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Effects of heavy-metal contaminants (Cd, Pb, Zn) on benthic foraminiferal assemblages grown from propagules, Sapelo Island, Georgia (USA)

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ABSTRACT

Benthic foraminifera have long served as indicators of environmental conditions – both natural and anthropogenically impacted. To better understand the responses of benthic foraminifera to specific heavy metal contaminants (Cd, Pb, Zn), assemblages of coastal benthic foraminifera were grown from propagules (tiny juveniles) in the lab with exposure to a single heavy metal over a range of concentrations, based on the US Environmental Protection Agency's Critical Maximum Concentration (CMC) values. Foraminiferal propagule banks were collected from relatively pristine mudflats, located on the southern end of Sapelo Island, Georgia (USA). Consistent with the findings of numerous field-based studies, foraminifera were found to respond negatively to Cd, Pb, and Zn. Overall, assemblages grown with exposure to higher concentrations of these metals are characterized by decreased abundances, species richness, and evenness. All of the acute responses observed in these metrics occur at concentrations equal to or somewhat higher than the USEPA's CMC values. Foraminiferal responses vary by metal, though the four most common species (two monothalamids: *Ovammina opaca* Dahlgren, *Psammophaga sapela* Altin Ballero, Habura, Goldstein; and two rotaliids: *Haynesina germanica* (Ehrenberg), *Ammonia tepida* (Cushman)) responded in a broadly similar fashion. The monothalamid species however may be more sensitive to high concentrations of each metal. Of the metals examined, exposure to Pb had the most deleterious effect, followed by Zn, then Cd. These four most abundant species appear to be more tolerant of Cd than the other metals. Zn was the only metal in the study that produced abundant aberrant test morphologies. *Ammonia tepida* grew abnormally more frequently than any other species encountered and exhibited a distinctive enlarged aperture as well as aberrant patterns of calcification, chamber arrangement, and enlarged pores. Abnormal tests were also found in *H. germanica* and a miliolid. The monothalamid species did not produce aberrant test morphologies. Results support the application of foraminifera as bio-indicators in polluted environments.

1. Introduction

Benthic foraminifera, abundant and sensitive to a wide range of environmental parameters, are strongly affected by chemical contaminants in coastal settings (see reviews: Alve, 1995; Martin, 2000 and papers therein; Scott et al., 2001; Nigam et al., 2006; Martinez-Colon et al., 2009; Armynot du Chatelet and Debenay, 2010; Frontalini and Coccioni, 2011). Responses to heavy-metal exposure may include the appearance of aberrant test morphologies (e.g., Alve, 1991; Yanko et al., 1994; Samir and El-Din, 2001; Olugbode et al., 2005; Polovodova and Schönfield, 2008; Caruso et al., 2011; Hart et al., 2017) and changes in assemblage species composition, diversity and abundance (e.g., Ellison et al., 1986; Yanko et al., 1998; Samir, 2000; Frontalini and Coccioni, 2008), among others. As a result, foraminifera have been promoted as effective bio-indicators in monitoring both coastal pollution (e.g., Coccioni, 2000; Scott et al., 2001; Armynot du Chatelet and Debenay, 2010; Frontalini and Coccioni, 2011) and the progress of recovery following environmental remediation (Alve, 1995; Alve et al., 2009; Hess et al., 2014). Furthermore, the persistence of mineralized tests of many taxa in sediments provides insight into the geohistorical record of both pollution and changing environments.

Understanding specific foraminiferal responses to pollution from field-based studies alone, however, presents a challenge. Environments, particularly those of the coastal zone, are ever-changing, and environmental parameters, such as salinity, temperature, and patterns of sedimentation, often shift in complex ways that may impact foraminiferal distributions, abundance, diversity, and the occurrence of aberrant test morphologies (e.g., Boltovskoy et al., 1991; Stouff et al., 1999; Geslin

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et al., 2000, 2002). Furthermore, impacted coastal settings often contain multiple contaminants that may behave differently according to sediment type, pH, salinity and various microbial processes, all of which have the potential to affect the bioavailability of a contaminant (Adriano, 2001; reviewed by Martinez-Colon et al., 2009). As a result, identifying the effect(s) of a specific contaminant or change in a single environmental parameter on foraminifera using field-based studies alone is often problematic (Armynot du Chatelet and Debenay, 2010). Laboratory-based, experimental studies therefore are needed to better understand the effects of a contaminant under conditions that are better constrained (e.g., Gustafsson et al., 2000; Le Cadre and Debenay, 2006; Nigam et al., 2009; Denoyelle et al., 2012; Frontalini et al., 2015; Nardelli et al., 2016; Frontalini et al., 2018a).

The goal of this study is to experimentally assess the responses of common coastal foraminifera, both assemblages and select individual species, grown with exposure to a single heavy metal (Pb, Cd, Zn). To accomplish this, we utilized the Propagule Method (Goldstein and Alve, 2011; Alve and Goldstein, 2014) to grow assemblages of coastal foraminifera from propagules (juveniles) with exposure to a single heavy-metal contaminant over a range of concentrations based on the United States Environmental Protection Agency's (USEPA) National Recommended Water Quality Criteria for Saltwater. This approach allows us to control several important environmental parameters (salinity, temperature, illumination, sediment source) while varying the contaminant concentration. Thus, we can assess the impacts of individual contaminants without most of the confounding effects of unpredictable fluctuations in environmental conditions or exposure to multiple contaminants. Furthermore, by exposing foraminifera to a contaminant during growth from very small juveniles (often just ~10–50 μm), we can better assess whether exposure produces aberrant test morphologies. Those responses to heavy metal exposure examined during the course of this study include: a) the impact on both abundance and species richness within experimentally grown assemblages, b) the contaminant concentrations, at least in broad terms, at which deleterious effects occur, c) species-specific effects and the identification of potential bio-indicator species, and d) the occurrence of aberrant test morphologies.

2. Materials and methods

Assemblages of foraminifera were grown from propagule banks present in the fine sediment fraction of mudflat sediments collected on Sapelo Island, Georgia (USA). Foraminiferal propagules, tiny juveniles that undergo dispersal both within and beyond the distribution of conspecific adults (Alve and Goldstein, 2003, 2010), are very abundant on Sapelo Island mudflats and can be used experimentally to address a variety of ecological questions regarding benthic foraminifera (Goldstein and Alve, 2011; Alve and Goldstein, 2014). Sapelo Island, Georgia was chosen as a field site because it is accessible and generally lacks anthropogenic pollution and disturbances. Sediment samples were collected from a mudflat near the Island's lighthouse (31° 23.384′N, 81° 17.072′W; Fig. 1), located at the southern end of the island.

