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Experimental Chronic Exposure of the Foraminifer *Pseudotriloculina rotunda* to Zinc

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Abstract. Miliolid (calcareous imperforated test) foraminifera have large diffusion all over the world in a wide range of marine environments, but their distributional pattern in relation to heavy metal pollution is not clearly understood yet. The aim of this study was to better understand the biological response of the miliolid species *Pseudotriloculina rotunda* to experimental chronic exposure at several zinc concentrations. The duration of the experiment was 10 weeks, and six different concentrations of zinc were tested between 0 and 100 mg/L. Increasing zinc concentrations led to increasing delay or to complete cease of the new chambers' construction, with consequences on growth rates and affected vitality and biomass variations at medium to high concentrations. Moreover, our results showed that, even at high concentrations, zinc did not cause macroscopic test deformities due to anomalous arrangements of chambers.

Key words: Foraminifera, zinc pollution, heavy metal, test deformations, survival, growth rates.

INTRODUCTION

Benthic foraminifera are abundant protozoa, occurring in a variety of brackish and marine environments, from coastal to deep areas (Murray 2006). They are generally abundant, they respond quickly to environmental changes because of a relatively short life- and reproductive cycles and they are easily sampled (Alve 1991, Hallock *et al.* 2003, Mojtahid *et al.* 2006, Schönfeld *et al.* 2012). These characteristics make them interesting candidates as bioindicators. Moreover, most of the species have calcareous or agglutinated shells which can be preserved in the sediment and represent an important source of information on past environments (Yanko *et al.* 1998; Coccioni 2000; Debenay *et al.* 2001, 2005; Samir and El-Din 2001; Geslin *et al*. 2002; Murray and Alve 2002; Armynot du Chatelet *et al.* 2004; Coccioni and Marsili 2005; Frontalini and Coccioni 2008; Romano *et al.* 2008; Bergamin *et al.* 2009; Schönfeld *et al.* 2012). For this reason, in the last decades the interest of the scientific community in benthic foraminifera as bioindicators has grown (Schönfeld *et al.* 2012). However, little is known about the effect of many toxicants on the biology and ecology of foraminiferal species.

Some environmental studies showed that heavy metals can deeply affect foraminiferal faunas, in terms of abundances and species richness (e.g. Romano *et al*.

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2008, 2009; Coccioni *et al*. 2009). Among foraminiferal species, however, miliolids showed contradictory responses to heavy metal pollution in different studies. For example Ferraro *et al.* (2006) and Frontalini and Coccioni (2008) suggested that they could be sensitive to heavy metals, because they were rare or absent in heavily polluted areas, while Samir and El-Din (2001), Romano *et al.* (2008), Cherchi *et al.* (2009) and Foster *et al*. (2012) found high abundances or even dominance of these species in heavy metal polluted areas. Moreover, the presence of a large number of deformed test of benthic foraminifera sampled in highly heavy metal polluted sites let several authors (e.g. Yanko *et al.* 1998, Samir and El-Din 2001, Ferraro *et al*. 2006, Frontalini and Coccioni 2008, Coccioni *et al.* 2009, Frontalini *et al.* 2009) suppose a possible causal-effect relationship between these occurrences and heavy metal contamination, and hypothesize the use of test deformities as biomarker (Coccioni *et al.* 2003, 2005).

The high complexity of natural environments, however, makes difficult to discern causal-effect relationships between variables. Culture experiments, therefore, represent the most effective and direct method to assess the effect of a single parameter whilst the others are controlled constantly (Llirós *et al.* 2008). With this aim several studies have recently investigated the effects of various pollutants on cultured benthic foraminifera (e.g. Saraswat *et al.* 2004, Le Cadre and Debenay 2006, de Nooijer *et al.* 2007, Nigam *et al.* 2009, Denoyelle *et al.* 2012).

In this study, we chronically exposed imperforated foraminifer *Pseudotriloculina rotunda* (Schlumberger 1893) (Fig. 1) to zinc at different concentrations, in order to study the biological and ecological response of this species to the metal and to assess its degree of tolerance.

