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Zinc incorporation in the miliolid foraminifer Pseudotriloculina rotunda under laboratory conditions / Nardelli, M. P.; Malferrari, Daniele; Ferretti, Annalisa; Bartolini, A.; Sabbatini, A.; Negri, A.. - In: MARINE MICROPALEONTOLOGY. - ISSN 0377-8398. - STAMPA. - 126:(2016), pp. 42-49. [10.1016/j.marmicro.2016.06.001]

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28/04/2024 09:48

Elsevier Editorial System(tm) for Marine Micropaleontology Manuscript Draft

Manuscript Number:

Title: Zinc incorporation in the miliolid foraminifer Pseudotriloculina rotunda under laboratory conditions

Article Type: Research Paper

Keywords: Foraminifera; Miliolid; Zn/Ca; LA-ICP-MS; culture experiments; pollution.

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Abstract: The incorporation rate of Zn into the calcareous tests of Pseudotriloculina rotunda was investigated in culture in order to evaluate the possibility to use Zn/Ca ratios as pollution proxy. Foraminifera were incubated at zinc concentrations up to 10-fold higher than unpolluted seawater (sea+10 mg Zn/L) during 70 days. New calcite was observed at Environmental Scanning Electron Microscopy (ESEM), for potential alteration of test structure. Laser ablation-Inductively Coupled Plasma-Mass spectrometer (LA-ICP-MS) was used to quantify Zn contents. The analyses revealed that test structure is not visibly altered by the presence of zinc. However, significant Zn incorporation is detected by LA-ICP-MS. The zinc partition coefficient, DZn, decreases at increasing Zn concentrations (from 4.03 ± 0.06 at control conditions to 0.2 ± 0.01 at the highest tested Zn concentration) and the zinc is incorporation into the calcite follows a power function.

Zinc incorporation in the miliolid foraminifer Pseudotriloculina rotunda under laboratory
conditions
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15 Highlights

1- Zinc incorporation into miliolid foraminiferal tests was experimentally tested; 2- *Pseudotriloculina rotunda* was cultured at several zinc concentrations for 70 days; 3- LA-ICP-MS analyses show that Zn
 is incorporated into calcite; 4- Partition coefficients (D_{Zn}) decrease at increasing Zn/Ca_{seawater} ratios.

20

ABSTRACT

21 The incorporation rate of Zn into the calcareous tests of *Pseudotriloculina rotunda* was investigated 22 in culture in order to evaluate the possibility of using Zn/Ca ratios as a pollution proxy. Foraminifera were incubated at zinc concentrations up to 10-fold higher than unpolluted seawater (sea+10 mg 23 24 Zn/L) during 70 days. New calcite was investigated under Environmental Scanning Electron 25 Microscope (ESEM), for potential alteration of test structure. Laser ablation-Inductively Coupled 26 Plasma-Mass spectrometry (LA-ICP-MS) was used to quantify Zn contents. The analyses revealed that 27 test structure is not visibly altered by the presence of zinc. However, significant Zn incorporation is 28 detected by the LA-ICP-MS. The zinc partition coefficient, D_{zn} , decreases at increasing Zn 29 concentrations (from 4.03±0.06 to 0.2±0.01) and the zinc is incorporated into the calcite, non-30 necessarily linearly.

31

32

KEYWORDS

33 Foraminifera; Miliolid; Zn/Ca; LA-ICP-MS; culture experiments; pollution.

34

35 1. Introduction

The recent worldwide legislation aims to restore the "pre-anthropic impacts" status in marine environments (e.g., WFD, 2000/60/EC and MSFD, 2008/56/EC in Europe). Information about the pristine faunas like those in pre-industrial times, however, are often impossible to obtain because of the scarcity (or lack) of reference stations that could still represent unimpacted present-day 40 conditions. Fossilizing organisms represent an excellent historical archive of environmental 41 conditions. Foraminifera are distributed worldwide in many different habitats, from brackish to 42 marine, and their fossil records create an excellent historical archive which can be used as proxies for 43 the reconstruction of past environments, such as pre-industrial ecological conditions (Schönfeld et al. 44 2012). A new approach involves the use of foraminiferal test geochemistry to assess the evolution of 45 pollutant (i.e., metals) concentrations through time. Incorporation rates of trace elements are widely 46 used as specific proxies in paleoceanography and paleoecology (e.g., Eggins et al. 2003; Hönisch and 47 Hemming 2005; Levi et al. 2007; Katz et al. 2010; Sabbatini et al. 2011), despite the possible bias 48 linked to the biological influence on calcification processes (i.e., vital effects). The need to calibrate 49 these proxies through culturing experiments was highlighted in the last decade by several authors (e.g., de Nooijer et al. 2007). This approach offers the advantage of changing one single variable 50 51 (while all the others are kept constant) in order to better evaluate the vital effect. These biological 52 aspects could be even more important for the incorporation rates of chemicals whose concentrations 53 exceed natural baselines due to human activity, and that could be potentially used as pollution 54 markers.

