilution of the sample (Chan et al. 1977; Lea and Boyle 1989; Vanhaecke et al. 1996; Townsend et al. 1998). Accuracy of Ba was determined against an ICP-MS certified standard. Accuracy of Cd and consistency of both elements was monitored with NASS-5 certified reference material (open ocean standard).

Replicate samples (multiple samples from the same tank and/or from multiple tanks) were collected from any given day and analyzed to determine the precision of our measurements as a pooled standard deviation. To verify physicochemical consistency among the four tanks, samples taken from each tank for each parameter were subjected to an analysis of variance (ANOVA, $\alpha = 0.05$).

Culture chamber setup and foraminiferal inoculation—Each culture chamber was preassembled dry and empty in a trace-metal clean laminar flow hood. The sealed culture chambers were transferred to the environmental room and connected to the culture system to allow for equilibration and to survey for leaks. Previous work showed that 150 to 200 foraminifera ranging in size from 90 to 150 µm provided sufficient calcite to clean and analyze by ICP-MS (Havach et al. 2001). In addition, individual *Uvigerina peregrina* were known to produce as many as 55 offspring (Chandler unpubl. data unref.). The foraminifera were sorted in shallow Petri dishes with a fine sable brush or pipette and kept in temperature-controlled water baths or on ice. All visible traces of sediment were removed before transfer to culture chambers. Isolated foraminifera were held in 6-well tissue culture plates containing food (see *Foraminiferal feeding*), minimal sediment, and the fluorescent calcite label calcein (Bernhard et al. 2004) for two to three weeks prior to experimental initiation. Calcein incubation caused newly precipitated calcite to fluoresce marking preexisting calcite (i.e., fluorescent pre-experiment) from experimentally precipitated calcite (i.e., nonfluorescent). Silica substrate was added to the culture chambers through a sampling port during a low-flow regime (0.3 mL min^{-1}). During this time, the top output port was closed, forcing seawater into the top of the culture chamber and down through the culture insert membrane, promoting even silica settlement onto the membrane. After the silica settled, calcein-labeled foraminifers were added under the same low-flow regime. Immediately after inoculation, normal flow conditions (3 mL min^{-1}) were reinstated.

Foraminiferal feeding—All foraminiferal cultures (bulk, calcein incubations, and experimental) were fed algae on a weekly basis. The algal cultures were grown to log phase in F/2 media. *Dunaliella tertiolecta* was concentrated (~200:1) by centrifugation and reconstituted in culture-system seawater. While using the low-flow regime, 100 µL of concentrated algae (~10^7 cells mL^{-1}) was introduced into each culture chamber via one of the top sample ports. The flow was ceased to allow the algae to settle onto the substrate surface for 1 h. This ensured that the algae were not immediately washed out of the culture chamber. After feeding, standard flow conditions were resumed.

Experimental completion—Earlier culturing experiments using natural sediments and some of these foraminiferal species indicated that it took four to six months for 30 to 50 pooled juveniles to obtain a shell mass adequate for cleaning, dissolution, and chemical analysis (Wilson-Finelli et al. 1998; Havach et al. 2001). After 3 months of culturing during Experiment 1, one culture chamber was removed from the system and all of its contents were sieved over a 75-µm nylon/acrylic sieve. Microscopic examination of the >75 µm fraction revealed that many foraminifera had reproduced; the juvenile foraminiferal population was ~10 times the original inoculated population. After an additional month, the remaining eight culture chambers maintained during the experiment were harvested, sieved, and briefly sonicated (~3 to 5 s) to disaggregate silica layers accumulated around the foraminifers. The isolated foraminifera were air-dried, sorted by species, measured for length, and stored for analysis.

During Experiment 2, four cultures were harvested after four months, and the remaining eight cultures were harvested after six to eight months. Each chamber was removed from the system without disturbing the substrate and immediately observed using a dissection stereomicroscope. Individual foraminifers were identified to species and as epibenthic or endobenthic, removed, air-dried, measured for length, and stored for later analysis.