Zinc is an essential metal in eukaryotic life, required for numerous important cellular functions, but when its concentration exceed a threshold, it turns toxic on biological systems (Haase *et al*. 2001, Valko *et al*. 2005, Díaz *et al*. 2006, Formigari *et al.* 2007) for example causing reactive oxygen species generation in ciliates (Rico *et al*. 2009), yeasts (Fujs *et al.* 2005) and microalgae (Tripathi *et al.* 2006). Despite a fairly abundant literature on several marine taxa (e.g. ciliates, algae, fishes), nothing is known about the influence of high zinc concentration on benthic foraminiferal biology. We chose zinc, among heavy metals, for two main reasons: it is one of the most diffused heavy metals in areas subjected to anthropic impact and it is one of the metals suspected to cause test deformations in foraminifera (e.g. Sharifi *et al.* 1991, Samir and El-Din 2001, Romano *et al.* 2008). In unpolluted seawater zinc concentration is generally < 10 µg/L (Eisler 1993) but in highly polluted areas concentrations up to 5 mg/L have been reported (Reddy *et al.* 2005).

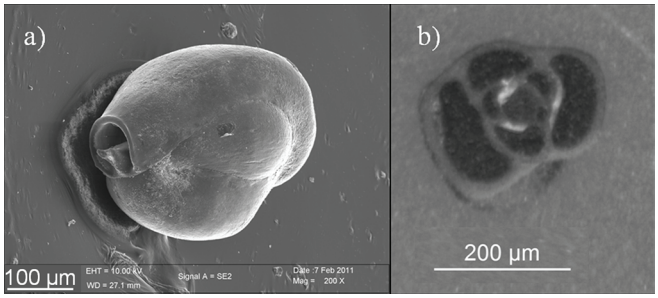

Fig. 1. a) SEM image of aperture view of a specimen of *Pseudotriloculina rotunda*. Magnification: 200 ×. Scale bar: 100 µm. b) Cross section of a specimen of *P. rotunda* under stereo microscope. Scale bar: 200 µm.

MATERIALS AND METHODS

Culturing procedures

Monospecific cultures of *P. rotunda* were inoculated with specimens picked from sediment samples. The samples were collected from an unpolluted coastal site situated in the central Adriatic Sea (Lat. 43°60.335 N, Long. 13°61.175 E) at 15 m water depth, using a Van Veen grab. Only the superficial sediment (0–0.5 cm layer) was collected with a spoon and stored in clean plastic jars with in situ seawater until laboratory. Then it was sieved (> 90 µm), using in situ seawater, to pick foraminifera. Cultures were maintained at the same environmental conditions measured at the sampling site: temperature (15 ± 0.5°C), salinity (38 ± 0.001 psu) and pH (8.02 ± 0.1) in microfiltered (0.42 µm) seawater and autoclaved sediment (63–150 µm) from the sampling site. They were fed with a mixture of *Chlorella marina* and *Dunaliella parva* every two weeks (approximately $5*10^4$ cell/cm^2). After the first reproduction event, juveniles were transferred to new jars and maintained at the same conditions for three weeks.

At the start of the experiment, the young specimens, all born in controlled experimental set, were picked using a fine brush, observed under a phase contrast optical microscope for pseudopodial activity as proof of vitality, then measured under optical microscope and randomly transferred in several sterile plastic jars, previously prepared for the experiment (see below for details).

The choice to use specimens born in culture was taken to be sure that they were all of similar ages and dimensions, already accustomed to experimental conditions (in terms of diet, temperatures and other parameters) and born in controlled conditions, in order to avoid results due to unknown pre-experimental conditions.

Experimental design

A simplified scheme of the experimental set is shown in Fig. 2. Five different zinc concentrations and one control (Ctrl) were tested. Two replicates, each containing six specimens, were exposed to each concentration for 10 weeks.

As nothing was known about the sensitivity of this species to zinc, six concentrations, increasing of a factor 10, were tested (Ctrl = 0, C1 = 0.01, C2 = 0.1, C3 = 1, C4 = 10 and C5 = 100 mg/L), in order to try the response on a wide range of concentrations. The highest concentration was chosen in order to include values of zinc at lethal concentrations as known for other protozoa, such as some species of marine ciliates (e.g. until 50 mg/L, Madoni *et al*. 1992).

Two control replicates were prepared with microfiltered seawater from the control site, not added with zinc. Specimens were individually checked for vitality (see paragraph *Foraminiferal observations*), test size and potential test deformities every week.

Experimental conditions

Twelve culture sets were prepared for the experiment. Sterile plastic jars were carefully cleaned with diluted (1:100 and 1:1000) ultrapure HCl (Sigma Aldrich) and rinsed with MilliQ water before the introduction of zinc solutions and miliolid specimens. A thin layer of artificial, previously washed with diluted HCl, coarse sediment (99.9% silicates, > 200 µm), which has a low affinity to metals compared to finer sediment (Förstner and Wittman 1981), was added to each jar: 1) to avoid the possibility that the absence of sediment could affect the development of the normal foraminiferal life cycle and 2) to limit the adsorption of zinc on sediment, which would have hampered the evaluation of its toxicity during the experiment. The expected zinc concentrations were obtained by means of dilutions of stock solutions of $ZnCl_2$ (> 99.999% pure, Sigma), acidified at pH 2, in microfiltered seawater. Zinc solutions were completely replaced every week.