Sediment was collected in October 2007, and experiments were established as described below. Additional sediment was collected in July and December 2008 to expand the study (include treatments with higher contaminant concentrations and potentially correct for a delay in analyzing water samples; see below). Sediment was collected by carefully scraping the upper few mm of the mudflat surface while avoiding the prominent, sulfidic subsurface. Sediment was sieved using two stainless-steel sieves (850 and 63 μm) and sand-filtered seawater (University of Georgia Marine Institute's seawater system) immediately after collection. The larger sieve removed detrital plant material and gastropods, while the smaller sieve separated the fine sediment fraction (< 63 μm), which includes the foraminiferal propagule bank, from coarser sediments (63–850 μm) that contain larger juvenile and adult in situ foraminifera. The < 63-μm fraction was retained and transported

Fig. 1. Sapelo Island, Georgia. Image from Google Earth. Sapelo Island is ~5 miles east of the coastal mainland. The Lighthouse mudflats are located at the southern end of the island (arrow). Inset shows location along the eastern U.S. coast.

to the University of Georgia. Sediments were allowed to settle for 2–3 days, after which the overlying seawater was siphoned away, and the sediment was then thoroughly mixed. Twenty-mL aliquots of the < 63-μm fraction were placed in experimental containers (polypropylene, 118-mL capacity, round with tightly fitting lids) along with 40 mL of artificial seawater (Instant Ocean; Aquarium Systems) that was diluted with distilled water to match the salinity at the initial time of collection (32‰). The same salinity was used for all experimental treatments and controls. The pH of Instant Ocean at this salinity is ~8.0. The sediment is fine-grained and clay-rich, though mean grain size was not determined.

Concentrations of heavy metal contaminants were added individually to the experimental containers based on the United States Environmental Protection Agency's National Recommended Water Quality Criteria, the Saltwater Criteria Maximum Concentration (CMC) (Cd 0.040 $mg\,L^{-1}$; Pb 0.210 $mg\,L^{-1}$; and Zn 0.090 $mg\,L^{-1}$; see http://water.epa.gov/scitech/swguidance/standards/criteria/current/index) and increased by an order of magnitude for four or more concentration levels of each individual heavy metal per treatment (Table 1). Each treatment included a replicate, and controls produced assemblages grown without exposure to a contaminant. The USEPA's guidelines specify that the CMC is the amount of a specific heavy metal that an "aquatic community can be exposed to briefly without resulting in an unacceptable effect". Heavy metals were added as dissolved chlorides with the exception of Pb, which was added as a carbonate, acidified prior to use. All assemblages were grown at a constant temperature of 18 °C and provided illumination on a 12-h cycle. After four weeks, the experimentally grown assemblages, including the controls, were harvested by sieving over a 63-μm sieve and preserved using 10% buffered formalin (buffered with sodium carbonate). All foraminifera harvested were included in the counts regardless of whether they were alive or dead at the time of harvesting because they had grown during the 4 weeks of the experiment. This study includes 40 samples (8 controls, 12 Cd treatments, 12 Zn treatments, 12 lead treatments). All samples

Table 1
Heavy metal concentrations at the onset and conclusion of the experiments. The latter are used for plots in Figs. 2 & 3.

Concentration ($mg\ L^{-1}$) at onset of experiment	October 2007 Concentration measured post-experiment	October 2007 Replicate	December 2008 Concentration measured post-experiment	December 2008 Replicate
Cadmium (detection limit = 0.0192 $mg\ L^{-1}$; BDL = below detection limit)				
0.024	0.04	BDL	0.03	0.02
0.24	0.36	0.36	BDL	BDL
2.4	3.60	0.55	BDL	0.02
24	11.20	10.36	BDL	0.03
240	39.60[a]	25.61[a]	0.87	0.62
2400	1582.37[a]	1599.31[a]	32.07	26.97
Zinc (detection limit = 0.027 $mg\ L^{-1}$)				
0.0432	BDL	BDL	BDL	BDL
0.432	BDL	BDL	BDL	BDL
4.32	BDL	BDL	BDL	BDL
43.2	1.01	0.12	BDL	BDL
432	170.14[a]	158.57[a]	BDL	BDL
4320	3429.38[a]	3449.18[a]	10.11	5.202
Lead (detection limit = 0.0275 $mg\ L^{-1}$)				
0.162	0.09	0.11	0.11	0.10
1.62	0.07	0.09	0.10	0.09
16.2	0.08	0.13	0.09	0.09
162	0.33	0.21	0.17	0.17
1620	2.10[b]	1.94[b]	2.10	1.94
16,200	13.97[b]	12.80[b]	13.97	12.80

[a] Samples were collected in July 2008.
[b] Samples were collected in December 2008.

were picked wet (no sample was air-dried), and foraminifera were identified, counted, and assessed for aberrant morphologies. These counts include monothalamid foraminifera.

At the time of harvesting, water in each culture container was decanted and stored in centrifuge tubes (Nalgene; 50 cc) for approximately a year prior to analysis of metals. After a year, water samples were filtered through a 0.45 μM membrane filter (Whatman, USA) and acidified to 10% with analytical-reagent grade HNO_3. The analytical determination of metals (Cd, Pb and Zn) was carried out using inductively coupled plasma optical emission spectrometry (ICP-OES; 4300DV; PerkinElmer-Sciex, Waltham, MA USA). For ICP-OES analysis, samples were dissolved and diluted in 0.16 M ultra high purity HNO_3. High-purity water (electrical resistivity > 18.2 MΩ cm) was produced with a Milli-Q system (Millipore, MA, USA).

Calibration was obtained with NIST traceable external standards. Standard solutions were prepared by diluting a 1000 $mg\ L^{-1}$ multi-element solution (ICP Multielement standards, PlasmaCal, Canada and Inorganic Ventures, USA) with 0.16 M ultra high purity HNO_3. Duplicate samples, blanks and internal calibration verification (ICVs) were analyzed at a rate of 15% of total samples and were accepted at a 90% accuracy rate.

The instrument detection limit (IDL) was calculated by obtaining 7 to 10 consecutive measurements of reagent blank solution. This parameter was calculated as the average of blank readings plus 3 times their standard deviation. All metal concentrations were determined in triplicates. Because nitric acid was not added to the October 2007 and July 2008 samples until October 2008, metals may have adsorbed to the Nalgene tubes, thus reducing their concentration prior to analysis. To address this problem, the growth experiments were repeated in December 2008, using freshly collected sediment, in an attempt to determine a correction factor to account for potential adsorption. These experiments were conducted using the same protocols described above with identical concentrations of heavy metals. When the December 2008 water samples were analyzed following the above procedures, metal concentrations were significantly lower than the October 2007 and July 2008 samples, which was not expected. Nonetheless, the trends in the data set are the same, in that the higher concentrations of heavy metals added experimentally yielded higher measured ICP-OES results. We expected to see higher concentrations of heavy metals in the ICP-OES analysis for the calibration suite of samples because they were treated and analyzed immediately after harvesting, thus reducing adsorption onto the walls of the Nalgene tubes and suspended fine sediment. This was not the case, and no correction factor was employed. Although the October 2007 and July 2008 water samples were left untreated they must contain at least the concentration measured a year after the conclusion of the experiment. Therefore, the overall trends are accurate and values should represent minimum values for the experimental heavy metal concentrations.

Images of specimens were obtained using the Scanning Electron Microscope (SEM) in the School of Veterinary Science and The Center for Ultrastructure Research (now Georgia Electron Microscopy) at the University of Georgia. Images of specimens with aberrant test morphologies were examined to characterize alterations in test morphology.