55 In this study we investigate the incorporation rates of Zn in the shell of the benthic miliolid foraminifer Pseudotriloculina rotunda (Schlumberger 1893). Among foraminiferal species miliolids 56 57 showed contradictory responses to heavy metal pollution in different studies. For example, 58 decreasing miliolid relative abundances in polluted (by both organic and inorganic chemicals) coastal 59 zones are reported and interpreted by some authors as a sensitivity index (e.g., Ferraro et al. 2006; Frontalini and Coccioni 2008). Other studies, on the other hand, suggest a strong tolerance of several 60 61 miliolid species to pollution, both in situ and under laboratory conditions (e.g., Samir and El-Din 62 2001; Romano et al. 2008; Cherchi et al. 2009; Foster et al. 2012; Nardelli et al. 2013).

The aim of the present study is to calibrate incorporation rates of Zn in miliolid foraminiferal shells and thus evaluate the usefulness of the Zn incorporation rate as an environmental proxy. Zn is, in fact, one of the most common pollutants associated with human activities (e.g., Callender and Rice

2000; Wuana and Okieimen 2011), that can be toxic for biological systems when its concentration
exceeds a threshold value (e.g., Haase et al. 2001; Valko et al. 2005; Díaz et al. 2006; Formigari et al.
2007). Nardelli et al. (2013) showed that inorganic Zn at concentrations higher than 0.1 mg/L can
cause biological stress in *Pseudotriloculina rotunda*, causing delay in calcification rates.

70 In this regard, our study also aims to test the hypothesis that the biological stress caused by high Zn 71 concentrations may influence metal incorporation rates as well. Zn incorporation was investigated 72 using the Laser Ablation Inductively Coupled Plasma Mass Spectrometer (LA-ICP-MS) analysis. 73 Moreover, morphological observation using the Environmental Scanning Electron Microscope (ESEM) 74 was employed to check for abnormalities in the organization/distribution of single shell crystallites. 75 Nardelli et al. (2013) previously suggested that Zn does not cause macroscopic test deformations in 76 miliolid foraminiferal shells, on the base of their observation of coiling patterns and chamber shapes 77 of *P. rotunda* specimens grown at increasing zinc concentrations, under the binocular microscope. 78 The aim of our ESEM analyses was to deepen these observations and check for calcite anomalies at 79 the crystallite level.

80

81 2. Material and Methods

82 2.1 Experimental set up

83 All the analyses were performed on specimens of Pseudotriloculina rotunda that grew at least one 84 chamber during 70-days exposure to six different Zn concentrations under laboratory conditions. Znenriched solutions were prepared adding respectively 0.01, 0.1, 1.0, 10, and 100 mg/L of Zn to 85 86 natural seawater (sea) from an unpolluted site (Portonovo, Adriatic Sea); Zn and Ca concentrations in 87 natural seawater were measured using Inductively Coupled Plasma Mass Spectrometer (ICP-MS). 88 Although precautions were taken during manipulations, the ICP-MS analyses revealed that the zinc 89 concentration of the natural seawater used for the experiment was higher than natural background. 90 In fact the zinc concentration measured on culture waters before the addition of zinc was 0.149±0.01 91 mg/L, while the zinc concentration of seawater from the sampling site was originally of 0.237±0.01 92 μg/L. However, as the same water was used to prepare all the zinc solutions for the different 93 treatments and, considering the very high concentrations of added zinc, we believe that this 94 contamination does not compromise the dataset. But, of course, this means that the lowest tested 95 seawater zinc concentration of the experiment (named "sea" hereafter) cannot be considered as a 96 control representative of unpolluted seawater conditions.

97 Temperature (15.0±0.5°C), salinity (38.0±0.001) and pH (8.0±0.1) were kept constant during the
98 experiment. Refer to Nardelli et al. (2013) for further details on culture settings and preparation of
99 Zn solutions.

As reported in Nardelli et al. (2013), none of the specimens produced new chambers at the highest tested Zn concentration (sea+100 mg/L), therefore only specimens coming from culture sets from treatments sea, sea+0.01, sea+0.1, sea+1 and sea+10 mg/L were investigated. Moreover, two samples were treated for a "passive Zn incorporation test": empty tests of *P. rotunda* were exposed to sea+10 mg/L Zn concentrations for two weeks in order to measure Zn passively adsorbed to calcite, without involving cellular-mediated mineralization processes.

