Combined carbonate carbon isotopic and cellular ultrastructural studies of individual benthic foraminifera: Method description

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[1] Carbon isotopes of foraminiferal tests provide a widely used proxy for past oceanographic environmental conditions. This proxy can be calibrated using live specimens, which are reliably identified with observations of cell ultrastructure. Observations of ultrastructures can also be used for studies of biological characteristics such as diet and presence of symbionts. Combining biological and isotopic studies on individual foraminifera could provide novel information, but standard isotopic methods destroy ultrastructures by desiccating specimens and observations of ultrastructure require removal of carbonate tests, preventing isotope measurements. The approach described here preserves cellular ultrastructure during isotopic analyses by keeping the foraminifera in an aqueous buffer (Phosphate Buffered Saline (PBS)). The technique was developed and standardized with 36 aliquots of NBS-19 standard of similar weight to foraminiferal tests (5 to 123 μg). Standard errors ranged from $\pm$ 0.06 to $\pm$ 0.85‰ and were caused by CO_2 contaminants dissolved in the PBS. The technique was used to measure $\delta^{13}C$ values of 96 foraminifera, 10 of which do not precipitate carbonate tests. Calcareous foraminiferal tests had corrected carbon isotope ratios of -8.5 to $+3.2$‰. This new technique allows comparisons of isotopic compositions of tests made by foraminifera known to be alive at the time of collection with their biological characteristics such as prey composition and presence or absence of putative symbionts. The approach may be applied to additional biomineralizing organisms such as planktonic foraminifera, pteropods, corals, and coccolithophores to elucidate certain biological controls on their paleoceanographic proxy signatures.

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1. Introduction

[2] Chemical and isotopic compositions of foraminiferal tests provide some of the most widely used proxies for paleoenvironmental reconstructions (e.g., Mg/Ca, Sr/Ca, $\delta^{13}C$, $\delta^{18}O$, $^{87}Sr/^{86}Sr$) [e.g., *Healey et al.*, 2008; *Hendry et al.*, 2009; *Wefer et al.*, 1994]. Of these, the $\delta^{13}C$ value of foraminiferal tests ($\delta^{13}C_{test}$) is an important means to assess paleoceanographic characteristics, particularly paleoproductivity and global carbon cycling. For example, carbon isotopic ratios have been useful to determine remineralization of organic carbon and its flux from organic carbon–rich sediments [e.g., *Hill et al.*, 2004; *Stott et al.*, 2002; *Zeebe*, 2007]. Carbon isotope ratios in benthic foraminiferal tests generally follow a relationship with $\delta^{13}C$ of the dissolved inorganic carbon ($\delta^{13}C_{DIC}$) in pore waters of marine sediments, although offsets from equilibrium values can be

caused by species-specific biological fractionation, commonly referred to as "vital effects" [e.g., *McCorkle et al.*, 1990, 1997; *Rathburn et al.*, 1996]. Because of the relationship between pore water $\delta^{13}C_{DIC}$ and $\delta^{13}C_{test}$, foraminifera are assumed to consistently record the carbon isotopic composition of the DIC in the water and thus can be used as a reliable proxy for environmental conditions at the time the foraminifera precipitated their carbonate tests. Other factors, such as food sources or postmortem diagenetic alteration, however, could alter the relationship between $\delta^{13}C_{test}$ and environmental conditions. Understanding these controls on $\delta^{13}C_{test}$ requires linked studies of foraminiferal biology and isotopic compositions of their tests.

[3] Recent studies have described environments where foraminifera have $\delta^{13}C_{test}$ values that differ from the composition of pore water more than expected from vital effects. Sites of extraordinarily light $\delta^{13}C_{DIC}$ values (to as low as about -60‰ [e.g., *Martin et al.*, 1997; *Torres et al.*, 2002]) occur at methane cold seeps, but benthic foraminifera thought to be living, or recently living, in methane seeps have been found to have $\delta^{13}C_{test}$ values that are as much as 40‰ out of equilibrium with the ambient pore water, with $\delta^{13}C_{test}$ values closer to bottom seawater values than ambient $\delta^{13}C_{DIC}$ [*Martin et al.*, 2004; *Rathburn et al.*, 2000, 2003; *Torres et al.*, 2003]. As a result, foraminifera within cold seeps commonly have isotope ratios similar to con-

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specific individuals living a few meters outside cold seeps regardless of the extreme differences in $\delta^{13}C_{DIC}$ values between the two locations [*Martin et al.*, 2004; *Rathburn et al.*, 2003]. The cause of this disequilibrium is unknown but could result from variations in the $\delta^{13}C_{DIC}$ values of microenvironments of the pore water, variations in isotopic compositions of foraminiferal food, or presence of symbionts in the foraminifera. Disequilibrium caused by food sources and/or presence of symbionts would complicate understanding of paleoenvironments estimated from $\delta^{13}C_{test}$ values. Although in general the measured carbon isotope values for seep and nonseep conspecific foraminifera are similar, seep foraminifera typically show a larger range of $\delta^{13}C_{test}$ values than their nonseep conspecifics. The wide range indicates that, although the seep foraminifera are close to equilibrium with seawater, ambient conditions of the seeps may influence foraminiferal $\delta^{13}C_{test}$ values [*Rathburn et al.*, 2003]. Additionally, examining isotopic variability and possible causes of disequilibrium of seep foraminifera with their environment requires the examination of individual foraminifera living in seeps.

[4] Understanding the relationship between environmental conditions and chemical and isotopic signals recorded in foraminiferal tests typically are based on identifying foraminifera thought to be alive at the time of collection to minimize postmortem alteration of the tests [e.g., *Corliss and Emerson*, 1990; *Fontanier et al.*, 2008] and to link observations of current environmental characteristics with the foraminifera thought to be calcifying in those conditions. Even live foraminifera may not accurately reflect current environmental conditions if the conditions vary at time scales shorter than foraminiferal life spans. Authigenic carbonate may precipitate on relict foraminiferal tests thereby potentially masking the original environmental signal recorded by the foraminifera. Contamination by authigenic carbonate is minimized for foraminifera that were alive at the time they were collected because of the limited amount of time for precipitation of authigenic carbonate on the tests.