Statistical analyses and graphical interpretation were performed using R version 2.5.1 (R Development Core Team, 2007). Individual scatter plots include abundance (total individuals in an assemblage), species richness (number of species in a sample), evenness as indicated by Simpson's Diversity Index ($D = (\Sigma p_i^2)^{-1}$; Simpson, 1949), and abundances of each of the four most common species vs. Cd, Zn, and Pb concentrations as measured by ICP-OES after the conclusion of the experiment. For concentrations that fall below the detection limits of the instrument (Cd 0.0192 L^{-1}, Pb 0.0275 L^{-1}, Zn 0.027 L^{-1}), half of the detection limit is used and plotted to the left of the line indicating the detection limit. Linear regressions and exponential regressions are fitted to each data set and the exponential regression is illustrated on the graphs. The slope of the exponential regressions and R^2 values are reported to illustrate the acuteness of contaminant effects. For this study, an R^2 value of 0.600, $p \leq .0005$ or greater is considered significant.

3. Results

Foraminifera respond negatively to high concentrations of Cd, Zn and Pb as indicated by trends in total abundances, species richness, Simpson's Index, and trends in the abundances of individual species (Figs. 2 & 3). Responses, however, were not uniform to these three metals. Of the metals examined, Pb produced the most acute effects. Overall, the four most common species responded similarly to each of these metals. However, the monothalamids (*Ovammina opaca* and *Psammophaga sapela*) appear to be somewhat more resilient to low concentrations of Cd, Zn, and Pb than the rotaliids (*Ammonia tepida* and *Haynesina germanica*) but suffer greater negative effects at the highest concentrations of all the metals in the study (Fig. 3).

The heavy metal concentrations added to the experiments at the start of the study are based on the USEPA's guidelines for saltwater environments. Those concentrations measured 1 year after the assemblages were harvested, however, are significantly less (Table 1). This may reflect adsorption onto sediment and organic particles during the 4-week experiment (e.g., Frontalini et al., 2018a), adsorption onto the container walls, and/or interactions with the living, non-foraminiferal microbiota. The heavy metal concentrations measured by ICP-OES should be regarded as minimal values that most likely declined over the course of the experiments.

3.1. Trends in overall abundance

Eight controls (no metals added) were evaluated (4 from October 2008, 2 from July 2009, and 2 from December 2009). On average, 1374 (range 1099–1571) individual foraminifera grew from propagules present in 20 mL of sediment in the controls. *Ovammina opaca*, *P. sapela*, *H. germanica*, and *A. tepida* dominate the assemblages in both the controls

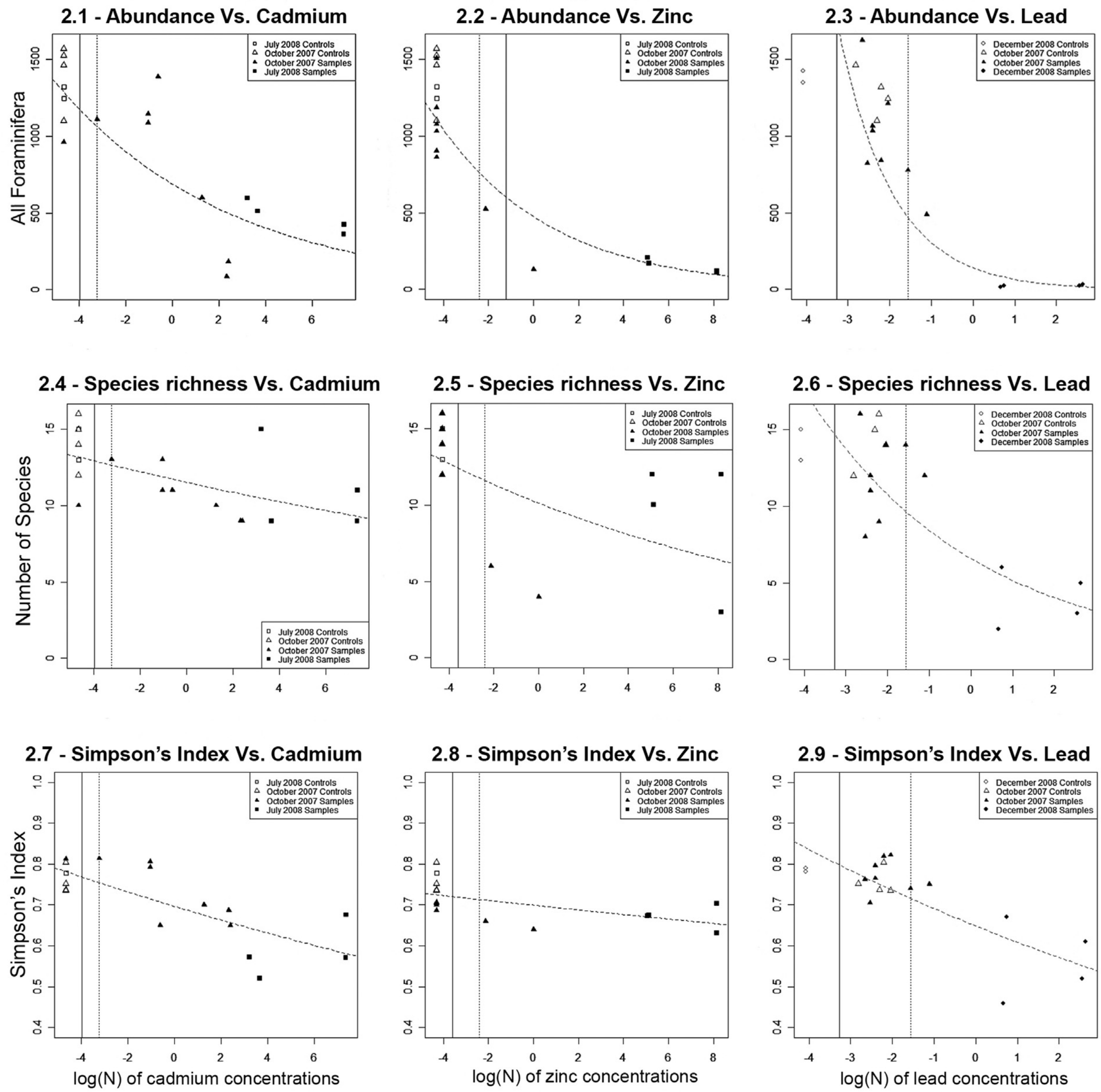

Fig. 2. Foraminiferal abundances (2.1–2.3), species richness (2.4–2.6), and Simpson's Index (2.7–2.9) plotted against the natural log of individual heavy metal concentrations. The dashed vertical line is the U.S. EPA CMC limit. ICP-OES measurements that are below the instrument detection limits are plotted to the left of the solid vertical line.

and experimental treatments. Along with *Buliminella elegantissima* and *Miliammina fusca,* these four species (Fig. 4) comprise at least 80% of most assemblages. Species that grew abundantly in the experiments are also abundant in the in situ assemblages at the sampling site (Goldstein and Alve, 2011; Weinmann and Goldstein, 2016).