Foraminiferal observations

Foraminiferal specimens were isolated in a petri dish for observations every week and then transferred to freshly prepared zinc solutions. Cytoplasm color, from yellow/brown to green (following Kitazato *et al.* 2003, de Nooijer *et al.* 2008), pseudopodial activity and the presence of cysts or particles of sediment around the specimens' apertures (Heinz *et al.* 2005) were all observed parameters to assess vitality. The need to observe the same specimens every week, in fact, prevented the possibility to use other vitality tests which imply the death of the specimen or persistent labeling (e.g. CTG, Bernhard *et al.* 2006; FISH, Borrelli *et al.* 2011). In addition, the weekly controls allowed to re-check the vitality of each specimen every week and we never observed any case of specimen not showing pseudopodial activity at one time, showing this activity at the following steps. Thus, once the pseudopodial activity was null and the specimens showed uncolored cytoplasm or empty test and absence of particles outside the aperture, they were considered dead and excluded from the total biomass count.

Pseudopodial activity and test size of every specimen were checked under contrast phase optical microscope (Nikon Eclipse E 600 POL). Test sizes (i.e. maximum specimen length of each individual) were measured every week at the same optical microscope (error ± 6 µm). Biomass was estimated at any weekly control times during the whole duration of the experiment, using the formula proposed by Altenbach (1985) and modified by Kurbjeweit *et al*. (2000) for miliolids (*Pyrgo* spp. and *Quinqueloculina* spp.): 3.606*0.0000001*X^2.49, where X is the measure (in µm) of the maximum specimen length. The biomass estimate, based on volumetric analysis is a method largely used for meiofaunal taxa (Danovaro *et al.* 2003). During these controls new formed chambers and potential deformities were also checked.

Statistical analysis

Statistical analyses were performed with R software, version 2.11.1. Spearman's rank correlation was carried out on survival data in order to investigate if the vitality was correlated to zinc concentrations. One-way ANOVA and Friedman test were used respectively to test the statistical difference in biomasses among replicates at T_0 and in biomass variations among treatments over experimental time (Sokal and Rohlf 1995). P-values < 0.05 were considered as significant. When the Friedman test resulted significant, the Schaich-Hamerle post-hoc test was performed.

A T-test for paired data was also performed for each treatment individually in order to test for significant biomass variations from T_0 and the end of the experiment (T_{10}). Finally the regression analysis was tested for biomass variation in relation to zinc concentrations (e.g. Salemaa and Monni 2003), using SPSS 13.0.

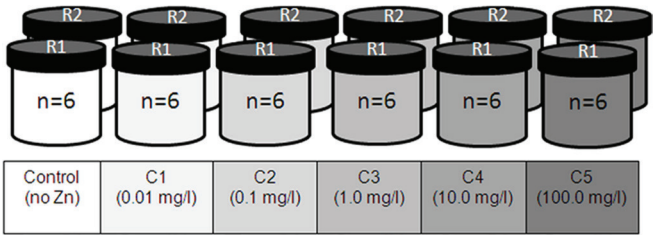

Fig. 2. Scheme of the experimental set up. R means replicate, n is the number of specimens for each replicate, C means zinc concentration.

RESULTS

Survival rates

Percentages of individuals which were still alive at the end of the experiment are shown in Fig. 3. At the maximum tested concentration C5 = 100 mg Zn/L, 100% of specimens were already dead after one week (T_1). At C3 and C4 (1 and 10 mg Zn/L, respectively), the vitality of 33% of specimens was negatively affected during the experiment, ceasing between weeks seven and ten (T_7 and T_{10}, see Table 1). At C2 = 0.1 mg Zn/L, only one specimen (corresponding to 8% of the