106

107 2.2 ESEM and LA-ICP-MS sample preparation

The analyzed foraminiferal tests (n=41) were washed with millipore water and dried at 40°C. The same cleaning procedure was performed on the two empty tests (n=2) used for the passive incorporation test. To perform both ESEM observations and LA-ICP-MS analyses, samples were fixed to aluminium stubs using conductive carbon adhesive discs. All samples analysed with an LA-ICP-MS were photographed under the ESEM before and after the analyses to check for the success of ablations (i.e., the correct chamber, no multiple chamber sampling, and no breakage of chambers – see Appendix, Figs. A.1a-c).

115

116 2.3 LA-ICP MS analysis: analytical protocol optimization

This study represents the first LA-ICP-MS investigation on miliolid foraminifera. Chemical 117 118 composition, micro-structure and chamber arrangements of miliolids strongly differ from other 119 benthic foraminifera more commonly analyzed with the LA-ICP-MS (i.e., Rotaliidae, e.g., de Nooijer et 120 al. 2007; Munsel et al. 2010; Dissard et al. 2010; or Buliminidae, e.g., Hintz et al. 2006; Barras et al. 121 2010). Miliolid foraminifera have a calcareous non-lamellar imperforate test consisting of calcite 122 needles randomly oriented in an organic matrix. They also possess a smoothly finished outermost 123 layer of well crystallized calcite, with rhombohedral crystal faces arranged parallel to the surface 124 (Debenay et al. 1998). Moreover, *Pseudotriloculina rotunda* creates chambers, each one-half coil in 125 length, adding the new ones in planes oriented at 120°, with only three final externally visible chambers (Loeblich and Tappan 1964). 126

For this reason it was necessary to optimize the existing protocols and to obtain the best ablation setting to be applied to our specimens. In particular, it was compulsory to prevent the laser from ablating the innermost (older) chambers. Several trials on foraminiferal tests were thus performed to find the most suitable combination of the instrument setting parameters. A detailed description of the followed procedures is given in Appendix A, and the values of the optimized parameter used for measurements are reported in Table 1. An example of successful sampling is shown in Figure 1.

133

134 2.3.1 Analytical standards preparation and instrument calibration

135 A mass spectrometer, like any measurement device, requires a suitable calibration procedure. When 136 laser ablation is employed, the interaction between laser and solid sample is complex and the response is dependent on the sample matrix. For this reason, two forms of calibration are 137 138 mandatory: i) a reference (internal standard) is required to compensate for changes in the quantity 139 of ablated mass, even when the concentration remains constant; ii) matrix-matched solid standards 140 (frequently referred to as "external standards") are necessary to calibrate laser ablation processes 141 and the instrument response. In fact, a relative measure of ablated mass can be achieved by 142 simultaneously measuring emission from the analyte and a common matrix element (internal standard). For absolute calibration of the LA-ICP-MS conditions, standards made of the same matrix
as the samples would be required, but are seldom available (e.g., Darke and Tyson 1994; Raith et al.
1996; Hathorne et al. 2003).

146 Previous studies on perforate calcareous species of foraminifera (or other organisms with calcareous 147 compounds) used NIST610-611 or NIST612-613 as external standards, together with internal standards (generally ⁴⁴Ca isotope) and "in-house made standards" (e.g., Hathorne et al. 2003; Eggins 148 149 et al. 2003; Montagna et al. 2007; Rathmann and Kuhnert 2008; Munsel et al. 2010). The same 150 standards were also used in our study in order to test their possible employment also for miliolids. 151 In-house made standards were obtained as hereafter described. Stock solutions with defined Zn 152 concentration were prepared using Zn ICP-standard solutions [1mg/mL in 2 (vol.%) HNO₃] and 153 Millipore water. A proper amount of each solution was added to a mixture composed by 400 mg of 154 ultrapure CaCO₃ powder (particle size less than 1 micron) and cellulose; to prevent CaCO₃ dissolution, 155 the pH of each solution was adjusted to 7.5 ± 0.1 using an appropriate amount of ammonia solution. 156 Each suspension was mixed and homogenized in an agate mortar and then dried at 30°C for 12 157 hours. The resulting powder was then re-homogenized in the agate mortar and pressed at 12 tons 158 into tablets of 12 mm diameter. Such "standard tablets" at different Zn concentrations were then 159 checked via LA-ICP-MS using ablation lines to verify whether Zn distribution was homogeneous. As 160 shown in Figure 2, the spectra resulting from these analyses revealed a fairly smooth plateau, 161 confirming a homogeneous distribution of the element into the standard tablets.

According to the literature (see above), NIST standards could be used as well. However, the difference in composition of the matrix (i.e., silica glass in NIST standards, Ca carbonate in miliolids) does not match the second requirement mentioned above. However, several measurements using NIST610 were performed in order to test the possibility to use a certified and easily acquired analytical standard. In particular, the same laser parameters (i.e., the same ablation conditions) were applied to both NIST610 and our samples (miliolids) but the response on the internal standard (⁴⁴Ca) was not satisfactory (see next paragraph for more details).