In all cases, assemblages grown with exposure to high concentrations of metals yielded fewer foraminifera than those of the controls. In Cd treatments, abundances are similar to those of the controls (Supplementary Data Table 1) at concentrations up to 0.55 $mg\,L^{-1}$, which is greater than the EPA's CMC value of 0.04 $mg\,L^{-1}$. Cd concentrations of 3.6 $mg\,L^{-1}$ (the next highest concentration measured) result in an acute effect on the total abundance, with reductions by half or more, and abundances decline still further at higher concentrations (Fig. 2.1; Supplementary Data Table 1). The slope of the exponential regression is -0.134 (Table 2).

Abundances in assemblages grown with exposure to Zn are comparable to those of the controls at concentrations up to at least the detection limit of 0.027 $mg\,L^{-1}$ for the ICP-OES (Fig. 2.2; Supplementary Data Table 1). An acute response was found at the lowest Zn concentration measured, 0.12 $mg\,L^{-1}$, which is just slightly greater than the EPA's CMC value of 0.09 $mg\,L^{-1}$. The reduction in foraminiferal abundances produced an exponential regression slope of -0.195 (Table 2).

Assemblages grown with exposure to Pb exhibit the most acute effects on abundance observed in this study. At low concentrations ($\leq$ 0.13 $mg\,L^{-1}$), total abundances in assemblages are high and generally comparable to those of the controls (Fig. 2.3; Supplementary Data Table 1). Concentrations of Pb at 0.21 and 0.33 $mg\,L^{-1}$ correspond to abundances reduced by approximately half in experimentally grown assemblages. The EPA's CMC value for Pb is 0.21 $mg\,L^{-1}$. Pb concentrations above these values correspond to a > 95% reduction in foraminiferal abundances (range from 17 to 35 individuals per assemblage). The slope of the exponential regression is -0.772, the steepest of the three metals tested (Table 2).

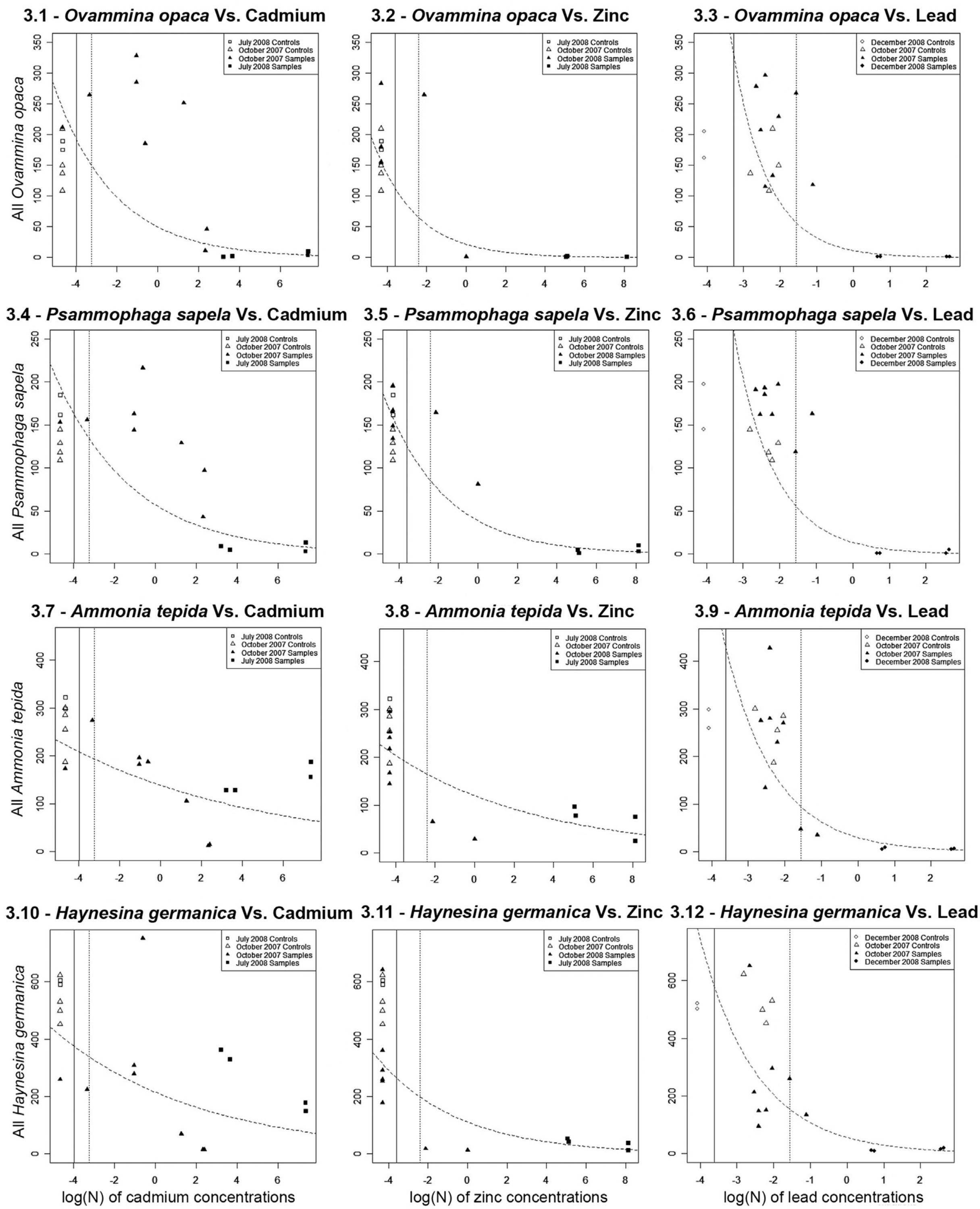

Fig. 3. *Ovammina opaca* (3.1–3.3), *Psammophaga sapela* (3.4–3.6), *Ammonia tepida* (3.7–3/9), and *Haynesina germanica* (3.10–3.12) plotted against the natural log of individual heavy metal concentrations. The dashed vertical line is the U.S. EPA CMC limit. ICP-OES measurements that are below the instrument detection limits are plotted to the left of the solid vertical line.

3.2. Species richness and evenness (Simpson's index)

To compare samples of uneven abundances, species richness (the number of species present in each assemblage) and Simpson's Diversity Index are used to describe the assemblages. Species richness in the controls ranged from 12 to 16. The lowest values for species richness (2–4) are found in assemblages grown with exposure to a heavy metal, particularly Pb and Zn. Trends, however, vary.

In assemblages grown with exposure to Cd, richness values are only slightly less (9–15 species) than in the controls (Fig. 2.4; Supplementary Data Table 1). As a result, no acute effect on species richness is observed in the Cd treatments, and the slope of the regression is only slightly negative (−0.029; Table 2).