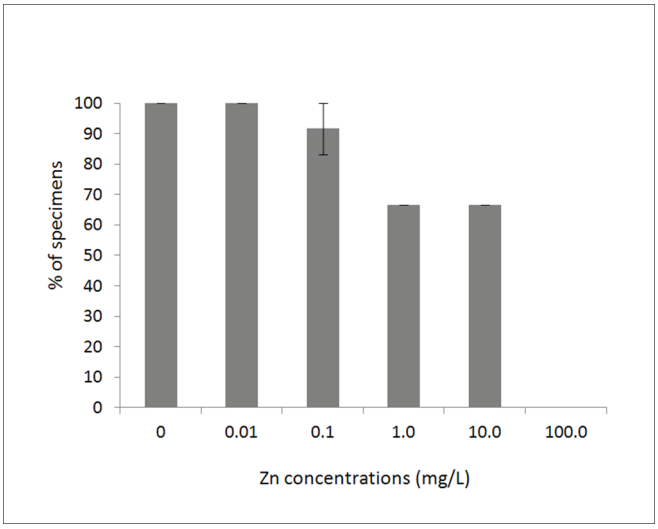

Fig. 3. Percentage of specimens still alive at the end of the experiment for each zinc treatment.

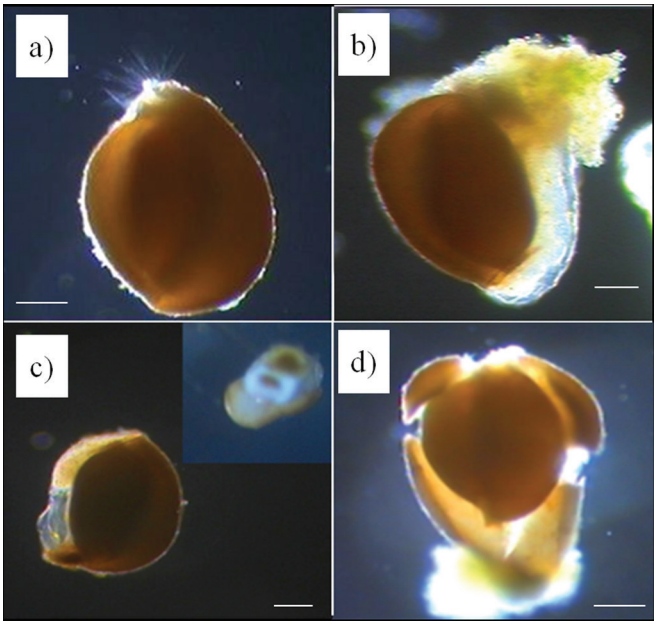

Fig. 4. a – pseudopodial activity; **b** – new chamber produced during the experiment; **c** – growth of abnormal test (in the right square a detail of the aperture of the same specimen); **d** – occurrence of a breakage event. Scale bar: 100 μm.

Table 1. Summary of main results of the experiment. T means time (corresponding to weeks). R1 and R2 are the two replicates performed for each zinc concentration.

	Control		C1 = Zn [0.01 mg/l]		C2 = Zn [0.1 mg/l]		C3 = Zn [1.0 mg/l]		C4 = Zn [10.0 mg/l]		C5 = Zn [100.0 mg/l]	
	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
First new chamber (time = week)	T_2	T_2	T_2	T_2	T_3	T_2	T_4	T_4	T_5	T_4	–	–
Number of specimens growing new chambers	6	6	6	6	6	6	5	3	4	3	0	0
Number of new chambers grown during the experiment	11	9	11	8	4	5	1	4	5	3	0	0
Number of specimens showing test abnormalities	0	0	0	0	0	0	0	1	0	0	–	–
Number of dead specimens (time of first death)	0	0	0	0	0	1(T_{10})	2(T_7)	2(T_{10})	2(T_7)	2(T_{10})	6(T_1)	6(T_1)

tested specimens) died during the last week (T_{10}). All specimens of Ctrl and C1 (0.01 mg Zn/L) survived the experiment, showing a normal pseudopodial activity during the whole duration (as in Fig. 4a).

The number of dead specimens resulted positively correlated to zinc concentration ($\rho = 0.97$, $p < 0.01$).

New chambers and test deformations

Main results are summarized in Table 1. All the specimens from controls, C1 and C2 (0.01 and 0.1 mg/L) treatments produced at least one chamber (Table 1, Fig. 4b). At higher concentrations C3 and C4 (1 and 10 mg/L), only some specimens grew new chambers. In particular, respectively for the two replicates, 5 and 3 specimens at C3, and 5 and 6 specimens at C4 grew at least one new chamber (Table 1). Not any new chamber was produced at C5 (100 mg/L).

The first new chamber grew after 2 weeks (T_2) in both Ctrl and C1; between the second and the third week (T_2, T_3) at C2; after 4 weeks (T_4) at C3. In the last treatment some tests were found broken during weekly controls. Finally, chamber growth occurred between the fourth and the fifth week (T_4, T_5) at C4 (10 mg/L).