In the light of these results, and also in agreement with Hathorne et al. (2003), only in house-made
Tablets were used as external standards for measurements here reported. In detail, nine in house
made standards were used with Zn concentrations ranging from 0 (blank, CaCO₃+cellulose+Millipore)
to 1050 ppm.

173

174 2.4 LA-ICP-MS: analyses of samples and data elaboration

Eleven specimens from "sea", five from sea+0.01 mg/L, six from sea+0.10 mg/L, ten from sea+1.0 mg/L, nine from sea+10 mg/L, were analyzed by the LA-ICP-MS. Moreover, the two specimens for the "passive sorption tests" were investigated as well. In few cases more than one ICP-MS analysis was carried out on the same chamber, otherwise one linear ablation per chamber was generally realized. Standards were ablated using exactly the same laser setting parameters used for foraminifera, and their concentrations were regularly measured during sampling procedure, in order to correct instrumental deviation.

⁶⁶Zn and ⁶⁸Zn isotopes, and ⁴⁴Ca were measured. A mix of helium (95%) and hydrogen (5%) were used
as reaction gas and a collision and reaction cell (KED) was used to minimize spectral interferences.
Both for calibration and for sample measurements, Ca and Zn concentrations were calculated
integrating over a time-interval of 1.00E+05ms in the flattest region of each spectrum of each
individual ablation profile using PlasmaLab[™] Software Package 2007.

For data analyses the relation between Zn/Ca ratios in calcite and seawater was observed. Partition coefficients for Zn were calculated for each set of Zn enriched cultures following the formula D_{Zn} = (Zn/Ca _{calcite})/(Zn/Ca _{seawater}).

190

191 **3. Results**

192 3.1 ESEM observations

Results from ESEM observations on structure and crystal organization of Zn-exposed foraminiferal tests did not reveal any obvious anomalies. All specimens showed the typical structure of miliolid tests (Figs. 3 a-d), characterized by organized crystals on the external test surface and disorganized crystals in an organic matrix in the inner part of the wall (Hay et al. 1963; Towe and Cifelli 1967; Haake 1971; Debenay et al. 1998).

ESEM observations were also performed to adjust laser ablation parameters (see Appendix A) and tocheck samples for correct ablation after LA-ICP-MS measurements.

200

201 3.2 LA-ICP-MS measurements

202 The main results of the LA-ICP-MS analysis are given in Figure 4 (a,b). The Zn seawater concentrations 203 are given in Zn/Ca mmol/mol to facilitate the comparison with Zn/Ca ratios in the calcite. The 204 different treatments (i.e., Zn concentrations) are indicated by the colors. The LA-ICP-MS analysis 205 revealed that Zn/Ca ratio increases linearly with water concentrations at least up to sea+1.0 mg/L 206 (Fig. 4b). The Zn/Ca shell concentrations vary between 1.11±0.02 mmol/mol for the "sea" treatment 207 and 1.77±0.04 mmol/mol of the treatment sea+1.0 mg/L (Tab. 2; see also Tab. A.2 in Appendix A for 208 the complete dataset). The measurements obtained on calcite produced at the highest tested Zn 209 concentrations (sea+10 mg/L), however, with average Zn/Ca values of 3.81±0.17 mmol/mol, suggest 210 the possibility that the linear trend, observed for seawater Zn concentrations lower than sea+1.0 211 mg/L, can turn towards a plateau for higher concentrations. Possible explanations of this trend are 212 discussed later.

The passive sorption test on empty shells incubated at sea+10 mg/L conditions showed very low Zn concentrations (Zn/Ca_{calcite}= 1.17±0.01 mmol/mol), comparable to the ones of samples from the "sea" treatment) (Zn/Ca_{calcite}=1.12±0.02 mmol/mol), and more than 3 times lower than living samples from highest tested zinc concentrations (sea+10 mg/L) (Zn/Ca_{calcite}=3.83±0.17 mmol/mol) (Fig. 4 and Tab. A.2 of Appendix A).