Results in treatments with Zn vary (Fig. 2.5; Supplementary Data

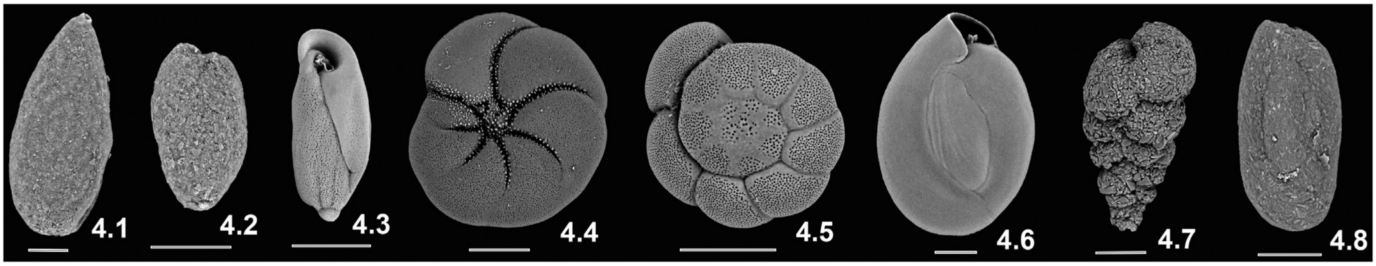

Fig. 4. Non-deformed test morphologies of common taxa grown in experimental assemblages. 4.1 *Ovammina opaca;* 4.2 *Psammophaga sapela;* 4.3 *Buliminella elegantissima*; 4.4 *Haynesina germanica*; 4.5 *Ammonia tepida*; 4.6 *Quinqueloculina jugosa*; 4.7 *Textularia nitens*; 4.8 *Miliammina fusca*. Scale = 25 µm.

Table 2
Values for R^2 and the slope of the exponential regressions.

Exponential Regression Values		
	R^2	Slope
Abundance: All Foraminifera		
Cadmium	0.4697**	−0.134
Zinc	0.8466***	−0.195
Lead	0.8232***	−0.772
Species Richness		
Cadmium	0.333**	−0.029
Zinc	0.2605*	−0.057
Lead	0.6071***	−0.245
Simpson's Index		
Cd	0.5598***	−0.025
Zinc	0.2912*	−0.008
Lead	0.5877***	−0.063
Ovammina opaca		
Cadmium	0.5574***	−0.34
Zinc	0.8403***	−0.46
Lead	0.7936***	−1
Psammophaga sapela		
Cadmium	0.6441***	−0.262
Zinc	0.8341***	−0.406
Lead	0.7403***	−0.902
Ammonia tepida		
Cadmium	0.166	−0.102
Zinc	0.5591**	−0.132
Lead	0.8263***	−0.737
Haynesina germanica		
Cadmium	0.2079*	−0.134
Zinc	0.5807**	−0.241
Lead	0.7399***	−0.646

* $P \leq .05$; ** $P \leq .005$; *** $P \leq .0005$.

Table 1). At a Zn concentration of 1.01 mg L^{-1} species richness totaled 4, whereas 12 species were recorded at a concentration of 3429.4 mg L^{-1} and 3 species at 3449.18 mg L^{-1}. Because of such variation, no acute response in species richness to Zn is identified.

In assemblages grown with exposure to Pb, species richness remained comparable to that of the controls up to concentrations of 0.33 mg L^{-1} (Fig. 2.6; Supplementary Data Table 1). Pb concentrations result in the most acute affect on species richness at 1.94 mg L^{-1} with a slope of −0.245 (Fig. 2.6; Table 2). Assemblages grown with exposure to Pb concentrations of ≥1.94 mg L^{-1} contain only 2–6 species.

Simpson's Index is used to evaluate species evenness in the controls and experimental treatments. In the controls, Simpson's Index varies from 0.738–0.80. In the Cd treatments, values ranged from a high of ~0.8 at exposures up to 0.36 mg L^{-1}, to a low of ~0.52 at higher concentrations (Fig. 2.7; Supplementary Data Table 1). Simpson's Index generally declines with increasing exposure to Cd. The resulting slope of the regression is −0.025 (Table 2) with no abrupt change.

Exposure to Zn concentrations up to 3449.18 mg L^{-1} had no significant effect on Simpson's Index (Fig. 2.8; Supplementary Data Table 1). At all concentrations of Zn (< 0.027–3449.18 mg L^{-1}), Simpson's Index values are only slightly less than those of the controls. As a result, the slope of the regression is −0.008 (Table 2).

Exposure to Pb resulted in the most acute effect on Simpson's index at 1.94 mg L^{-1}, though the slope of the regression is only slightly negative, −0.063 (Table 2). In treatments with Pb concentrations of 0.33 mg L^{-1} or less, Simpson's Index values are broadly comparable to those of the controls. At higher concentrations, (1.94 mg L^{-1} or greater) Simpson's Index measures just 0.46–0.67; 0.46, the lowest values recorded in the study (Fig. 2.9; Supplementary Data Table 1).

3.3. Species trends

The four most abundant species that grew in the controls and treatments include two monothalamids: *Ovammina opaca* and *Psammophaga sapela*, and two rotaliids: *Ammonia tepida* and *Haynesina germanica*. All four species are abundant in the controls and treatments with relatively low concentrations of metals. All four species show precipitous declines in abundance in treatments with high concentrations of metals (Fig. 3; Supplementary Data Table 1). Overall, these four species show similar trends in abundance relative to metal concentrations with the exception of Cd (see below), but respond differently to different metals.

These four species appear to be more tolerant of Cd than to either Zn or Pb (Fig. 3). With exposure to low concentrations of Cd (0.0192–3.6 mg L^{-1}), both monothalamid species had abundances that were greater than or similar to those of the controls. High abundances, therefore, occurred in treatments that exceeded the EPA's CMC value of Cd (0.04 mg L^{-1}). Treatments that included higher concentrations of Cd (10.36 mg L^{-1} or above), however, hosted far fewer monothalamids (Figs. 3.1, 3.4). The abundance patterns of the rotaliid species (Figs. 3.7, 3.10) differed from those of the monothamids in that abundances are generally lower in all treatments compared to those of the controls, and abundances declined with increasing concentrations of Cd. However, abundances remained significant (generally > 100 individuals per treatment) even at Cd concentrations that exceed the EPA's CMC by 4 orders of magnitude.

Both the monothalamids and rotaliids are much less tolerant of Zn and Pb at concentrations above the EPA's CMC (0.09 and 0.21 mg L^{-1} respectively) (Fig. 3). However, *Ammonia tepida* appears to tolerate exposure to Zn better than the other taxa. Though population sizes are much reduced at high concentrations of Zn, 75 individuals grew in a treatment with a concentration (3449 mg L^{-1}; measured after the conclusion of the experiment) that was ~6 orders of magnitude above the CMC value. *Haynesina germanica* showed a similar trend in abundance with exposure to Zn, but with smaller population sizes. Pb had a strikingly acute effect on all four of the common species. *Ovammina opaca* is absent in treatments with Pb concentrations above 0.33 mg L^{-1}, and just a few individuals of *Psammophaga sapela* were recorded in treatments with high Pb concentrations. Likewise, just a few individuals of both of the rotaliids were recorded in treatments with high concentrations of Pb.