[5] Whether or not individual foraminifera are alive at the time of collection has been assessed using multiple techniques (reviewed by *Bernhard* [2000]), including stains (e.g., rose Bengal [*Walton*, 1952]), more recently with fluorescent labeling (CellTracker Green [*Bernhard et al.*, 2006]), and through observations of ultrastructural features of the foraminiferal cytoplasm using transmission electron microscopy (TEM) [*Bernhard*, 2000]. Staining and labeling are simple, easy to use, and leave the test available for measurements of paleoenvironmental proxies such as $\delta^{13}C_{test}$. These techniques can result in false identification of living specimens, however, if staining or labeling results from foraminiferal cytoplasm remaining in the carcass following death or if the test is colonized by other organisms, such as bacteria. During necrosis, changes occur in the structure of organelles, and thus the presence of intact organelles is a clear indication that foraminifera were alive when collected [*Bernhard*, 2000].

[6] Organelles can be observed using TEM of the foraminiferal cytoplasm, which requires chemical fixation causing death of the foraminiferan, removal of the carbonate tests using a weak acid such as ethylenediaminetetraacetic acid (EDTA), staining, and embedding the cytoplasm in epoxy resin [e.g., *Flegler et al.*, 1993]. This process, up to epoxy embedding and including removal of the carbonate tests, must occur while the foraminifera remains in liquid so that its cytoplasm is not desiccated causing destruction of the ultrastructure. Conversely, standard techniques for isotopic and chemical analyses of foraminiferal tests by mass spectrometry require extraction of CO_2 gas from the carbonate tests after drying the foraminifera, removing organic material with hydrogen peroxide, and dissolving the remaining tests with phosphoric acid. This process destroys or alters foraminiferal ultrastructures, so the characteristics of cellular ultrastructures cannot be determined subsequent to standard isotopic analyses.

[7] In this paper, we describe a procedure that allows the mass spectrometric measurement of foraminiferal calcite while preserving foraminiferal cytoplasm for TEM observations of cell ultrastructure. Specifically, we describe technique development, precision and accuracy, and some preliminary results. The technique allows measurements of $\delta^{13}C$ values of individual foraminiferal tests linked to unequivocal determination of whether the specimen was alive at the time of collection, evaluation of the individual's biological characteristics, such as food vacuole contents and the presence of symbionts, and assessment of diagenetic alteration of the test carbonate. Results of ultrastructural analysis and detailed comparisons of isotope ratios and ultrastructural analyses will be presented in a separate contribution (J. M. Bernhard et al., Combined carbonate carbon isotopic and cellular ultrastructural studies of individual benthic foraminifera: 2. Assessing controls of apparent disequilibrium in hydrocarbon seeps, submitted to *Paleoceanography*, 2009).

2. Materials and Procedures

[8] Foraminiferal-bearing sediments were collected using push cores deployed by the ROV *Jason* in July 2007. Materials were obtained from cold seeps and sediments surrounding the seeps at Clam Flats (approximately 36° 44′N, 122° 16′W; ~1000 m water depth) in Monterey Bay, California. Within an hour, the uppermost centimeter of each sediment core was extruded from the underlying sediments and fixed in ~3.0% glutaraldehyde buffered with 0.1M Na-cacodylate acid (pH 7.2 [e.g., *Bernhard*, 2000]), thereby killing the foraminifera (Figure 1). Samples were kept cold (~5°C) for about eight months until further processing. To aid in identifying cytoplasm-containing specimens, a saturated solution of rose Bengal (aqueous) stain was added to the fixative solution approximately one week prior to isolating the specimen from sediments.

[9] Foraminifera were not picked until immediately prior to introduction to the mass spectrometer to preserve cytoplasm from desiccation and to prevent decalcification of the tests in the mildly acidic Phosphate Buffered Saline (PBS) (Figure 1, see below). Samples were wet sieved using a 63 μm mesh and individual rose-Bengal stained specimens were removed from the coarse fraction with the aid of a binocular microscope. Although recent work has indicated
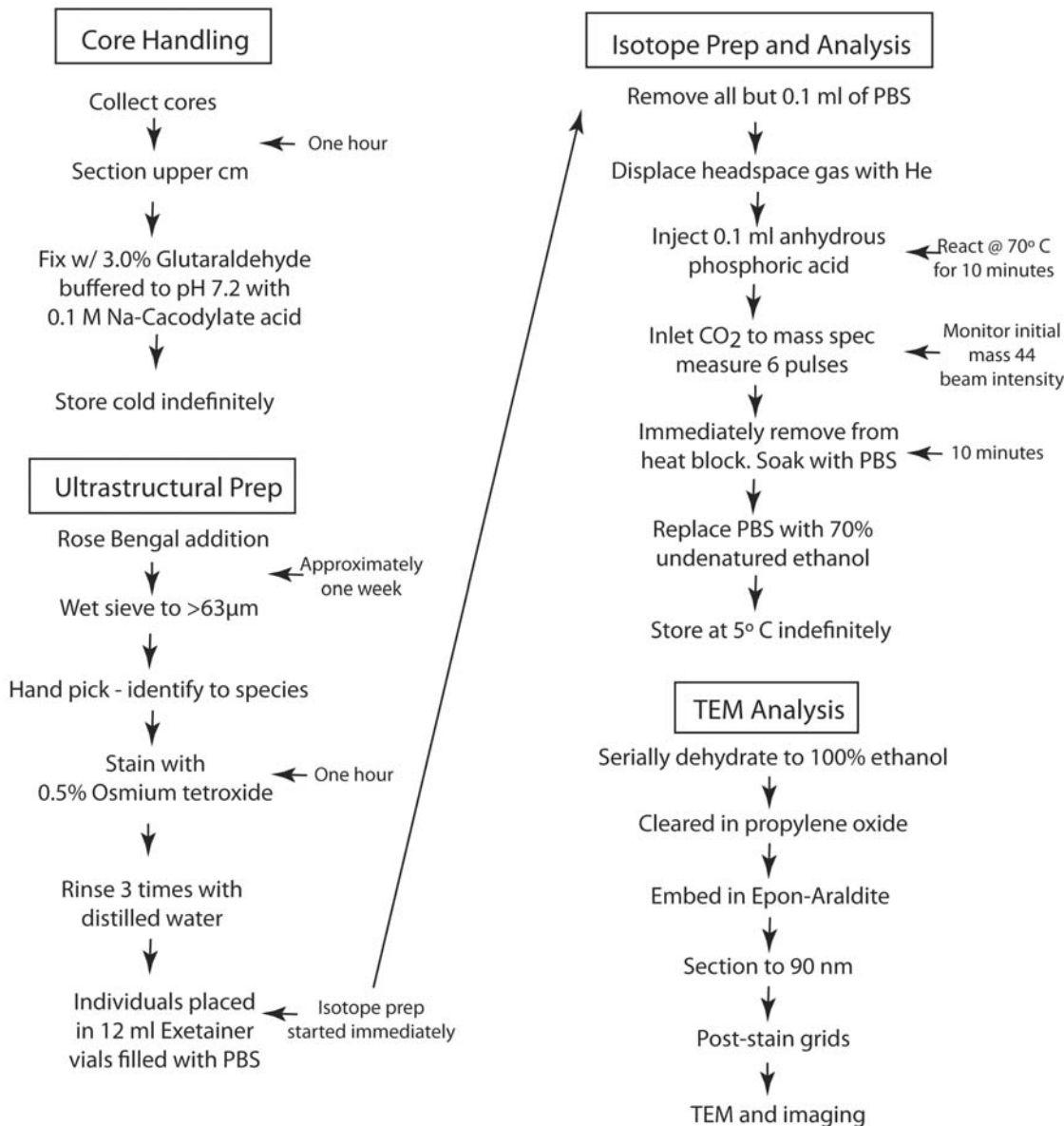
Core Handling