218 The calculated Zn partition coefficients (D_{Zn}) for each Zn treatment (indicated by the colors) are given 219 in Figure 5a. The average D_{zn} (±standard deviation) is given in numbers. Because of inhomogeneous 220 variance, the Kruskal-Wallis test and Mann-Whitney pairwise comparisons post-hoc test were 221 performed to determine whether the difference between partition coefficients was significant 222 among treatments (p-value < 0.001). The results showed that the Zn partition coefficients were all 223 significantly different among treatments. The obtained average values of D_{Zn} varied between 224 4.03±0.06 at "sea" conditions and 0.2±0.01 at sea+10 mg/l Zn concentration (Fig. 5) (total average 225 D_{Zn} = 2.22±1.56). The observed decrease of D_{Zn} at increasing seawater Zn concentration is well 226 described by a power function (R²=0.997; p-value<0.001; see Fig. 5a). However, due to the difficulty 227 to assess whether the results obtained for the highest tested concentration (sea+10 mg/L) are real or 228 affected by neglected precipitation of zinc oxides/hydroxides (see discussion paragraph 4.2) we 229 reported in fig. 5b only the D_{Zn} obtained at Zn treatments lower than this concentration. Even 230 without the last concentration, the data are still well described by a power function (R^2 =0.998; p-231 value<0.001) and the regression equation slightly change from $y = 1.5552x^{0.713}$ to $y = 1.4826x^{0.775}$.

232

233 4. Discussion

234 *4.1 ESEM structural observation of tests*

235 Miliolids are often regarded as pollution sensitive organisms. Several studies on test deformity 236 induced by environmental contamination suggest that miliolids are more easily affected by test 237 deformities than other foraminifera. For example, Samir and El-Din (2001) found that the majority of 238 deformed tests collected in the El-Mex Bay (Egypt) were miliolid shells. Also Sharifi et al. (1991) 239 reported that deformed foraminiferal tests from the Southampton coastal area contained much 240 higher Cu and Zn concentrations than non-deformed specimens, suggesting again a responsibility of these metals for test deformations. However, Nardelli et al. (2013) tested in laboratory conditions 241 242 the biological effects of several Zn concentrations on P. rotunda and, despite the fact that some 243 specimens calcified new chambers at Zn concentrations up to 10 mg/L, no obvious deformations due 244 to Zn exposure were observed, in terms of chamber arrangements or general shape of the 245 foraminiferal shells. Our ESEM observations confirm these results because the calcite produced 246 during Zn exposure showed the typical aspect and arrangement known for the species (as described 247 by Debenay et al. 1998). Then, Zn exposure does not appear to be, by itself, a cause of abnormal 248 calcification for P. rotunda, either at the macro or micro scale. A possible explanation for the fact that 249 Zn is often found in anomalous tests is its possible covariance with other pollutants (for example 250 other metals) or environmental parameters, which can be alone the real cause of test deformation or 251 have synergic behaviors to zinc and enhance its toxicity (as demonstrated, for example, for Co and Zn 252 by Bresson et al. 2013). In fact, even if Zn is one of the most cited heavy metals, potentially 253 responsible for test deformation, many other pollutants and anthropogenic events were related to 254 test deformities, e.g. Cd, Cr and Ti (Yanko et al. 1998), Cu (Le Cadre and Debenay 2006), 255 hydrocarbons and oil spills (e.g., Vénec-Peyré 1981; Vénec-Peyré et al. 2010), rapid changes of 256 salinity and hypersalinity (e.g., Eichler-Coelho et al. 1996; Sousa et al. 1997; Stouff et al. 1999; Geslin 257 et al. 2002). The absence of visible test anomalies can be coherent with the hypothesis that Zn 258 incorporation into biomineralized calcite may occur, as already observed on non-biogenic calcites 259 (e.g., Elzinga and Reeder 2000; Temmam et al. 2000), after substitution of Ca or Mg to form 260 isomorphic Zn carbonates, as suggested by Madkour and Ali (2009).

261

262 4.2 Zinc incorporation

Despite the absence of deformations or visible abnormalities in the specimens analyzed in this study, high Zn/Ca contents were detected in the internal wall of the last chamber of the test by LA-ICP-MS analysis. The incorporation rates of Zn into the calcite do not seem to be constant at increasing Zn concentrations. Zn incorporation, in fact, linearly increases at lower concentrations (from sea to sea+1.0 mg/L, Fig. 4b), but the measures obtained at Zn concentrations higher than sea+1.0 mg/L, even if represented by the results from only one Zn treatment, suggest that at higher concentrations (i.e., sea+10 mg/L) the trend determined at low concentrations (up to sea+1.0 mg/L) is no longer
respected (Fig. 4a). The decreasing Zn incorporation rate in the shell is reflected by the partition
coefficients that are lower for higher Zn seawater treatments (Fig. 5a,b).

272 Considering that we worked at high zinc concentrations we cannot avoid to take into account that 273 zinc oxides/hydroxides precipitation could have been important at the highest zinc concentration we 274 tested (sea+10 mg/L). In fact, if we roughly estimate the expected Zn²⁺ saturation based on the pH of 275 our cultures (8.0±0.1) and neglect all potential influences of kinetics, we obtain a concentration of 276 saturation of 4.37E-05 M, which is 3 times lower than the concentration of zinc we added to this 277 treatment (see Tab. A.2 in Appendix A). Zinc oxides/hydroxides precipitation could therefore at least 278 partially explain the decrease of Zn incorporation at sea+10 mg/L condition.