Assessment

Long-term physicochemical stability—During each experiment the variation in temperature, salinity (S), total alkalinity (small-volume [SVTA] and high-precision [HPTA]), and pH were less than 2% (Table 1; Fig. 3). In addition, the four reservoir tanks remained well-mixed during each experiment. For example, simultaneous measurements of salinity, SVTA, and pH among the four tanks were not significantly different (S: $P = 0.95$, SVTA: $P = 0.79$; pH: $P = 0.42$). Likewise, the variation in measured HPTA, DIC, [Cd], and [Ba] among the four tanks was not significant (HPTA: $P = 0.73$; DIC: $P = 0.32$; Cd: $P = 0.75$; Ba: $P = 0.32$). The differences between highest tank mean and lowest tank mean ($\Delta\overline{X}$) for salinity, SVTA, pH, [Cd], and [Ba] among the four tanks were equal to or smaller than the analytical precision (S: $\Delta\overline{X} \cong 0.1$ ‰, SVTA: $\Delta\overline{X} \cong 0.01$ meq kg^{-1}, pH: $\Delta\overline{X} \cong 0.015$, Cd: $\Delta\overline{X} \cong 32$ pmol kg^{-1}, Ba: $\Delta\overline{X} \cong 4$ nmol kg^{-1}). Method precision, as the pooled standard deviation, of each measurement is reported in Table 1.

Inspection of the pH and SVTA time-series indicated no variation (within the precision of these methods) in the course of the each experiment. Both of these small-volume measurements proved useful in week-to-week monitoring of the stability of the system. However, these data indicated a need for greater precision to provide better constraint on the evaluation of the carbonate system chemistry (i.e., partial pressure of CO_2 [pCO_2], calcite saturation index [Ω], and [CO_3^{2-}]). Beginning in the second half of Experiment 1, larger volume samples were collected for the analysis of HPTA and DIC. These analyses improved the accuracy and precision for evaluation of the carbonate system chemistry (Fig. 3). With the analytical precision of these few measurements, however, there are no statistically significant variations in the calculated carbonate system chemistry (Fig. 3D, 3F). These results demonstrated the system's stability. The small variations in system parameters, among the four tanks and during the course of each experiment, are unlikely to influence foraminiferal test chemistry. However, future experiments will need to assess the impact of small environmental fluctuations on stable isotope and elemental proxies. The relatively high TA, DIC, and pH, compared to our sample sites, were the result of the initial seawater filtration over crushed coral. Future experiments will lower the TA, DIC, and pH to reflect environmentally representative values.

The DIC $\delta^{13}C$ varied in a seasonal pattern (Fig. 3B). Insufficient sampling during Experiment 1 prevented immediate recognition of this effect, but expanded sampling during Experiment 2 provided compelling evidence that the seawater DIC $\delta^{13}C$ signal reflected a seasonal variation in atmospheric CO_2 $\delta^{13}C$.

Culture seawater $\delta^{18}O$ was stable throughout each experiment (Fig. 3B). Phase change is the primary influence on oxygen isotopic fractionation, therefore minimizing evaporation and condensation minimizes the change in seawater $\delta^{18}O$. Continuous seawater aeration with saturated air maintained a consistent water import/export to the system. The rise in $\delta^{18}O$ between Experiment 1 and 2 was due to the partial water change with Gulf Stream water.

Seawater [Ba] measurements during both experiments did not deviate more than 7% of the overall reservoir averages (Fig. 3E). The culture [Ba] was higher than field bottom-water concentration (~40 nmol kg^{-1}), but was very similar to shallow porewater concentrations (~120 nmol kg^{-1}) and concentration in older bottom waters (Lea and Boyle, 1989). The precision of the seawater Cd method during the experiments suffered due to low concentrations, high isobaric interferences in the culture seawater, and high-solids-derived "ghost-peak" interferences during analysis on the ICP-MS. Within the measured analytical precision, [Cd] did not vary during the course of each experiment. Ongoing method development should improve the precision of this measurement. The [Cd] was representative of bottom-water concentrations at our field sites and is not uncommon for Atlantic bottom waters (Boyle 1992).