Collect cores

↓ ← One hour

Section upper cm

↓

Fix w/ 3.0% Glutaraldehyde
buffered to pH 7.2 with
0.1 M Na-Cacodylate acid

↓

Store cold indefinitely

Ultrastructural Prep

Rose Bengal addition

↓ ← Approximately one week

Wet sieve to >63μm

↓

Hand pick - identify to species

↓

Stain with
0.5% Osmium tetroxide ← One hour

↓

Rinse 3 times with
distilled water

↓

Individuals placed
in 12 ml Exetainer ← Isotope prep started immediately
vials filled with PBS

Isotope Prep and Analysis

Remove all but 0.1 ml of PBS

↓

Displace headspace gas with He

↓

Inject 0.1 ml anhydrous
phosphoric acid ← React @ 70° C for 10 minutes

↓

Inlet CO_2 to mass spec
measure 6 pulses ← Monitor initial mass 44 beam intensity

↓

Immediately remove from
heat block. Soak with PBS ← 10 minutes

↓

Replace PBS with 70%
undenatured ethanol

↓

Store at 5° C indefinitely

TEM Analysis

Serially dehydrate to 100% ethanol

↓

Cleared in propylene oxide

↓

Embed in Epon-Araldite

↓

Section to 90 nm

↓

Post-stain grids

↓

TEM and imaging

Figure 1. Flowchart of procedures used to obtain linked isotopic and ultrastructural data for individual foraminifera.

isotopic differences between foraminifera at different size ranges [*McCorkle et al.*, 2008; *Schmiedl et al.*, 2004], here we picked specimens > 63 μm because of the small number of rose-Bengal stained specimens and to test the impact of size on the technique. After being identified to species, the foraminifera were photographed, poststained for one hour in 0.5% osmium tetroxide (e.g., osmicated), and rinsed three times in distilled water. Individual foraminifera were placed in 12 ml septum-topped Exetainer sample vials that were filled with PBS, and within one hour were prepared for mass spectrometric analysis.

[10] Preparation for mass spectrometry was started by removing all but about 0.1 ml of PBS using disposable transfer pipettes. Each Exetainer vial was then sealed with an airtight septum and the air headspace was displaced with

ultrapure He at atmospheric pressure by inserting two syringes through the septum, one of which was connected to a pressure-regulated He gas tank through a ten-port manifold (to allow simultaneous displacement of headspace gas of 10 samples) and the other open to the atmosphere. Helium was allowed to flow through the Exetainer vials for 10 min to ensure that all air was displaced from the headspace. Some PBS evaporated during the gas exchange, but each specimen remained completely immersed during the entire procedure.

[11] Standards were weighed into the same style 12 ml Exetainer vials and 0.1 ml of PBS was added to the standards prior to their measurement. In total, 36 aliquots of the National Bureau of Standards (now National Institute of Standards and Technology) reference material NBS-19 (TS-

Table 1. Beam Intensities of Reagent Blanks

Reagents Analyzed	Intensity (mV)
Helium only	4
Helium and acid	35
Helium and acid	12
Helium, acid, 100 ml PBS	50
Helium, acid, 100 ml PBS	50
Helium, acid, 100 ml PBS	45
Helium, acid, 100 ml PBS	45
Helium, acid, 100 ml PBS	40
Helium, acid, 100 ml PBS	40
Helium, acid, 200 ml PBS	45

limestone) [*Friedman et al.*, 1982] were measured to standardize the technique. Twelve aliquots were measured to develop the technique; the remainder were measured interspersed with the samples as check standards and to calibrate the reference gas. The mass of the standards ranged from 5 to 123 μg to mimic the expected mass of the foraminiferal tests. Weights of foraminiferal tests could not be determined because they contained cytoplasm that had been osmicated and the specimen remained wet throughout the procedure. Instead, the mass of CO_2 that evolved from the samples, standards, and blanks were compared by monitoring the maximum intensities of the first pulse of mass 44 beam intensity. Average weight of all the standards was 29.6 μg, and all but eight standards were less than 35 μg. Ten blanks were measured on the He gas, PBS solution and on the phosphoric acid by introducing one or more of the reagents to the Exetainer vials without any carbonate material (Table 1). Measured foraminifera included 86 rose Bengal-stained specimens from 13 species with calcite tests, and 10 specimens of a tectinous (lacking a calcitic test) species. The mass 44 beam intensity ranged from 60 to 4732 mV for the foraminifera and from 232 to 5791 mV for the standards. The mass spectrometer was set to provide isotope ratios only if the mass 44 beam intensity was > 100 mV.