279 However, this is not the first time that a similar decreasing trend for metal incorporation is observed 280 on benthic foraminifera and this could be also partially due to biological reasons. For example 281 Munsel et al. (2010) report similar observations for incorporated Ni in A. tepida's calcite. The 282 incorporation of the metal is linear at low experimental concentrations and drops down when 283 foraminifera are exposed to Ni concentrations 20-fold higher than natural seawater. The authors 284 suggest that this could be due to biological effects. In fact, according to them, Ni at high 285 concentrations could have toxic effects on foraminiferal cells and inhibit calcification. Toxic potential 286 of some highly concentrated metals could trigger their cellular expulsion or blocking mechanism (for 287 example metallothioneins-mediated) and prevent their incorporation in proportion to their sea 288 water concentrations. This hypothesis is also valid for our study. Nardelli et al. (2013) reported 289 delayed growth rates (i.e. calcification) of Pseudotriloculina rotunda exposed to Zn concentrations 290 higher than sea+1.0 mg/l and ascribed the reduced calcification rate to biological effects of high zinc 291 concentrations on foraminiferal cell, invoking similar processes. Thus we think that the potential 292 biological influence on the Zn incorporation observed at sea+10 mg/l in the present study cannot be 293 excluded. Some other possible explanations are hypothesized by Mewes et al. (2014) who also 294 observed an exponential decreasing partition coefficient for Mg/Ca at increasing seawater metal 295 concentrations following a power regression for two rotaliid foraminifera (Ammonia aomoriensis and 296 Amphistegina lessonii): (a) presence of two different CaCO₃ layers, (b) involvement of two different 297 biomineralization pathways. The first hypothesis depends on the possibility, shown for some 298 foraminifera, to precipitate two different calcite phases, with two different Mg/Ca calcite ratios, 299 independently of a migration of the foraminifera through different chemical environments (in the 300 water column or in the sediment). This pathway was described by Bentov and Erez (2005) and 301 particularly for A. lessonii and Orbulina universa by Branson et al. (2013). However, as far as we 302 know, this biomineralization mechanism has not been described and does not fit with the existing 303 calcification models for miliolid foraminifera. In fact the existing biomineralization models for miliolid 304 foraminifera propose two kinds of calcification mechanisms: some miliolid foraminifera form bundles composed of an array of oriented crystals. Each crystal is enveloped by organic material, and then 305 306 bundles are passed through the cell membrane by exocytosis. Other miliolids, instead, accrete pre-307 formed crystals and matrix materials onto extracellular surfaces to form a loosely packed wall 308 structure in a "stack-of-bricks" process (e.g., Berthold 1976; Hemleben et al. 1986).

The second hypothesis considers the involvement of different biomineralization pathways of Mg incorporation during calcification. This hypothesis derives from the biomineralization model proposed by Nehrke et al. (2013) for rotaliids foraminifera, but there is no evidence that the same processes occur into miliolid foraminifera. Therefore also this hypothesis does not seem to be valid to explain our results. Thus, the oversaturation of the highest tested zinc solution and/or the biological effect of potentially toxic zinc concentrations on the biomineralization process remain, to us, the more realistic hypothesis to explain the low zinc incorporation at sea+10 mg/L treatment.

A part from the exponential decrease of partition coefficient, another interesting point highlighted by our results is the apparent positive intercept of D_{Zn} (Zn/Ca_{calcite} vs Zn/Ca_{seawater}) regression (Fig. 4b). This result suggests a difference in Zn incorporation for this miliolid species compared to all the rotaliid foraminifera for which D_{Zn} was previously estimated (e.g. Marchitto et al., 2000; Bryan and Marchitto, 2010). In fact, despite the highly variable D_{Zn} estimated for the different species,

Marchitto et al. (2000) and Bryan and Marchitto (2010), report a Zn/Ca_{calcite} vs Zn/Ca_{seawater} regression 321 line passing from the origin of the axes for all the measured rotaliids foraminifera. Similar results, 322 323 showing a positive intercept on the y-axis, reported in Mewes et al. (2014) for Mg/Ca of two rotaliid foraminifera (Ammonia aomoriensis and Amphistegina lessonii), were hypothesized by the authors to 324 be possibly due to purely chemical reasons: sorption of Mg²⁺ to mineral surfaces would be stronger 325 than sorption of Ca²⁺ (Mucci and Morse 1983) and then increased Mg²⁺ (adsorbed on calcite) would 326 327 locally increase Mg/Ca, and then explain the positive y-axis intercept, especially at low seawater 328 Mg/Ca. Due to its divalent nature and its ionic radii smaller than Ca^{2+} , this kind of mechanism is also 329 possible for Zn²⁺ (Elzinga and Reeder, 2000). In this regard, the results of our sorption test (fig. 4) 330 seems to suggest that, at least at the highest tested concentration (sea+10 mg/L), Zn sorption is 331 negligible compared to the fraction incorporated into the calcite of living foraminifera incubated at 332 the same Zn concentrations. This suggests that this kind of mechanism could have low influence on Zn enrichment of calcite. However, the test was performed only at the highest Zn treatment and it is 333 334 not enough to draw any definitive conclusion about this aspect.