Culturing success—During both experiments, many foraminiferal species remained viable, and two species reproduced (Table 2). Mixed-species cultures from Experiment 2 yielded reproduction by *Bulimina aculeata* and *Rosalina vilardeboana* in approximately 4 months. Calcite from juvenile *B. aculeata* (mostly 200 to 220 µm in length) comprised 90% to 95% of the harvested carbonate. In addition, the fluorescently labeled inoculated foraminiferal calcite provided clear indication that specimens of many species precipitated calcite dur-

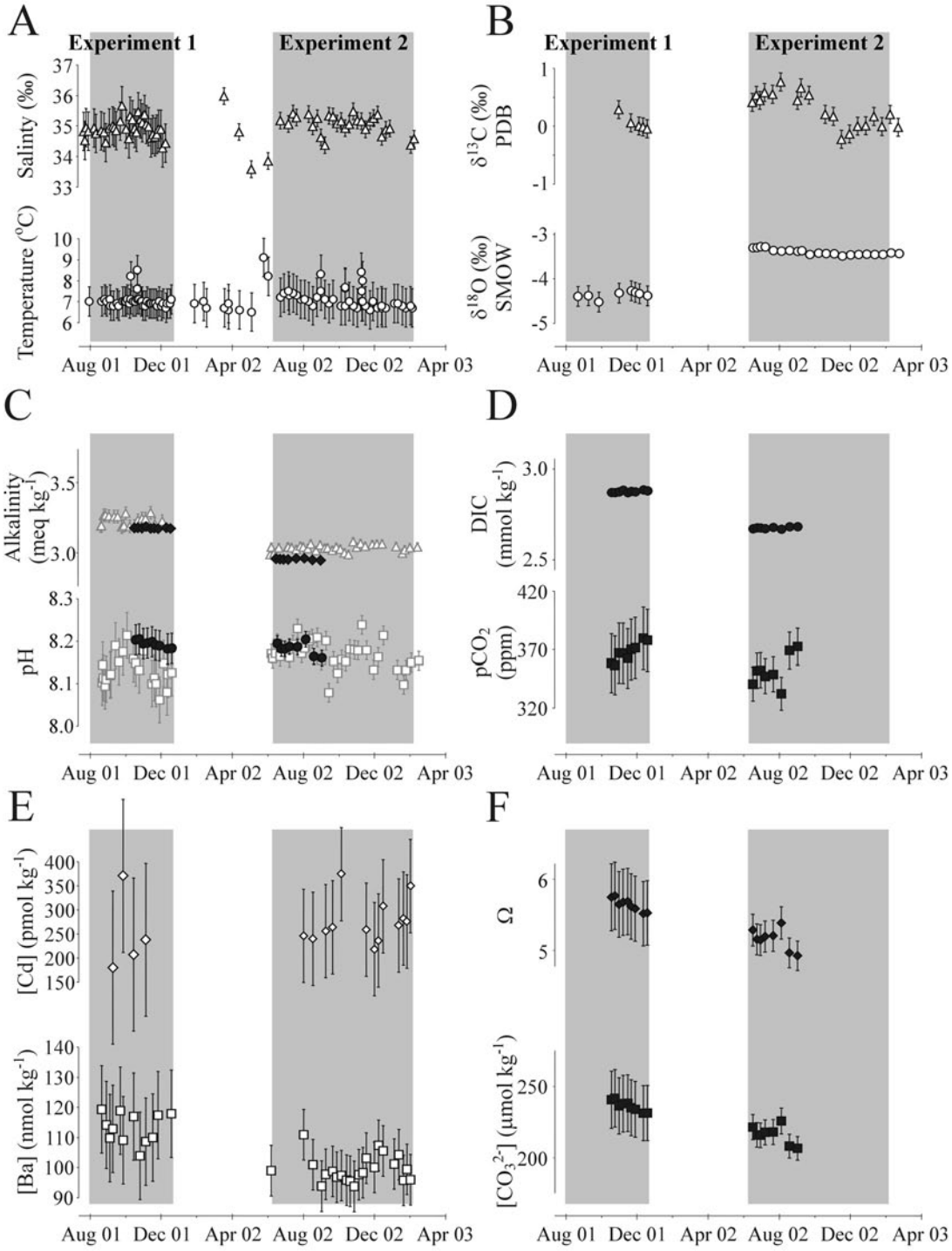

Fig. 3. Physicochemical stability of culture system during Experiments 1 and 2 (gray areas). Salinity and temperature (A), stable carbon and oxygen isotopes (B), carbonate chemistry (C, D, and F), and trace metals Cd and Ba (E) are shown. Error bars on individual measurements are 2 standard deviations around a mean of multiple, same-day measurements. Error bars that appear absent are smaller than the plotted symbol. The pCO_2 (D), calcite saturation index (Ω) (E), and CO_3^{2-} (E) were calculated using HPTA (C) and DIC (D) measurements (solid symbols) with CO_2SYS (Lewis and Wallace 1998) using the constants reported by Roy et al. (1993) and Dickson (1990). Measured temperature and salinity variations were assumed not to add error to these calculations. In (C), open gray triangles represent SVTA (4 mL), whereas black solid diamonds represent HPTA (100 mL). Open gray squares represent measured pH values on the seawater pH scale; solid black circles represent calculated pH (seawater scale) from HPTA and DIC measurements. Measured pH instrumental drift was corrected using a tris buffer, however, other factors (e.g., probe stability, probe age) during Experiment 1 lowered the pH measurement precision.

Table 2. Number of specimens collected at experimental termination from reproducing species

Experiment 1												
Culture chamber	1	2	3	4	5	6	7	8	9*			
Number of inoculated specimens	85	100	100	100	100	100	100	100	85			
Days in culture	84	119	120	120	113	113	107	108	94			
Bulimina aculeata (>500 µm†)	18	34	60	99	50	16	13	62				
Bulimina aculeata (260 to 500 µm†)		437	922	1042	144	219	45	57				