[12] Carbon dioxide was evolved from standards and foraminifera by injecting 0.1 ml of anhydrous phosphoric acid through the vial's septum using a glass gastight syringe (Figure 1). Standards and foraminifera were reacted off-line with the acidified PBS for 10 min in an aluminum reaction block of a GasBench II preparation device at 70 ± 0.1°C. After 10 min, no visible reactions could be seen in either the samples or standards and it was assumed the carbonate material had dissolved completely. Immediately after this reaction time, the headspace gas was introduced into a Finnigan-MAT DeltaPlusXL continuous flow mass spectrometer using the automated GasBench II inlet system at the University of Florida. Six pulses of sample gas were measured sequentially during analysis, which took an additional 10 min, and the isotope ratios of all six pulses were averaged. After the measurement of $\delta^{13}C_{test}$ values, the Exetainer vials were immediately removed from the reaction block and filled with PBS solution. The foraminifera were allowed to soak for 10 min. After soaking, all but about 0.1 ml of solution was pipetted from the Exetainer vials, which were then filled with 70% undenatured ethanol to stabilize the sample until specimens were further prepared for TEM analysis. Direct transfer from PBS to

70% ethanol has been used previously in ultrastructural studies [*Colquhoun and Rieder*, 1980].

[13] At WHOI, specimens were serially dehydrated to 100% ethanol, cleared in propylene oxide and left overnight in an equal volume of propylene oxide and uncatalyzed Epon-Araldite epoxy resin at room temperature, following standard procedures [e.g., *Bernhard*, 2000]. Final transfers and embedding also followed standard procedures. Blocks were sectioned into 90 nm sections, placed on either Formvar slot grids or mesh grids lacking a support film. Sections were poststained with a saturated uranyl acetate solution in 50% ethanol and examined with either a Zeiss 902A TEM or a Zeiss 10CA TEM.

3. Assessment

3.1. Standardization

[14] The measured isotopic composition of the standards varied from −3.01‰ to +2.24‰ relative to the Peedee belemnite (PDB) and showed a dependence on the weight of the standards and the mass 44 beam intensity. We assume a similar relationship occurs for the carbonate of the foraminifera, and thus to correct for the weight dependence of the isotope ratios, the $\delta^{13}C$ values of the standards were plotted versus their mass 44 beam intensity (Figure 2). The mass 44 beam intensity showed a strong positive linear relationship between the weight of the standard and the intensity of the beam, with an R^2 value of 0.94 (Figure 3). This relationship indicates that the mass spectrometer was unaffected by variations in the amount of CO_2 introduced to the instrument and suggests that corrections for differing mass of foraminifera can be made on the basis of the mass 44 beam intensity. The relationship between mass 44 beam intensities and $\delta^{13}C$ values of the standards showed a constant value in the isotope ratio at beam intensities greater than 2000 mV. A constant ratio was also found for beam intensities between 1000 and 2000 mV, but it was lower and more scattered than for beam intensities > 2000 mV. A constant decline in isotope ratio was found for beam intensities < 1000 mV (Figure 2a). The linearity between beam intensity and mass of the standard, but the nonlinear relationship between isotope ratio and amount of gas introduced to the mass spectrometer, suggests that introduction of contaminant CO_2 may cause the decrease in the $\delta^{13}C$ values of small standards, as discussed below.

[15] None of the blank measurements had sufficient CO_2 to yield an isotopic ratio, and thus it is impossible to directly identify the isotope ratio of the blank (Table 1). The strong decrease in $\delta^{13}C$ values of the standards with beam intensities below 1000 mV suggests that CO_2 contributed from the blank contaminant is isotopically light (Figure 2). The He carrier gas alone produced a mass 44 beam intensity of only 4 mV, indicating it produced only minor contamination. Little CO_2 should remain dissolved in the anhydrous phosphoric acid, but blanks containing only phosphoric acid produced beam intensities of 12 and 35 mV (Table 1). Additional gas evolved from the PBS following acidification, ranging up to 50 mV with an average value of 45 ± 4 mV. Although PBS does not contain any carbonate salts, it was not degassed prior to its use and thus the contaminant
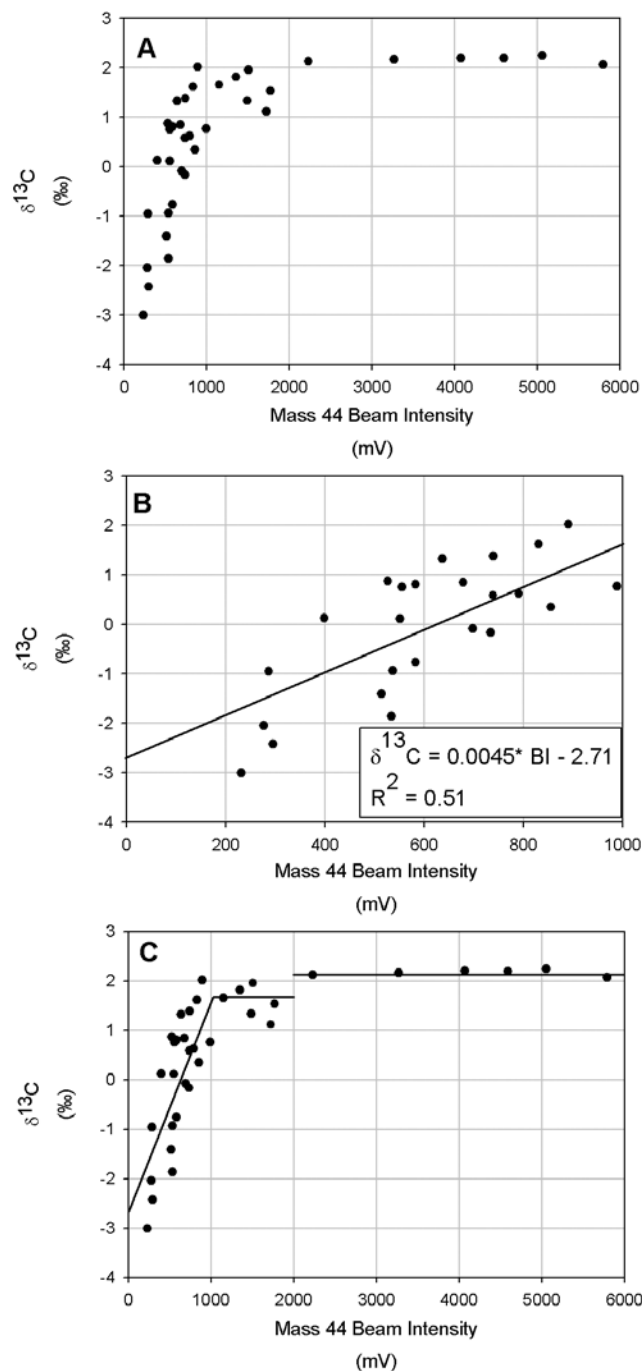
Figure 2. (a) Mass 44 beam intensity versus $\delta^{13}C$ values of all measured standards. (b) Mass 44 beam intensity versus $\delta^{13}C$ values for standards between a beam intensity of 250 and 1000 mV. (c) Mass 44 beam intensity versus $\delta^{13}C$ values showing standard values used to correct measured values of specimens.