Only at Ctrl and C1 (0.01 mg Zn/L) some individuals grew more than one chamber (minimum 1, maximum 4). At higher concentrations, just one specimen in replicate 2 at concentration of 10 mg Zn/L grew two chambers during the experiment; the others grew 1 chamber as maximum.

Despite the high total number of new formed chambers in all treatments (except C5), only in one case an obvious test deformation was observed. One specimen exposed to intermediate concentration C2 (0.1 mg/L), presented a double aperture (Fig. 4c), formed during week 3 (T_3) (Table 1). It has to be remarked, moreover, that anomalous breakages events occurred during some of the weekly observations of specimens at concentration C3 (1 mg/L, Fig. 4d). These events did not affect in any case the vitality of the concerned specimens, which still showed pseudopodial activity. In some cases new chambers were re-constructed after these accidental breakages, giving sometimes somehow abnormal chambers as result. However, this kind of abnormalities was easily discriminated.

Biomass variation

The ANOVA test, performed on data of biomasses at T_0, confirmed that specimens randomly attributed to the various zinc treatments had statistically equal biomasses (p-value > 0.05) at the start of the experiment. The biomass variation for each treatment during the experiment is shown in Fig. 5.

The Friedman test, performed to assess potential differences among treatments in test sizes variations during the whole experimental time, resulted significant (p-value < 0.05, Fig. 6). The post-hoc test revealed that the difference (p-value < 0.05) was significant between test sizes at Ctrl and concentrations C2, C3, C4 and C5 (respectively 0.1, 1.0, 10.0 and 100.0 mg/L) and between C1 (0.01 mg/L) and C5 (100.0 mg/L), while differences among specimens exposed to intermediate