Bulimina aculeata (<260 µm†)	77	526	917	1772	505	991	139	461	47			
Rosalina vilardeboana	6	32	6	39	51	31	2					
Experiment 2												
Culture chamber	1	2	3	4	5	6	7	8	9	10	11	12
Number of inoculated specimens	60	3	10	57	71	70	59	78	97	97	95	59
Days in culture	119	209	119	210	216	208	119	120	173	173	171	173
Inoculated with *Bulimina aculeata*	No	No	No	No	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes
Inoculated with *Rosalina vilardeboana*	Yes	No	No	Yes	No	Yes	No	Yes	No	No	No	Yes
Bulimina aculeata (>500 µm†)						28	18		45	58	27	
Bulimina aculeata (260 to 500 µm†)					219	60	105		27	8	16	2
Bulimina aculeata (<260 µm†)					52	22	66				10	
Rosalina vilardeboana (>500 µm†)							15	45				
Rosalina vilardeboana (260 to 500 µm†)	19	265	8	233	306	47	7	19	19	35	27	10
Rosalina vilardeboana (<260 µm†)	29	251	7	274	176	137	5	37	14	10	9	48

*Monoculture of *Bulimina aculeata* that did not have reproduction. It was inoculated with <30-day-old juvenile, not adult, specimens.
†Parenthetic size fractions listed are at the time of culture harvest.

ing both experiments.

Experiment 2 had less reproductive yield than Experiment 1, but the results of Experiment 2 directly addressed concerns about competition raised in Experiment 1. Based on the culturing results from Experiment 1, *B. aculeata* was omitted from five of the Experiment 2 cultures to investigate other species' ability to grow and reproduce without competition from *B. aculeata*. Other than *R. vilardeboana*, species did not reproduce in the absence of *B. aculeata*.

To promote harvest of larger specimens from Experiment 2, only four cultures were terminated after four months (similar to Experiment 1) while the remaining eight were harvested after six to eight months. Cultures maintained for longer than four months produced larger *B. aculeata* compared to conspecifics from four-month-old cultures (ANOVA, $P = 0.04$). Cultures maintained for four months, on average, yielded 29% > 220-µm-long specimens (excluding inoculated adults) and 71% < 220-µm-long specimens. Cultures maintained for over four months, on average, yielded 86% > 220-µm-long and 14% < 220-µm-long specimens.