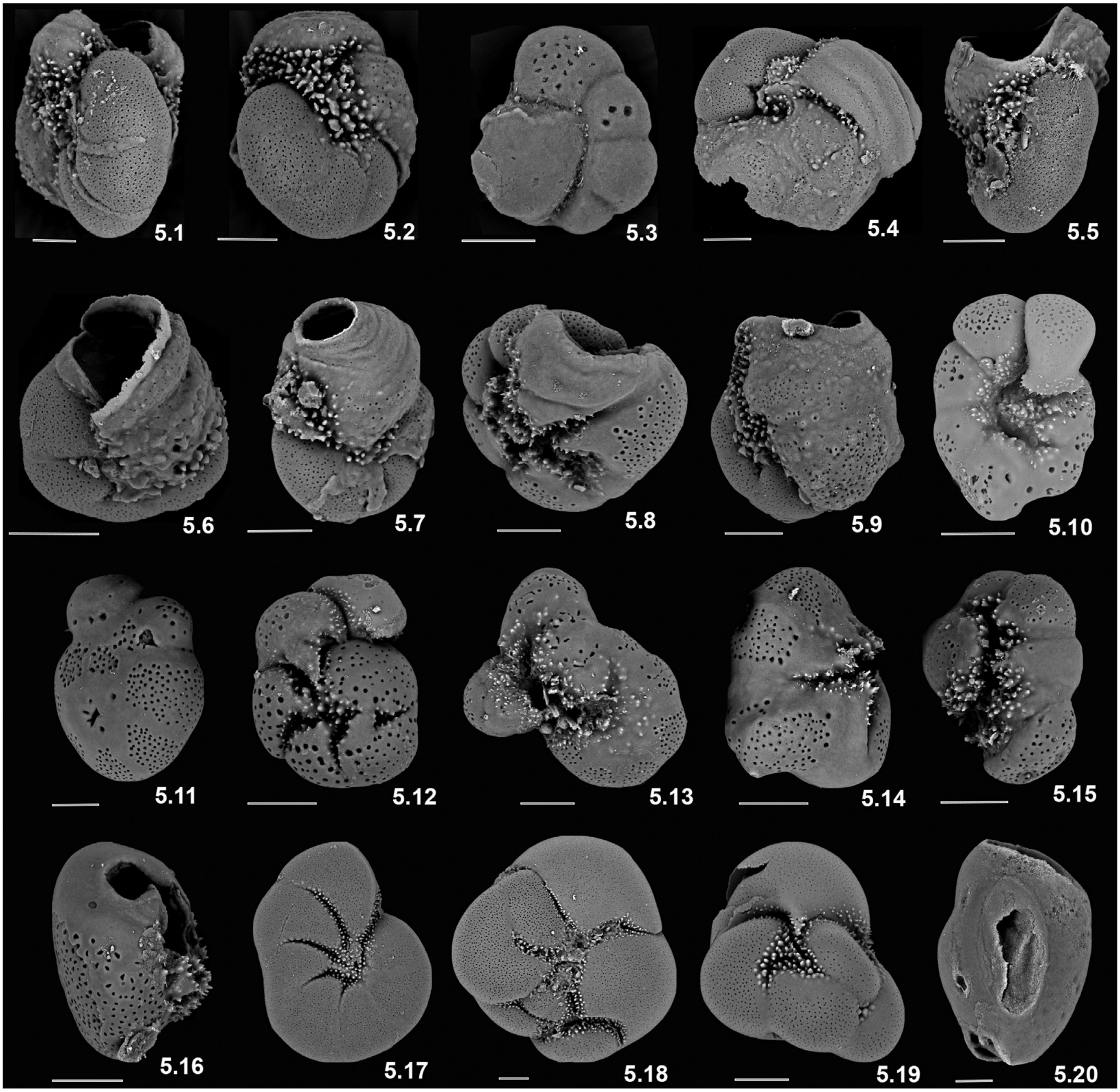

Fig. 5. Aberrant test morphologies of calcareous foraminifera that were grown with exposure to Zn. Deformities in *Ammonia tepida* (5.1–5.16) include: extremely enlarged apertures, irregular calcification, enlarged pores, misshapen chambers, and/or increased pustules. Deformities in *Haynesina germanica* (5.17–5.19) include twisted and distorted chamber arrangement and misshapen chambers. Miliolid deformities (5.20) include enlarged aperture, misshapen chambers. Scales = 25 μm. No scale is available for 5.17.

3.4. Test deformities

Deformed tests occurred in the controls at a low frequency (0.91–0.27%), which is comparable to levels reported for naturally occurring assemblages (Alve, 1991; Boltovskoy et al., 1991). Of the heavy metals examined, high proportions of abnormally constructed tests occurred only in those assemblages grown with exposure to Zn (Fig. 5). However, concentrations of Zn do not positively correlate to the percent of the deformed shells in the assemblage (Fig. 6). Zn concentrations of $< 0.027\ mg\,L^{-1}$ result in 12.2% of the assemblages having aberrant test morphologies, the highest observed in this study. Higher concentrations of Zn ($158.57–3449.18\ mg\,L^{-1}$) result in lower percentages of deformed tests, 0.9–2.7%. However, far fewer individuals grew at these higher concentrations.

Some species had much higher frequencies of deformed tests than others. The monothalamids, *Ovammina opaca* and *Psammophaga sapela,* produced no aberrant test morphologies in any of the experiments. Exposure to $Zn < 0.027\ mg\,L^{-1}$, however, produced abnormal morphologies in several calcareous species: 54.4% of *Ammonia tepida* specimens, 24% of *Haynesina germanica*, and 25% of *Quinqueloculina jugosa* (Fig. 5). Very few, < 0.1%, of multilocular agglutinated foraminifers produced deformed tests. Following the terminology of Alve (1991), the aberrant test morphologies found in *A. tepida* are best described as an enlarged aperture with extreme deformation forming

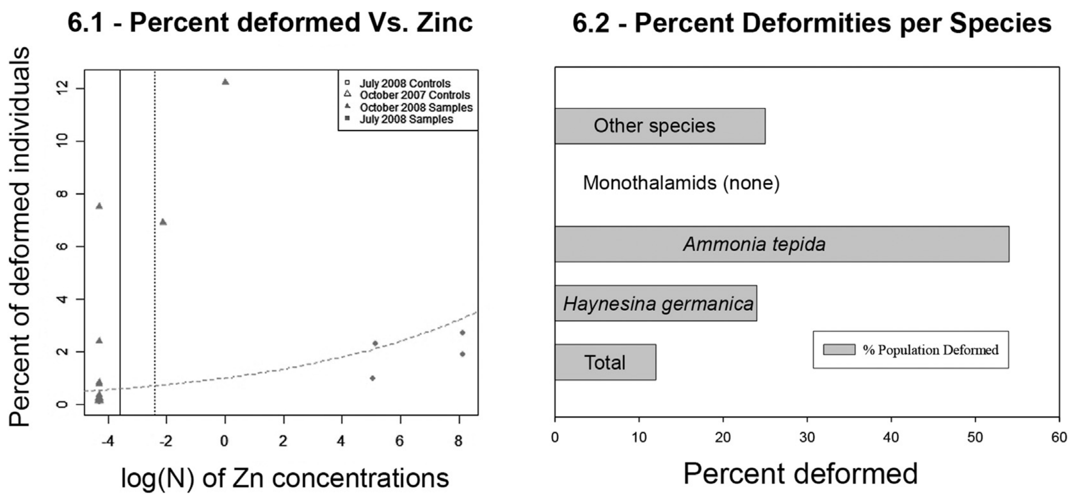

Fig. 6. Fig. 6.1: Frequency of aberrant test morphologies grown with exposure to Zn. Most deformities occurred with exposure to low concentrations of Zn. Fig. 6.2: Percentage of aberrant test morphologies in different calcareous species grown with exposure to Zn at a concentration of 2.82 mg L^{-1}.

pustules and aberrant regions of calcification (Fig. 5). In addition, some specimens exhibit sutures that are covered in part with an additional layer of calcite (Fig. 5) compared to a normal test (Fig. 4).