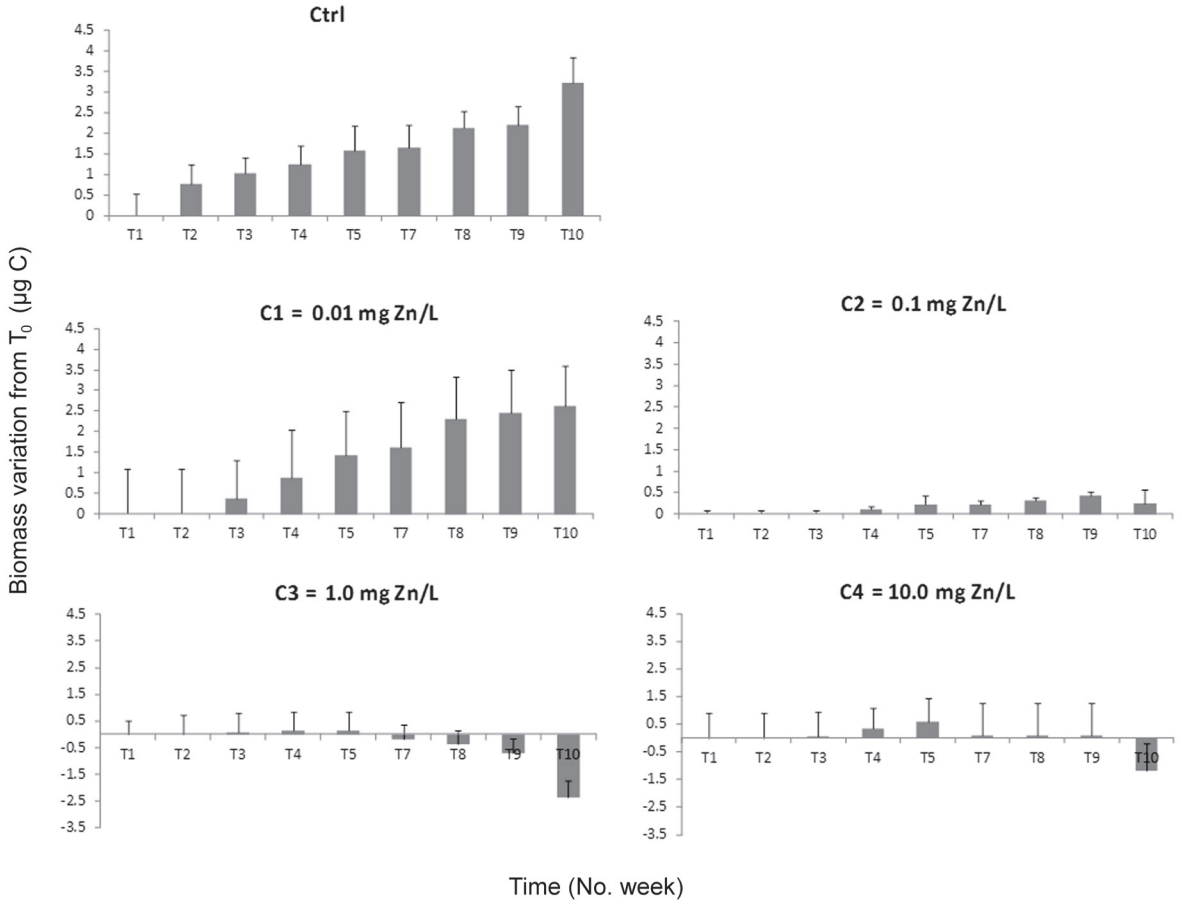

Fig. 5. Biomass variations at each experimental time for each zinc concentration (T_x–T_0). At C5 = 100 mg/L biomass variation was zero. Time (T) is expressed as weeks from the start of the experiment.

concentrations (C2, C3, C4) and between the ones at Ctrl and C1 were not statistically significant.

The total test size variation (calculated as size at T_{10} – size at T_0) for each treatment separately, as mean values of the two replicates, is shown in Fig. 7. Regression analysis on these data revealed that there is a logarithmic relation between biomass variation and zinc concentration (ANOVA, p-value < 0.05). The T-test for paired data performed to test the significance of the biomass variation from T_0 of each treatment resulted significant (p-value < 0.05) for Ctrl, C1, C3 and C5 zinc concentrations. Biomass variation at T_{10} was statistically higher at Ctrl and C1 (0.01 mg/L) compared to the initial biomass, it was not statistically different for intermediate concentrations C2 and C4 (0.1 and 10 mg/L), and statistically lower for C5 (100 mg/L). At C3 (1 mg Zn/L) the significance of test size decrease was due to tests breakage events.

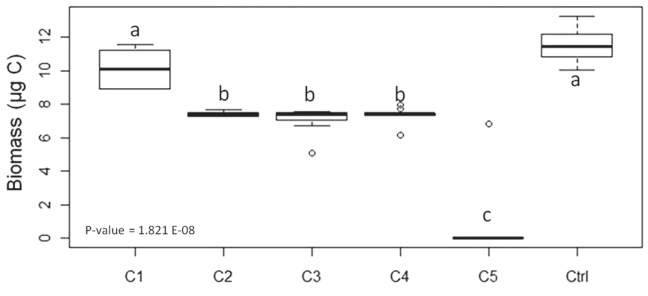

Fig. 6. Result of Friedman test on biomass variation stratified by time at each zinc concentration. P-value < 0.05. Letters (a, b, c) represent groups resulted significantly different by post-hoc test. Objects with the same letter are statistically equal among them and significantly different from the ones with different letters.

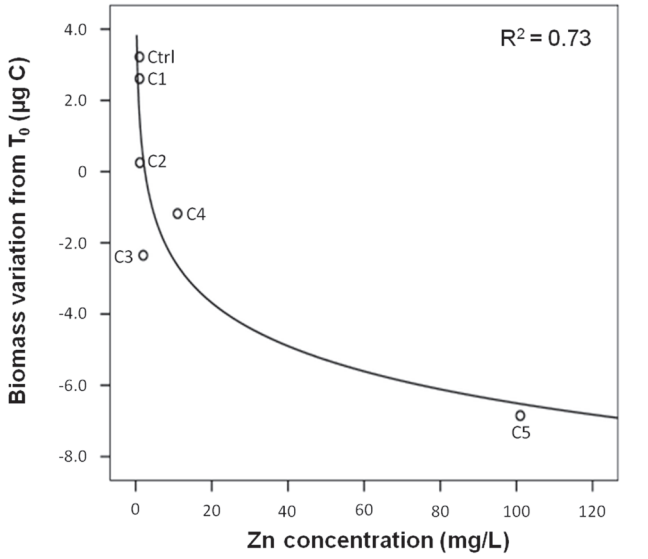

Fig. 7. Total biomass variation (T_{10}–T_0) at each zinc concentration. Results of regression analysis are shown (P-value < 0.05).

DISCUSSION

Zinc is an essential metal, structural compound of many enzymes (Vallee and Falchuk 1993). Some studies on ciliates (i.e. *Tetrahymena pyriformis*) demonstrated that phenomena of adaptation of these organisms to an excess of zinc occur (Nilsson 1989), consisting on a prolonged generation time due to the metabolic cost of the intracellular handling of zinc excess.