335 Another hypothesis, invoked by Langer et al. (2006) to explain the enriched Sr/Ca ratio in the calcite 336 of the coccolithophore Emiliania huxleyi, could also explain the Zn/Ca enrichment we observe for 337 Pseudotriloculina rotunda. Similarly to miliolid foraminifera, coccolithophores precipitate calcite into specific intracellular vesicles. According to the authors, the intracellular calcite precipitation yields an 338 339 accumulation of Sr in the coccolith vesicle until Sr steady state is achieved. Then, the observed Sr/Ca 340 of the coccolith calcite would be an integral value that arises from the sum of Sr/Ca over the time required for the formation of one coccolith. This is suggested to explain the enriched Sr/Ca ratios 341 342 found into the coccolithophore compared to theorical ones expected by purely chemical 343 precipitation. According to us, and keeping into account the results of our sorption test, this 344 hypothesis seems more convincing to explain the apparent positive y-axis intercept of figure 4.

345

346 4.3 Implications for reconstructing past seawater zinc concentrations

347 As zinc is one of the most diffused pollutants related to the industrial activity (e.g., Callender and 348 Rice 2000; Wuana and Okieimen 2011) a proxy for its concentrations in historical times could 349 represent an important tool to study the evolution of marine ecosystems under anthropic stress. This 350 is particularly interesting in view of the animated debates on the possible boundary for the recently 351 proposed new epoch of the Anthropocene (e.g., Crutzen 2002; Zalasiewicz et al. 2011). The 352 accelerated increase of some industrial-derived pollutants is, in fact, one of the proposed points to 353 establish the start of this epoch. Our results suggest a possible reliable identification and 354 interpretation of one of these pollutants in the historical sediment record. In fact, except for the D_{2n} 355 estimated for the sea+10 mg/L that could have been influenced by Zn oversaturation problems (as 356 discussed above), we are confident that the D_{Zn} estimated for *Pseudotriloculina rotunda* in this study 357 can be applied for past seawater concentrations reconstructions. The partition coefficients we 358 obtained are different from the ones previously estimated for other foraminiferal species (mainly 359 Rotaliids), but rely on the variability range of values found for different species of rotaliid 360 foraminifera (e.g. ~3 for U. peregrina to ~22 for C. pachyderma reported by Bryan and Marchitto, 361 2010) and they represent the Zn/Ca of the seawater where the calcite was biomineralized. Zn/Ca 362 contents into miliolid calcite could therefore allow reconstruction of concentrations of this metal in 363 the past seawater.

364 The obtained D_{Zn} for each tested zinc concentrations (fig. 5a) show statistically significant differences. 365 For this reason we would suggest not to average all the values but rather using the equations given in 366 figure 4b and 5b for Zn seawater reconstructions. For the highest tested zinc concentration, for which the estimated D_{Zn} could have been highly driven by zinc oxides/hydroxides precipitation and 367 368 physiological cell problems due to potential toxic effects of zinc, our results are more difficult to 369 apply and need further investigation to be confirmed. For the use of this proxy in potentially 370 contaminated environments, the possible toxic effect of Zn at high concentrations on P. rotunda 371 could be an important boundary constraint. A possible solution to overcome this problem could be 372 the use of multiple species, with different thresholds of tolerance to zinc.