Whereas the *B. aculeata* specimens produced were lighter mass than field-collected specimens, they were predominantly immature "babies" (2 to 5 chambers, < 220 µm; Experiment 1) and "juveniles" (5 to 10 chambers, ~230-450 µm; Experiment 2). These specimens did not have enough time to develop and mature during the two experiments.

We did not observe any morphology variation compared to field-collected specimens during our experiments. The immature "baby" *B. aculeata* were short and disproportionately wide for their length, compared to larger individuals. We found no similarly sized specimens in our field-collected samples with which to compare these cultured individuals. However, the larger "juveniles" from Experiment 2 had normal morphology compared to similarly sized field-collected specimens.

Bulimina aculeata were found on the substrate surface (epibenthic) as well as within the 2-mm-thick substrate layer (endobenthic). Collection methods did not allow for precise measurement of each foraminifer's three-dimensional position, but specimens did not appear to be evenly distributed over the entire substrate volume. No clear distribution pattern (i.e., epi- versus endobenthic) was observed, however, for any species.

Also, in Experiment 2, juvenile to adult *Rosalina vilardeboana* occurred in all cultures, whether or not the culture was inoculated with them. Within the cultures, many *R. vilardeboana* were attached to other individuals of the same or different species. It was common for 3 to 4 juveniles to be attached to a single adult.

Small nematodes colonized the cultures by the end of the Experiment 2. These nematodes likely dwelled within the entire culture system. Each culture had dozens of nematodes that secreted mucous, which loosely bound the silica particles together. The nematode populations may have inhibited large-scale reproduction of *Bulimina aculeata* and/or other species in Experiment 2; nematodes were not observed at the end of Experiment 1. A future goal is to modify the culture system to limit nematode recruitment.

As reported by Bernhard et al. (2004), the fluorescent com-

pound calcein showed no evidence of acute or chronic toxicity to various benthic foraminiferal species. Because two foraminiferal species reproduced prolifically, it is unlikely that calcein toxicity is a factor in our experiments. Also, initial measurements of minor-element:calcium ratios (Mg/Ca, Sr/Ca) in calcein-labeled field-collected specimens do not indicate variation in the expected-element:calcium ratios of *Bulimina aculeata* (Hintz unpubl. data unref.; Rosenthal et al. 1997; Lear et al. 2002).

Bulimina aculeata seemed particularly well-suited to these culture conditions, as evidenced by its prolific reproduction in Experiment 1. *Rosalina vilardeboana* was particularly opportunistic evidenced by traveling through the culture system and settling in areas where other foraminifers were absent. Food availability was certainly not a limiting factor to foraminiferal reproduction, but the green alga *Dunaliella tertiolecta* may not be ideal as the sole food source for all foraminiferal species.

Foraminiferal life spans vary and are not known for most species. While we know that individuals survive at least eight months in culture, but do not necessarily reproduce, it is possible that longer time periods are required to obtain foraminiferal reproductive events in cultures maintained at temperatures representative of in situ bathyal conditions. *Cibicidoides pachyderma* reproduced in limited numbers after nine months at 10°C (Havach 1998). Perhaps lengthening the culture periods and eliminating competition from fast-growing *B. aculeata* and *R. vilardeboana* may promote reproduction in other bathyal species.

There may be a trace constituent necessary for reproduction that is absent in our artificial seawater system. Even though 30% and 51% of the culture seawater during Experiments 1 and 2, respectively, was Gulf Stream water, future experiments will further limit and probably eliminate artificial seawater use.

Method modification assessment—Initial experiments not presented here with single-species benthic foraminiferal cultures used a different, simplified culture chamber flow scheme. While reproduction was observed in these experiments (Havach 1998), culturing at 10°C required almost nine months. As with our experiments, *Bulimina* spp. was most prolific, but *Cibicidoides pachyderma* also reproduced, albeit in only 1 culture. After these initial experiments, significant improvements were made to the culture chamber to lower the seawater residence time without greatly increasing seawater velocity. These modifications further ensured that the culture chambers were well mixed, limiting microhabitat formation. Greater reproductive yield in the current experiments compared with those of Havach (1998) demonstrated the improvement of the described culture-chamber design.