4. Discussion

Many field studies report that while pollution may strongly influence species trends in foraminifera, other environmental factors affect the assemblages as well, including substrate, salinity and hydrodynamics (e.g., Coccioni, 2000; Cearreta et al., 2002; Geslin et al., 2002; Hayward et al., 2004; Ruiz et al., 2004; Romano et al., 2008). In this study, we were able to control such environmental factors and better assess the responses to each contaminant over a range of concentrations. In general, our results are consistent with those of numerous field studies in that we found extreme reductions in abundances and diversity with exposure to high concentrations of a contaminant. We also found abnormally formed calcareous tests, but only those grown with exposure to Zn. This study also identifies several unique responses to individual heavy metals. Of the metals studied, Cd, even at the highest concentrations, has the least acute affect on total abundances. The response reflects a persistence of opportunistic species (*Haynesina germanica* and *Ammonia tepida*) corresponding to a decline in the monothalamids at the highest concentrations examined. Foraminifera are very sensitive to Zn at low concentrations. Exposure to higher concentrations of Zn produced variable results with larger than expected populations of the rotaliids, but very few monothalamids. Ultimately, high concentrations of Pb have the greatest acute effect on all species, both rotaliids and monothalamids. Similarly, Frontalini et al. (2018a) reported reduced foraminiferal abundances and diversity in microcosms treated with Pb (concentrations of 10,100,200, and 500 µg L^{-1}, and 1, 5, and 10 mg L^{-1}).

Bioavailability is the potential of living organisms to take up chemicals from food or the abiotic environment (Adriano, 2001). In general, dissolved substances are more labile and bioavailable than solids, and uptake of a contaminant occurs through the solution phase (Traina and Laperche, 1999). In all of the metal-spiked experiments conducted in this study, dissolved metals were measured in significant concentrations after the conclusion of the study, and foraminifera therefore were exposed to a single metal in the solution phase for the entire duration of the experiment. The negative impacts on foraminiferal abundance and species richness found with increasing metal concentrations further indicates that each of the metals examined was bioavailable over the course of the experiment.

Total metal concentration alone, however, is not an adequate predictor of bioavailability or toxicity, because this is further influenced by its chemical state or (chemical) speciation (Traina and Laperche, 1999). This speciation may explain why foraminifera appeared less sensitive to cadmium than zinc or lead. Whereas cadmium exists largely as free Cd^{2+} at pH < 6, increasing quantities of $CdHCO_3{}^{+}$, and a neutral carbonate complex, $CdCO_3{}^{0}$, are expected to occur as pH increases (Adriano, 2001). These cadmium carbonate species would be expected in seawater (including artificial seawater) which generally has a pH of ~8.1–8.2. The pH of Instant Ocean at a concentration of 32‰, as used in this study is ~8.0. Possibly, these carbonate forms of cadmium are less bioavailable and/or less toxic to foraminifera than Cd^{2+}. Chemical speciation, however, was not determined in this study; an extensive study examining its effect on foraminiferal toxicity is an interesting future line of investigation. The bioavailability of zinc and lead are influenced by pH, but had a more toxic impact than did cadmium.

Reduced foraminiferal growth rates with exposure to heavy metals have been reported in several studies. Le Cadre and Debenay (2006), for example, reported the cessation of growth in *Ammonia beccarii* in response to exposure to Cu at high concentrations (400 µg L^{-1}). Denoyelle et al. (2012) reported slower rates of chamber addition in *Ammonia tepida* with increased concentrations of Cd. Nardelli et al. (2013) likewise reported reduced rates of chamber formation or cessation of growth in the miliolid *Pseudotriloculina rotunda* grown with exposure to elevated concentrations of Zn. Reduced growth rates may be a generalized response of foraminifera when exposed to toxic heavy metals. In this current study, at least a few foraminifera grew in all treatments, even those with very high concentrations of Cd, Zn, or Pb.

Field-based studies report decreases in diversity with exposure to heavy metals (e.g., Coccioni, 2000; Armynot du Chatelet et al., 2004; Kfouri et al., 2005). In this study, both species richness and Simpson's index (reflecting evenness) are reduced in assemblages grown with exposure to high concentrations of Pb. However, with exposure to Zn and Cd there was no acute effect (as indicated by the shape of the regression line) on species richness and Simpson's Index. The overall decline in species richness is a result of the absence of monothalamids and some less common taxa at higher concentrations. Simpson's values decline in treatments with higher concentrations of Cd and Pb, reflecting a general decline in evenness.

Few studies address the response of monothalamid foraminifera to anthropogenic pollution. *Ovammina opaca* and *Psammophaga sapela* are not mentioned in pollution studies, most likely a result of research location (though both genera are widely distributed) and sample preparation methods. These species would have been absent if the samples had been dried. However, both monothalamids have the potential to be good bio-indicators. In treatments of high concentrations of Cd, Zn, or Pb, very few if any monothalamids grew. However, in treatments with low metal concentrations, the monothalamids thrived and even exceeded the abundances found in the controls. This may be an opportunistic response resulting from slight declines in the dominant rotaliids or other microbial competitors. Reduced abundances of agglutinated taxa with exposure to heavy metals have been documented in many field studies (e.g., Banerji, 1992 especially with Zn; Debenay et al., 2001; Bergin et al., 2006), and results of the present study are consistent with those findings.

Ammonia tepida is an opportunistic species that is tolerant to a variety of environmental conditions (e.g., Almogi-Labin et al., 1992; Alve and Murray, 1999). *Haynesina germanica* reportedly is also resistant to pollutants, including metals, and is recognized as an opportunist (Debenay et al., 2001; Armynot du Chatelet et al., 2004). Our results concur: *H. germanica* and *A. tepida*, are the most resilient of the common mudflat species when exposed to high concentrations of Cd, Zn, and Pb. The presence of calcareous species and absence of agglutinated species suggest that a calcareous test may be beneficial in environments with heavy metal contamination.

Previous studies report an increase in opportunistic/tolerant foraminiferal taxa at polluted sites (e.g., Watkins, 1961; Samir, 2000; Luan and Debenay, 2005;Di Leonardo et al., 2007; Motjahid et al., 2008; Romano et al., 2008; Valenti et al., 2008). These studies attribute these increases to the exploitation of sediment type, food abundance, and habitat space as less tolerant species decline. In the present study, several major environmental parameters were controlled, leading us to suggest that the increased availability of habitat space and perhaps other resources, coupled with lack of competition allow opportunistic/tolerant species to grow.