The present experiment was a first step to test the tolerance of a benthic foraminiferal species to zinc as potential toxic element, and its implications on growth and the occurrence of deformed shells. This last aspect is particularly interesting because several authors have interpreted test abnormalities as indicators of heavy metal contaminations (e.g. Samir and El-Din 2001, Coccioni *et al.* 2005, Frontalini and Coccioni 2008, Bergamin *et al.* 2009, Romano *et al.* 2009). Coccioni *et al.* (2005), with the Foraminiferal Abnormality Index (FAI), also proposed the use of (high) percentages of abnormal tests as indicators of environmental pollution. Even if the specialists are debating on the possibility to use abnormalities as bioindicators of pollution, their occurrence in environmental samples were not yet definitely ascribed to a particular pollutant and, moreover, it seems it could be also related to natural environmental stress (e.g. salinity changes; Stouff *et al.* 1999, Geslin *et al.* 2002). Therefore, every particular effort in this sense could add useful information.

Survival rates

Pseudotriloculina rotunda showed high tolerance to zinc. Most of the specimens survived until zinc concentrations of 10 mg/L. This tolerance is comparable with that of other protozoa, for example ciliates. Díaz *et al.* (2006) estimated LC_{50} (Lethal Concentration resulting in 50% of mortality of the experimental population) of 40 to 150 mg/L zinc for some species of soil ciliates whereas Madoni *et al.* (1995) evaluated zinc LC_{50} between 0.2 and 85 mg/L in protozoa from activated sludge communities. Different zinc lethal concentrations esti-

mated for several metazoa both belonging to meio- and macrofauna were below or similar to the values estimated for *P. rotunda*. For example Hagopian-Schlekat *et al*. (2001) estimated LC_{50} of ~ 0.4 mg/L (after 96 h) for the harpacticoid copepod *Amphiascus tenuiremis*; Ramakritinan *et al.* (2012) an LC_{50} of 31 and 13 mg/L (after 48 h) respectively for the snail *Cerithedia cingulata* and the bivalve *Modiolus philippinarum*.

Considering that in chronic contamination conditions the degree of tolerance is generally much lower compared to acute stress (e.g. Bechmann 2009, Kenaga 2009), we can conclude that, compared to other benthic species, the tolerance of *P. rotunda* to zinc could be considered even more important. Despite this, some negative effects of the metal on vitality were already observed at lower concentrations. In fact, zinc already caused some deaths at concentrations of 0.1 mg/L. Actually, during the experiment, 8 to 33% of individuals died at intermediate concentrations (0.1, 1.0 and 10 mg/L), and 100% of specimens at the highest tested concentration (100 mg/L). Statistical analyses on percentages of dead specimens revealed their significant correlation to zinc concentrations. Similar effects were also noticed by Denoyelle *et al.* (2012), resulting in reduced pseudopodial activity after a 30 days long contamination of cultured foraminifera (*Ammonia tepida*) with cadmium, fuel oils and drilling muds. This aspect (i.e. the effects of chronic exposure) is very important especially for policy purposes, when limits for chemicals in sediments are established, basing on results obtained from short-term experiments. However, it must be considered that our study does not take into consideration many environmental variables that could decrease the effects of zinc, as for example the presence of other metals (e.g., cadmium as described by Díaz *et al.* 2006), or, in marine environments, the increase of salinity or the presence of fine grain-sized sediment, which can reduce the toxic effects of this metal.

New chambers and test abnormalities

The biomineralization process of our experimental model resulted to be affected by zinc. The production of the first new chamber was delayed by increasing zinc concentrations, in particular at concentrations higher than 1 mg/L. The number of chambers grown during the experiment never exceeded 1 (except in a single case) at zinc concentrations higher than 0.1 mg/L (Table 1). This result is consistent with the results by Le Cadre and Debenay (2006), who noticed a delay in chamber formation in the calcareous perforated species *Ammonia tepida* and *Ammonia beccarii* contaminated with copper. Similarly Saraswat *et al.* (2004) and Nigam *et al.* (2009) found a negative correlation between foraminiferal growth rates and mercury at several concentrations. The authors concluded that this outcome could explain the dwarfism often described in foraminifera assemblages from polluted areas (e.g. Yanko *et al.* 1994, Samir and El-Din 2001). In both the mentioned experiments performed with mercury concentrations, however, the production of new chambers was often accompanied by the occurrence of deformed chambers. The mercury concentrations causing these occurrences were different in the case of gradual (180 ng Hg/L; Saraswat *et al.* 2004, Nigam *et al.* 2009) or sudden (< 150 ng Hg/L; Nigam *et al.* 2009) exposure of the specimens to the metal. Moreover they found much higher numbers of deformations and abnormalities during sudden exposure to mercury than during gradual contamination.