Culture-system maintenance with ambient (outdoor) air was an improvement from previous use of pressurized (medical-grade) air (Chandler unpubl. data unref.). More specifically, the addition of continuous large volume saturated atmospheric air to the culture system controlled $\delta^{13}C$, pCO_2, and O_2, and provided vigorous mixing within each tank to maintain seawater homogeneity. This benefit was offset, however, by the variation of atmospheric pCO_2 and CO_2 $\delta^{13}C$ that was reflected in the seawater carbonate chemistry and DIC $\delta^{13}C$. The ~1‰ variation in $\delta^{13}C$ observed over the course of Experiment 2 was still less than the $\delta^{13}C$ variation observed in previous shorter-duration, benthic-culturing experiments, which varied by > 3‰ (Wilson-Finelli et al. 1998).

Over the course of the experiments, culture harvest was accomplished in two ways. The first method removed an entire culture, sieved its contents over a 75-µm nylon/acrylic sieve, and briefly sonicated the coarser fraction to break up any silica constructs formed around foraminifers. This allowed quick and efficient separation of foraminifers from the substrate, but often damaged the newly calcified terminal chambers of adults and juveniles. The second method removed each culture without disturbing the substrate; examination of the culture and substrate using a stereomicroscope allowed identification of individual epibenthic and endobenthic specimens. While time intensive, this method offered the best specimen preservation and cataloging of individual positions with respect to the substrate-water interface, which may be a critical factor for certain proxies (e.g., Cd).

Discussion

The culturing approach described here provides sufficient physicochemical control to allow rigorous testing of isolated environmental and physiological (i.e., vital) effects on trace metal and stable isotope incorporation by benthic foraminifera. This approach limits variation in proxy uptake associated with varying water chemistry, sediment composition, and food supply.

Foraminiferal reproductive cues are not well understood, and there may be unknown signals missing from this ultra-clean system. Our foraminiferal stock culture system contains natural sediment cultures in a recirculating, filtered artificial seawater system (Chandler and Green 1996); adult and juvenile *Bulimina* spp., *Uvigerina peregrina, Cibicidoides* spp., *Melonis pompiloides, Bolivina* spp., *Discorbinella* spp., and *Hoeglundina elegans* are typically found in these cultures, indicating that all these species are able to reproduce and grow in laboratory environments. When adults of three species were maintained in silica monocultures, *Bulimina* spp. was the only taxon that reproduced consistently, while *C. pachyderma* reproduced with limited success (Havach 1998). The multispecies cultures in our experiments yielded consistent reproduction in *B. aculeata* and *Rosalina vilardeboana*.

Our culturing approach is important for three reasons: (1) Benthic foraminifers can be maintained to allow reproduction in a stringently controlled environment for assessments of paleoproxy fidelity in their calcite. (2) Pre-experimental calcite can be easily distinguished from experimentally produced calcite when inoculated specimens are labeled with the fluores-

cent compound calcein. (3) The use of non–trace-metal-contaminating silica substrate instead of natural sediment minimizes or removes confounding sediment microhabitat effects, allowing assessment of vital effects. This successful culturing method will allow future experimental assessment of different environmental effects (e.g., trace element concentrations, stable isotope ratios, carbonate chemistry, feeding regimes) on the incorporation of geochemical proxies into foraminiferal calcite. It is important to keep in mind, however, that the apparatus and culturing conditions described here are artificial and are not intended to completely reproduce natural conditions. Instead, the system is designed to regulate specific environmental parameters that are highly variable in the field. Stringent constraints are necessary to isolate and verify the parameters that affect foraminiferal calcite chemistry in order test the reliability of sequestered marine geochemical proxies.

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Submitted 27 August 2003
Revised 2 December 2003
Accepted 10 March 2004