standards with beam intensities between 250 and 1000 mV suggests that the isotope ratio of the blank is around −2.7‰. This value is about 4‰ heavier than expected from the $\delta^{13}C$ value of atmospheric CO_2 of approximately −7‰ [e.g., *Clark and Fritz*, 1997], but is poorly defined because of scatter in the data. At beam intensities of > 2000 mV, the blank contributes < 2.5% of the total signal. Because the value of the blank is similar to the value of the tests, a large amount of contaminated gas would be required to alter the measurements. At lower beam intensities, the blank contributes an increasingly larger fraction of the measured isotope ratio, but this contribution from the blank can be corrected to beam intensities as low as 250 mV based on the standard curve (Figure 2a; see below).

[16] The standard curve shown in Figure 2a was divided into three segments based on the measured values of the standards, their associated beam intensities, and the best fit for linear regression analysis of the data (Figure 2c). The segments included samples with beam intensities > 2000 mV (n = 6), between 1000 and 2000 mV (n = 6), and between 250 and 1000 mV (n = 24). The average isotope ratio of the standards with mass 44 beam intensities > 2000 mV (standard weights between 45 and 123 mg) was +2.16 ± 0.06‰. The defined value for NBS-19 is +1.95‰ [*Coplen*, 1995], indicating that a correction factor of −0.21‰ must be applied to samples with beam intensities > 2000 mV. There was considerable scatter between beam intensities of 1000 to 2000 mV (standard weights between 18 and 65 mg); these standards yielded an average $\delta^{13}C$ value of +1.56 ± 0.31‰ and require a factor of +0.39‰ to correct to a value of +1.95‰ for NBS-19. For beam intensities > 1000 mV, we use the standard deviation of the standards as an estimate of the accuracy for the measurements. For beam intensities > 2000 mV, the accuracy is estimated to be ± 0.06‰ and for beam intensities between 1000 and 2000 mV, the accuracy is estimated to be ± 0.31‰.

[17] Standards yielded isotope ratios that ranged from −2.43 to +2.01‰ for beam intensities ranging from 250 to 1000 mV (weights between 5 and 27 μg). Although the data

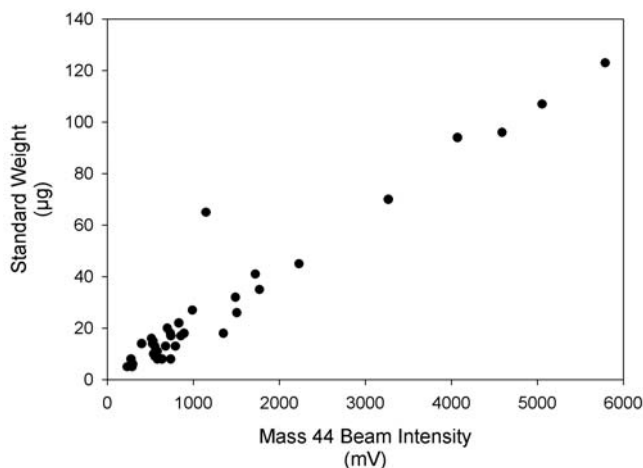could come from atmospheric CO_2 dissolved in the solution. Alternatively, some atmospheric contamination may leak into the Exetainer vials when the septum is punctured to acidify the samples. Extrapolation of linear regression of the

Figure 3. Mass 44 beam intensity versus weight of standard NBS-19.

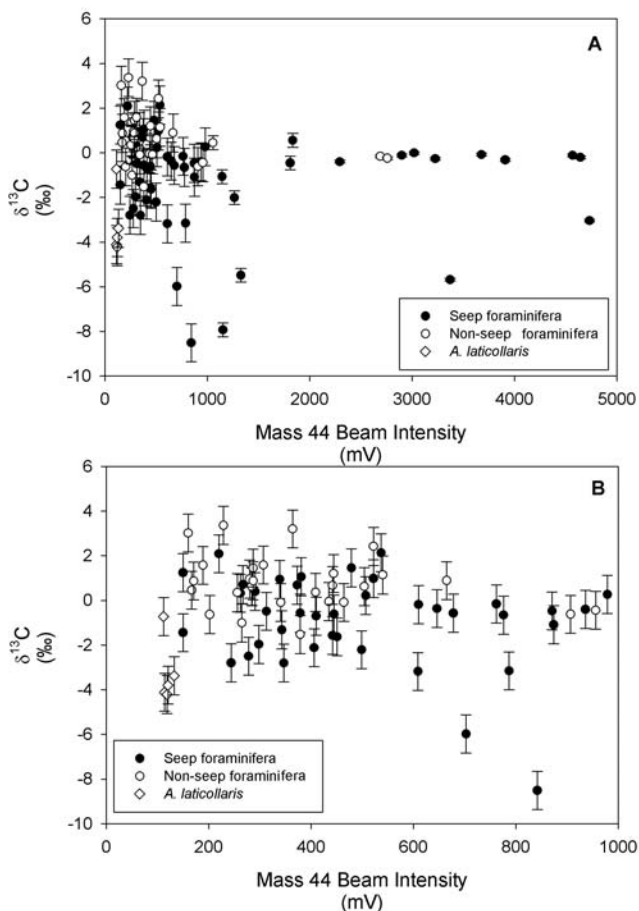**Figure 4.** The $\delta^{13}C_{test}$ values of foraminifer versus mass 44 beam intensity. (a) All measured specimens. (b) Samples with beam intensity between 100 and 1000 mV. All values were corrected using equation (2).