In our experiment with zinc, instead, even if the specimens were directly exposed to the final expected concentration, no deformation, in terms of chamber arrangement or morphology, were noticed, except for a specimen from one of the lower tested zinc concentration (0.1 mg/L), which presented two apertures after a new chamber construction (Fig. 4b).

This result strongly suggests that zinc does not cause macroscopic test deformations as already stated by Hayward *et al*. (2004), who did not found test deformities in fossil foraminifera from environments with zinc concentrations even higher than the experimented ones. Instead it partially disagrees with Samir and El-Din (2001), who suggested a correlation between environmental heavy metal concentrations, including zinc in the list of responsible metals, after observing high concentrations of the metal in abnormal tests of the miliolid species *Quinqueloculina seminula* from a polluted site along the Mediterranean Egyptian coast.

It has to be noticed, however, that the anomalous observation, during the weekly controls, of breakage events in some specimens treated with 1 mg/L of zinc, suggests the anomalous reduction of new chambers' thickness. The fact that these occurrences and the only abnormal test were observed at low-intermediate zinc concentrations but not at higher contaminations, is consistent with several studies (e.g. Alve and Olsgard 1999, Le Cadre and Debenay 2006) which stated that test deformations under high heavy metal concentrations occur less often than under medium pollution loads. However, the fact that in all replicates just one macro-

scopic deformation occurred does not allow a complete discussion about the event, but it still suggests that zinc is not responsible, by itself, of the production of obviously abnormal tests.

Biomass variation

As previously presented, the present study showed that increasing zinc concentrations have a significant influence on growth rates, slowing down the construction of new chambers. This obviously affects also biomass variations.

In both control and at lower zinc concentration C1 (0.01 mg/L) the biomass grew across all the experimental time, whereas at higher concentrations it stopped or even decreased (Fig. 5). This is also noticeable looking at the total biomass variation at the experiment end (T_{10}–T_0, Fig. 7). In fact, the total biomass variation after 10 weeks of zinc treatment was significantly higher both at control and 0.01 mg/L concentrations; instead, it did not vary significantly at intermediate concentrations and, decreased significantly at higher concentrations, showing a logarithmic regression with increasing zinc concentrations. Friedman and post-hoc tests, performed on all biomass data, showed that there was a significant difference only between biomass variation in control and all tested zinc concentrations higher than 0.1 mg/L and between the lowest (C1 = 0.01 mg/L) and the highest (C5 = 100 mg/L) tested concentrations. This suggests that after the threshold concentration of 0.1 mg/L, zinc negatively affected the organisms, producing a stop or a decrease in biomass variation. This is also consistent with the reduced observed survival rates. Similar observations were also reported for microalgae. Reduced growth rates were observed, in fact, for the three diatoms *Skeletonema costatum*, *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* after the exposure to 0.05, 0.25 and 25 mg/L of zinc, respectively (Jensen *et al.* 1974).

The biomass decrease at high zinc concentrations (> 10.0 mg/L) can be ascribed to extreme cellular disease, as it was consequent to the interruption of pseudopodial activity and the death of the specimens. On the other hand the growth stop at the other intermediate concentrations (0.1–1 mg/L) was mainly due to the delay in new chamber construction. This affection of the biomineralization processes is suggested to be due to cellular disease and/or to the ability of zinc to inhibit $CaCO_3$ precipitation, as demonstrated by Ghizellaoui *et al*. (2007).

CONCLUSION

Zinc showed to highly affect growth and vitality of the miliolid foraminifer *P. rotunda* when present in concentrations higher than 0.1 mg/L, in long-term experimental conditions.

Starting by this concentration decreased survival rates and delayed production of new chambers, thus biomass variations, were observed with effects always more intense at increasing zinc concentrations.

The biomass showed significant variations only at control and 0.01 mg/L zinc concentrations (increase) and at the highest (100 mg/L) tested concentrations (decrease), while it did not vary at intermediate concentrations, due to the delayed biomineralization. Despite biomineralization processes resulted influenced by the presence of zinc, no macroscopic deformation (i.e. abnormal chamber morphology or arrangement) was observed, except for one isolated case, occurred at intermediate zinc concentration (1 mg/L). Although further investigations are necessary to clarify the cause of the occurrence of breakage events during the weekly controls, our results strongly suggests that zinc, even at high concentrations, does not cause macroscopic test deformities.

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