373 5. Concluding remarks

374 Zn/Ca ratios in calcite of Pseudotriloculina rotunda are linear function of Zn/Ca in seawater at lower 375 Zn/Ca seawater and appear to turn into a power function at Zn seawater concentrations higher than 376 sea+1.0 mg/L. Further studies are needed to quantify the possible influence of zinc 377 oxides/hydroxides precipitation on the results we obtained at this higher experimental condition. 378 However, even if we omit the last point from the dataset, the results show that Zn is incorporated in 379 equilibrium with seawater concentrations and that partition coefficients significantly decrease at 380 increasing seawater zinc concentrations following a power function. The result of passive sorption 381 test performed on dead specimens incubated in water with high Zn concentration suggests that most 382 of the zinc incorporation is mediated by the cell and that chemical passive sorption is putatively 383 negligible (Tab. A.2). These results suggest the suitability of Zn/Ca into foraminiferal calcite as an 384 environmental proxy. Zinc contents of calcite can be in fact considered representative of the 385 concentrations in seawater at the time of their calcification, and relatively independent of post-386 mortem conditions occurring in the depositional area. The analysis of Zn/Ca contents into the calcite 387 could therefore allow the reconstruction of past zinc concentrations in seawater. Then, present-day 388 knowledge about tolerance limits of foraminifera (e.g., de Freita Prazeres et al. 2011; Nardelli et al. 389 2013) and other organisms to Zn seawater concentrations would allow us to study potential 390 ecological effects induced by high concentrations of this metal in environmental systems in the past. 391 However further studies would be needed for a more precise calibration of Zn/Ca as a proxy. In 392 particular the study of potential synergic, additive or antagonistic effects of several chemicals and/or 393 of environmental variables (e.g., salinity, temperature, alkalinity..) on Zn incorporation should be 394 deeply investigated. Moreover, further calibration will be very useful in the future to fill the gap for 395 concentrations between 1 and 10 mg/L and between 0.1mg/L and natural unpolluted seawater zinc 396 concentrations (e.g., one order of magnitude lower).

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- 398

399 Acknowledgements

400 The authors thank the reviewers, G.J. Reichart and an anonymous one for their important remarks 401 and encouraging comments that strongly improved the manuscript. Thanks to the Polytechnic 402 University of Marche (Italy) for funding the research. We thank the Centro Interdipartimentale 403 Grandi Strumenti (CIGS) of the Università di Modena and Reggio Emilia (Italy) for the facilities and 404 Daniela Manzini and Maria Cecilia Rossi for the technical support. Thanks to Dr. Silvia Illuminati and Prof. Scarponi for providing bottom seawater samples for background Adriatic seawater zinc 405 406 concentration data. Many thanks to Christine Barras and Frans Jorissen for their valuable suggestions 407 and discussions on a first draft of the manuscript.

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535 Tables

Tab. 1 Optimized laser parameters. Linear ablation, carried out on the flatter part of the last chamber

proceeding always from the centre to the aperture of the chamber, was preferred to spot ablation.

538 See Hathorne et al. (2003) for comparison and SI-1 for further information. A pre-ablation step was

539 introduced to clean the surface from potential contaminations before data acquisition.

540

	Laser Intensity (%)	Frequency (Hz)	Ablation line width (μm)	Duration (s)	Main results	
Pre-ablation	10	1	55	230	Sampling only of external chamber, without crash; good signal intensity at the detector.	
Ablation	50	4	55	230		

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Tab. 2 Mean Zn/Ca_{seawater}, Zn/Ca_{calcite} and D_{zn} values (±standard deviations) for each Zn treatment.

Treatment	Zn/Ca _{seawater} (mmol/mol)	Zn/Ca _{calcite} (mmol/mol) (± s.d.)	D _{zn} (±s.d.)
sea (control)	0.278	1.122 (±0.028)	4.034 (±0.059)
sea+0.01 mg Zn/L	0.297	1.152 (±0.012)	3.880 (±0.042)
sea+0.1 mg Zn/L	0.465	1.216 (±0.011)	2.616 (±0.024)
sea+1.0 mg Zn/L	2.145	1.771 (±0.037)	0.826 (±0.017)
sea+10.0 mg Zn/L	18.948	3.813 (±0.171)	0.201 (±0.009)

544

Figures

Fig. 1 Example of successful laser sampling on last chamber of P. rotunda



- Fig. 2 Spectra resulting from the ablation lines measured on home-made standard tablets at different
- Zn concentrations to verify if Zn distribution was homogeneous. The highest and the lowest curves
- represent the spectra measured respectively on 1050 ppm and 150 ppm zinc home-made standards.
- CPS means counting per second.



Fig. 3 ESEM micrographs of *P. rotunda*'s test. a) View of external wall of a specimen from control cultures (sea); b) detail of crystals on the external wall of the same specimen; c) View of external wall of a specimen from sea+10 mg Zn/L contaminated cultures and d) detail of internal part of the wall, with disorganized crystals of the same specimen.



Fig. 4 Zn/Ca ratios in *Pseudotriloculina rotunda* calcite in function of Zn/Ca in seawater. a) All the samples are included (the colors correspond to the different Zn treatments). The best fitting of power function to predict (Zn/Ca)_{calcite} to (Zn/Ca)_{seawater} relationship is shown in blue and compared to the linear model (black dashed line). b) This is an enlargement of the left corner of figure 4a where the lower Zn/Ca points are showed separately.



570

571 Fig. 5 Partition coefficients measured for each zinc treatment. The averages D_{zn}± standard deviation

572 for each treatment are given in numbers.



Supplementary Data for online publication only Click here to download Supplementary Data for online publication only: Appendix A.pdf