have considerable scatter, they show a linear relationship (Figure 2b). Regression analysis of the data yielded a linear relationship between the measured $\delta^{13}C$ values ($\delta^{13}C_{meas}$) and beam intensity (BI) of

$$\delta^{13}C_{meas} = 0.0045*BI - 2.71 \qquad (1)$$

with an R^2 value of 0.51. This equation represents the best fit for the data, and inclusion of standard isotope ratios into the linear regression with beam intensities > 1000 mV degraded the fit of the relationship. This relationship was used to correct the measured value of the standard to +1.95‰ using

$$\delta^{13}C_{corr} = \delta^{13}C_{meas} + (1.95 + 2.71) - (BI*0.0045) \qquad (2)$$

where $\delta^{13}C_{Corr}$ is the corrected value for measured $\delta^{13}C$ value. Following corrections using equation (2), the average value of standards was +1.94 ± 0.85‰. For samples in this range of beam intensities, the accuracy is thus estimated to be ± 0.85‰.

3.2. Sample Analyses

[18] Of the 96 foraminifera that were measured for $\delta^{13}C$, 10 were cultured in the laboratory, 56 were from cold seep sites, and 30 were from sediments surrounding the cold seeps (Figure 4). The cultured foraminifera were *Allogromia laticollaris*, which is a tectinous species (i.e., lacks a carbonate test [*Arnold*, 1955]). This species was included to observe the effects of phosphoric acid on the foraminiferal cytoplasm and to estimate potential effects of foraminiferal cytoplasm on isotopic analyses. Four of the *A. laticollaris* specimens each produced beam intensities < 100 mV and thus no isotope ratios were generated. The beam intensity of the remaining four specimens ranged between 112 and 133 mV. The average beam intensity was 102 mV for all nine samples, suggesting that approximately an equal amount of CO_2 was produced by the foraminifera and the PBS and phosphoric acid blank.

[19] The origin of CO_2 from the *A. laticollaris* samples is unclear. The 10 min phosphoric acid treatment is unlikely to have oxidized organic carbon of the foraminifera to CO_2 in the oxygen-free headspace of the Exetainer vials. Water within the foraminifera could contain dissolved CO_2, but the amount is probably vanishingly small. Approximately 6 orders of magnitude less CO_2 (by moles) would be liberated from water in foraminiferal cytoplasm than liberated from a 50 μg foraminiferal test, assuming foraminiferal cytoplasm weighed around 50 μg and contained 50 wt. % water with DIC concentrations of around 2 mmol. *A. laticollaris* has been found to contain refractory solid inclusions containing Ca and P and several other species of calcifying benthic foraminifera have been found to contain similar Mg, Ca, and P-bearing inclusions [*Bentov and Erez*, 2005; *West et al.*, 1995]. These inclusions could have up to 6 mol % CO_2 and would be soluble in acid if they are similar in composition to apatite [*Deer et al.*, 1966]. They are enveloped by cytoplasm, but because cytoplasm is soluble, the inclusions could also dissolve, generating CO_2. Each of these sources of CO_2 could contribute to the blank from the *A. laticollaris*, but there could also be other unknown sources. As described below, calcifying foraminifera produce considerably more CO_2 than the tectinous *A. laticollaris*, and its isotopic ratio appears to be close to the value of the carbonate mineral. Consequently, blank contributed from cytoplasm is unlikely to greatly alter values obtained from the foraminiferal tests.

[20] Ten individual calcifying specimens produced beam intensities that ranged from 150 to 244 mV. Although isotope data were reported by the mass spectrometer for these samples, the beam intensities were considered too low to be correctable (e.g., Figure 2b) and these values were not included in analyses of the data (Bernhard et al., submitted manuscript, 2009). Each species with beam intensities between 150 and 244 mV (*Bolivina* sp., *Bulimina mexicana*, *Buliminella tenuata*, *Chilostomella* sp., *Cibicides wuellerstorfi*, and *Gavelinopsis* sp.) provided additional specimens that produced sufficient CO_2 for analyses, so that paired isotope ratios and ultrastructure of these species could be evaluated.

[21] The corrected $\delta^{13}C_{test}$ values ranged from −1.5‰ to +3.2‰ (*Praeglobobulimina spinescens* and *B. mexicana*,

respectively) in nonseep calcareous specimens and from $-8.5‰$ to $+2.1‰$ (*Globobulimina pacifica* and *C. wuellerstorfi*, respectively) in seep specimens. Foraminifera with > 2000 mV beam intensity and accuracies of $± 0.06‰$, were restricted to *C. wuellerstorfi* (n = 10), *B. mexicana* (n = 1), and *G. pacifica* (n = 1). These specimens had corrected $\delta^{13}C_{test}$ values that ranged from -5.7 to $0.0‰$. An additional 7 specimens yielded beam intensities of between 1000 and 2000 mV and thus $\delta^{13}C_{test}$ values could be measured with an error of around $± 0.31‰$. These specimens included *Gavelinopsis* sp., *Uvigerina peregrina*, *G. pacifica*., and *C. wuellerstorfi*, and had corrected $\delta^{13}C_{test}$ values ranging from -7.0 to $+0.6‰$, providing significant differences between the $\delta^{13}C$ values of the samples (Figure 4a). The majority of the samples (n = 57) had beam intensities that ranged from 250 to 1000 mV and thus were measured with an estimated error of $± 0.85‰$ (Figure 4b). Although this error is about an order of magnitude higher than standard $\delta^{13}C$ techniques, the corrected isotope ratios range from -8.5 to $+3.2‰$, indicating that observed variations in the ratios are significant.