Previous studies on foraminifera and pollution that focused on sewage outfalls report that the diet of foraminifers may contribute to changes in abundance and diversity (e.g. Watkins, 1961; Bandy et al., 1964; Topping et al., 2006). *Haynesina germanica,* common in the present study, is known to sequester diatom chloroplasts (e.g., Lopez, 1979; Austin et al., 2005; Goldstein and Richardson, 2018). These chloroplasts remain photosynthetic and provide nutrition to the foraminifera (Lopez, 1979). Furthermore, the cell body of *H. germanica* lacks sediment-bearing food vacuoles (Goldstein and Richardson, 2018) and therefore cannot be characterized as a detritivore or deposit feeder. True to its generic name, *Psammophaga sapela* ingests numerous sediment grains, particularly heavy minerals, as well as diatoms (Altin Ballero et al., 2013). *Ammonia tepida* ingests diatoms and clay particles associated with bacteria (Goldstein and Corliss, 1994). The most abundant foraminiferal species encountered in this study therefore have different diets. However, the rapid decline of all species, with the exception of *A. tepida* and *H. germanica* in Cd treatments, suggests that diet per se is not the most important limiting factor.

The most common species encountered in this study included monothalamids and rotaliids, but not miliolids, which generally are not abundant on Sapelo mudflats and marshes. We are therefore unable to assess responses of species belonging to this important and diverse clade of foraminifera, though this is addressed in a separate study (Smith and Goldstein, 2019).

The EPA's National Recommended Water Quality Criteria for Saltwater, CMC (Cd $0.040\,mg\,L^{-1}$, Pb $0.210\,mg\,L^{-1}$, and Zn $0.090\,mg\,L^{-1}$) are the highest concentration of a heavy metal that an aquatic community can be exposed to "without resulting in an unacceptable affect." All of the acute responses observed in total abundance, species richness, and abundances of the four most common species are at concentrations equal to or somewhat higher than the EPA standards. This supports previous reports that identify coastal foraminifera as useful bio-indicators. However, additional experimental work is needed to better constrain metal concentrations that cause acute effects on benthic foraminifera and make further comparisons with field-based studies (e.g., Martinez-Colon et al., 2018).

Abnormally formed tests have often been reported in association with heavy-metal contamination in field-based studies. The underlying causes of the abnormal morphologies however, are often ambiguous because environmental conditions vary widely within the coastal zone, and typically multiple contaminants are present. Still other field-based studies or reviews link deformed tests with fluctuations in naturally occurring environmental parameters, including salinity, temperature, pH, and grain size (e.g., Almogi-Labin et al., 1992; Boltovskoy et al., 1991; Geslin et al., 2000; Debenay et al., 2001). Such environmental fluctuations were controlled in the present study.

Ammonia tepida produced aberrant morphologies more frequently than any other species, but only with exposure to Zn. The most common type of aberrant morphology observed in this study has been characterized as an "enlarged aperture" and has been reported in both calcareous and agglutinated species (Alve, 1991). Possibly, the extremely enlarged apertures found in this study may be a specific response to Zn exposure, though additional study is necessary. *Haynesina germanica* and *Quinqueloculina jugosa* also produced aberrant morphologies though at lower frequencies than *A. tepida*. In a field-based study focused on the western Baltic, Polovodova and Schönfield (2008) likewise reported higher frequencies of aberrant morphologies in *Ammonia* than in other taxa. Abnormally formed monothalamid tests were not observed in this study, perhaps because they lack a mineralized test and appear to have a rather simplistic test morphology.

Several biological factors have been proposed to account for abnormally formed tests with exposure to heavy metals. Yanko et al. (1998), for example, attributed abnormally formed chambers to the effects of heavy metals on the cytoskeleton. The cytoskeleton and associated reticulopodia construct a glycoprotein anlagen in the shape of the forming chamber. Calcification then occurs on this organic structure (e.g., Angell, 1967; Hemleben et al., 1977).

Alternatively, exposure to heavy metals may affect calcification itself. A variety of trace elements can be included in the foraminiferal test/calcite under natural conditions (e.g., Lea and Boyle, 1989; Fritz et al., 1992). Several studies report higher concentrations of heavy metals in foraminiferal tests (including Cd, Zn, and Pb) retrieved from environments with elevated heavy metals (e.g., Rathburn et al., 2008; Romano et al., 2008; Bloundi et al., 2009), and deformed tests from sites with heavy metal contamination reportedly have higher heavy metal concentrations than normal tests (Sharifi et al., 1991; Samir and El-Din, 2001), suggesting that the incorporation of metals into the calcite lattice results in deformation. However, mixed results have been reported from culture experiments. Le Cadre and Debenay (2006) reported an increasing proportion of deformed tests in *Ammonia* grown with exposure to elevated levels of Cu. Nardelli et al. (2016) did not find deformed tests in the miliolid *Pseudotriloculina rotunda* grown with exposure to Zn, but did find disorganized crystal growth (illustrated with SEM) in the tests of such specimens. Exposure to heavy metals also impact the cell body of foraminifera with either deformed (Le Cadre and Debenay, 2006) or normally formed tests (Frontalini et al., 2015, 2018b), including thickening of the organic lining and proliferation and modifications to lipids, among other changes.

Zinc is known to affect calcification in abiotic systems where it can retard or halt calcification when present in high concentrations (Meyer, 1984; Ghizellaoui et al., 2007). Ghizellaoui et al. (2007) found that just $1\,mg\,L^{-1}$ of Zn inhibited 100% of abiotic calcification in experimental studies; lower concentrations ($< 0.2\,mg\,L^{-1}$) reduced calcification by 20%. Furthermore, the presence of Zn resulted in the growth of significantly smaller crystals. In speleothems, another abiotic system, Caddeo et al. (2011) suggested that the presence of elevated concentrations of Zn (and possibly Pb) may account for the unexpected deposition of aragonite rather that calcite. The extent to which such abiotic processes may influence abnormal shell growth in foraminifera warrants further study.

5. Conclusions

Coastal environments are ever-changing, complex systems, many of which have been strongly impacted by anthropogenic activities. The anthropogenic effects on coastal foraminifera have been widely documented in field-based studies and to a lesser extent with laboratory-based controlled experiments. Several foraminiferal species have been identified as potential bio-indicators for coastal pollution. Yet, experimental studies are necessary to better assess how foraminifera respond to selected pollutants under controlled conditions and to refine the applications of foraminiferal bio-indicators. In this study assemblages of coastal foraminifera grew from propagules (small juveniles) under lab-controlled environmental conditions with exposure to a single heavy metal (Cd, Zn, or Pb) over a range of concentrations. Results show that: 1) foraminifera respond negatively to Cd, Zn and Pb as reflected in abundances, diversity, and evenness, however, responses vary by species and metal. 2) Zn causes distinct aberrant test morphologies, particularly in *Ammonia tepida*. 3) Pb has the most acute effect on total abundance, species richness, evenness, and abundances of the four most abundant species. 4) As indicated in other studies, *Ammonia tepida* and *Haynesina germanica* are potentially valuable bio-indicators for Zn, Cd and Pb. 5) The monothalamids *Ovammina opaca* and *Psammophaga sapela* are also potential bio-indicators in anthropogenically stressed environments. However, the lack of a hard test lowers the chances of fossilization and reduces their usefulness of studying the onset and recovery of anthropogenic pollution in the geohistorical record.

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Appendix A. Supplementary data

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