[22] As noted, because the foraminifera were placed in buffered glutaraldehyde within 1 h of core collection, all cellular ultrastructure of live foraminifera is preserved until microscopic examination using TEM (Figure 1). Ultrastructural analysis revealed that most organelle types, including mitochondria, nuclei, Golgi bodies, peroxisomes, vacuoles, food vacuoles, lipid bodies, and other items, including chloroplasts and externally attached bacteria can be distinguished (Figure 5), although not necessarily in every specimen examined. Some of the specimens in which organelles were highly degraded and where amorphous material occurred died prior to collection, rather than having been lysed by acidification during analyses (Figure 5d). At the present time only a limited number of specimens have been analyzed for their ultrastructure. Additional ultrastructural analysis is required to provide sufficient numbers of specimens for statistically relevant evaluation of biological and environmental controls of their $\delta^{13}C_{test}$ values (Bernhard et al., submitted manuscript, 2009).

4. Discussion

[23] Paired information on a given foraminiferal test's carbon isotopic signal and its cellular ultrastructure provides insights into biological controls on isotopic compositions of foraminiferal carbonate. These insights are gained in part from knowing for certain that the foraminifera were alive at the time of collection and thus are unaffected by overgrowths of authigenic carbonate that would alter the primary isotopic signal. Furthermore, the ultrastructural analyses can provide information on diet of the foraminifera [e.g., *Goldstein and Corliss*, 1994], and if symbionts are present. All of these biological factors could influence the isotopic composition of the foraminiferal tests.

[24] As noted, environmental interpretations utilizing this technique are forthcoming (Bernhard et al., submitted manuscript, 2009), but preliminary analyses show that the foraminiferal ultrastructures observed are interpretable and, in some cases, novel (Figure 5). For example, the large

vacuole veiled with peroxisomes has not been observed previously (Figure 5c), although peroxisome-endoplasmic reticulum complexes are common in certain benthic foraminifera [*Bernhard and Bowser*, 2008]. Although the use of phosphoric acid to decalcify specimens is harsher than the conventionally used EDTA, osmication prior to decalcification sufficiently stabilized the cell membranes from the phosphoric acid treatments so that they could be sectioned and observed (Figure 5). Direct transfer from phosphoric acid to PBS and subsequently directly to 70% ethanol also increased staining intensity compared to conventional methods, so it is difficult to directly compare TEM images of conventionally processed specimens and those obtained from examination of this phosphoric acid-treated material.

[25] Some general remarks can be made about the mass spectrometry results obtained using this new procedure. Foraminifera from within seeps had nearly twice the average beam intensity (1227 mV) compared to foraminifera from nonseep sediments (660 mV). These differences in beam intensities indicate larger foraminifera were collected from within the seep sites than nonseep sites, and thus the overall lower $\delta^{13}C_{test}$ values observed for foraminifera within the seeps are not a result of an artifact of blank contamination from small samples or fractionation to lighter isotopes in small foraminifera [e.g., *McCorkle et al.*, 2008]. The isotopically lighter values of seep foraminifera are far from equilibrium with $\delta^{13}C_{DIC}$ values of the pore waters, which are as low as about $-40‰$ [*Martin et al.*, 1997]. The isotopic values of the nonseep specimens are similar to values found elsewhere. Disequilibrium of seep $\delta^{13}C_{test}$ from the seep $\delta^{13}C_{DIC}$ could result from vital effects, such as diet, or from presence or absence of symbiotic bacteria, but complete evaluation of these biological attributes require further analyses of cellular ultrastructure from hundreds of specimens, more than we have currently analyzed.

[26] This method has great potential to be expanded to different habitats, organisms, and chemical proxies and could be used for simultaneous studies of tissues and $\delta^{13}C$ values of carbonate-forming organisms such as planktonic foraminifera, corals, pteropods, juvenile mollusks, echinoderms, and coccolithophorids. Expansion of the technique to other organisms would be limited by their modes of growth and whether organisms and their skeletal structures require microdissection prior to analyses. The method could be also modified for ICP-MS analyses on solutions used to dissolve the foraminiferal tests to obtain linked metal/Ca ratios, $\delta^{13}C$ values of individual foraminiferal tests, and TEM ultrastructural analysis. The primary issues that would need to be resolved for this refinement include the level of blank contamination from the reagents and the concentration of the metals originating from the foraminifera in the solution. Both of these issues would require ultrapure reagents, limiting reagent volumes, and measurement of blanks on all reagents.

[27] The technique we describe cannot be used for the measurement of $\delta^{18}O$ values of foraminiferal tests or other carbonate-producing organisms. Although the $\delta^{18}O$ value of foraminiferal carbonate is an important paleoenvironmental tracer, measured $\delta^{18}O$ values of both the samples and standards are contaminated by the large volumes of water in
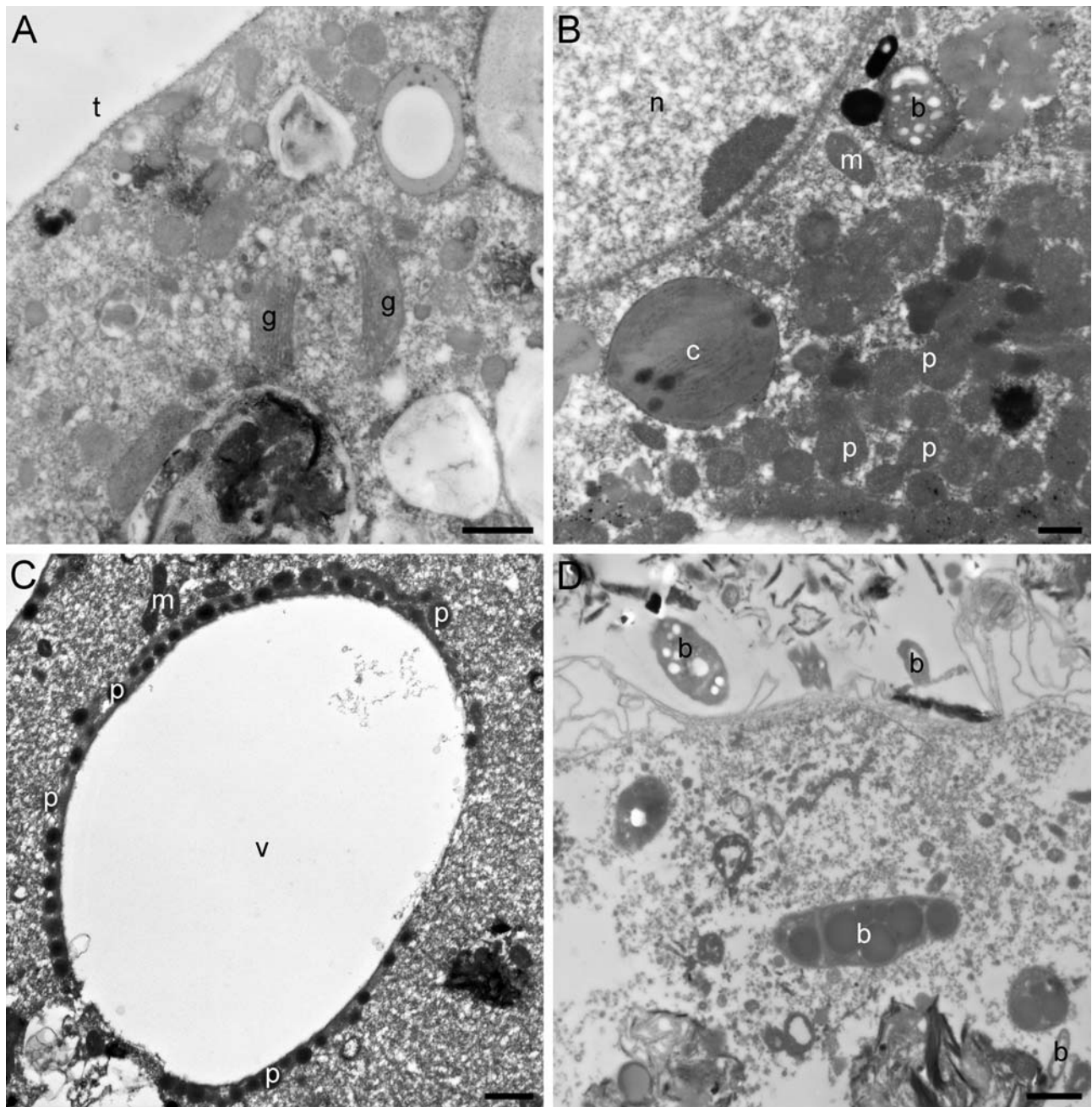
Figure 5. Transmission electron micrographs of benthic foraminifera processed for paired isotope-ultrastructure analyses. These specimens were small and thus had an estimated accuracy of ± 0.85‰ for their $\delta^{13}C$ values. (a) *Uvigerina peregrina* from a nonseep site; $\delta^{13}C = -0.6‰$. Ectoplasm showing paired Golgi bodies (g); t is location of test prior to decalcification. (b) *Buliminella tenuata* from a seep site; $\delta^{13}C = -2.1‰$. Endoplasm showing numerous peroxisomes (p), mitochondria (m), an endobiotic bacterium (b), a sequestered chloroplast (c), and a portion of nucleus (n). (c) *Buliminella tenuata* from a nonseep site; $\delta^{13}C = 0.6‰$. Endoplasm showing a large vacuole (v) fringed with peroxisomes (p). (d) *Bulimina mexicana* from a seep site; $\delta^{13}C = -0.7‰$. Varied morphotypes of bacteria (b) and remnant foraminiferal cytoplasm indicate that the bacterial ultrastructure was intact while identifiable organelles are absent; this foraminiferal cytoplasm was highly degraded at the time of fixation. Scale bar for Figures 5a and 5b is 0.5 μm; scale bar for Figures 5c and 5d is 1.0 μm.

PBS. Measured values of $\delta^{18}O$ for the NBS-19 standard values ranged from around +6 to around +11‰, considerably higher than the established value for NBS-19 of −2.2‰ [*Gonfiantini*, 1984].

[28] The primary limitations of the technique described here stem from the volume of gas that is evolved from small foraminifera and contamination from blanks. Nonetheless, even with blank contamination, 17 (or about 20%) of the samples required minimal corrections to their $\delta^{13}C$ values and had an accuracy of 0.31‰ or better. The limitations caused by contamination could be improved through collection of larger specimens, but this restriction would limit the species and range of individual sizes that could be measured. Comparison of isotopic compositions and ultrastructures at different size fractions could be important for assessing previously observed differences in isotopic ratios of species depending on specimen size [*McCorkle et al.*, 2008; *Schmiedl et al.*, 2004]. Multiple conspecifics from discrete samples could be dissolved simultaneously to increase the volume of evolved CO_2, but pooling individuals increases the risk of mixing live and dead foraminifera (e.g., Figure 5), preventing comparison of an individual's ultrastructure with its isotope ratio. The limitation from contamination may be improved through bubbling inert gas such as He through the PBS to remove CO_2 contamination. Reducing the volume of PBS could also lower the blank, but this modification would increase the risk of desiccating the foraminifera, thereby destroying their ultrastructure.

[29] Simultaneous measurement of $\delta^{13}C_{test}$ values and information on foraminifera's biology, including unequivocal identification of live specimens, presence or absence of symbionts, and observations regarding prey type, provides unprecedented information about controls, individual variability, and significance of an important paleoenvironmental tracer. We look forward to modifications of the method to make it even more powerful and applicable to a wider range of habitats, organisms, and proxies.

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