Proceedings of the 12th International Coral Reef Symposium, Cairns, Australia, 9-13 July 2012
15F Benthic foraminifera on coral reefs

Impact of metal exposure in the symbiont-bearing foraminifer *Amphistegina lessonii*

Martina de Freitas Prazeres, Samantha Eslava Martins, Adalto Bianchini

Instituto de Ciências Biológicas, Universidade Federal do Rio Grande – FURG, Av. Itália km 8 CEP 96201-900, Rio Grande, RS, Brazil
Corresponding author: mdfprazeres@gmail.com

Abstract. Ca^{2+}-ATPase activity was evaluated in adult holobionts of *Amphistegina lessonii*. Foraminifer and seawater samples were collected at sites located inside (Porcos Bay-POR, Dois Irmãos Shoal-LDI and Buracão-BUR) and outside (Santo Antonio Harbor-PSA and Biboca Beach-BIB) the "Fernando de Noronha" Marine National Park (FNMNP) (Northeastern Brazil). Individuals from BUR site were also transferred alive to the laboratory and exposed to Zn (0, 25, 42, 68 and 93 µg dissolved Zn/l). Ca^{2+}-ATPase activity was measured in both field- and laboratory-collected individuals. Higher enzyme activity was observed in foraminifers collected inside the FNNMP. The highest activity was observed in LDI foraminifers (7.4±1.4 mmol Pi/mg protein/min), whereas those collected at the BIB site showed a significantly ($p<0.01$) lower activity (0.7±0.3 mmol Pi/mg protein/min). Metal (Cd, Cu and Zn) concentration in seawater samples were strongly and negatively correlated to Ca^{2+}-ATPase activity. In laboratory-exposed individuals, Ca^{2+}-ATPase activity increased at 25 µg Zn/l, but was inhibited at Zn concentrations higher than 42 µg Zn/L, declining to an activity as low as 0.12 ± 0.02 nmol Pi/mg protein/min ($p<0.01$; $r^2=0.97$). Some metals are known to inhibit Ca^{2+}-ATPase activity and consequently calcium uptake. Ca^{2+}-ATPase plays a significant role in calcification. Therefore, its inhibition would affect negatively this process in symbiont-bearing foraminifers and other calcifying organisms, resulting in more fragile tests and consequently individuals more vulnerable to increasing threats arising from global changes and ocean acidification. Our results indicate that holobiont Ca^{2+}-ATPase activity is a good biomarker of metal exposure, allowing the detection of local stressors and implementation of management directives for coral reef preservation.

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Key words: Calcium uptake, Coral reef, Fernando de Noronha, Foraminifera.

Introduction

Large benthic foraminifers (LBF) are shelled protists that shelter endosymbiotic algae, sharing characteristics with hermatypic corals (Hallock et al. 2006). They have been used worldwide as important bioindicators of water quality in coral reef environments (Hallock et al. 2003; Uthicke and Nobes 2008, Prazeres et al. 2012). Also, LBF are contributors to the $CaCO_3$ (calcite and aragonite) cycling in the ocean (Tambutté et al. 2011), and 8% of the global foraminiferal reef carbonate is estimated to be produced by these species alone (Langer et al. 1997).

Ca^{2+}-ATPase is a membrane-bound enzyme which plays an important role in calcium homeostasis and is found to be ubiquitous in animal cells (Pattnaik et al. 2007). Foraminifers use the activity of this enzyme to transport Ca^{2+} across the membrane enclosing the calcifying fluid in order to initiate calcite precipitation (Nooijer et al. 2009). In LBF such as *Amphistegina lobifera*, the calcification process occurs in the extra-shell cytoplasmic space where $CaCO_3$ is precipitated over the existing shell (Erez 2003). Any disturbance in the Ca^{2+} balance, Ca^{2+}-ATPase activity or ion channel transport may disrupt the biomineralization process, leading to the formation of thinner, weakened and anomalous shells.

Coral reefs are among the ecosystems threatened by the increasing input of chemical contaminants into marine waters (Peters et al. 1997). Increasing concentration of metals has been found in reefs from Brazil, such as the Fernando de Noronha Archipelago (Prazeres et al. 2012). Toxicity of heavy metals in aquatic animals may be associated with effects on enzyme activities (Pattnaik et al. 2007). For example, metals such as Cd and Zn are shown to inhibit Ca^{2+}-ATPase activity in fish, causing perturbation in Ca^{2+} metabolism (Hogstrand et al. 1996).

In foraminifers, Prazeres et al. (2011) observed bleaching, lower antioxidant capacity and lipid peroxidation in *A. lessonii* individuals acutely exposed to 93 µg dissolved Zn/L. Also, field-sampled individuals from Fernando de Noronha Archipelago (Northeastern Brazil) revealed high degree of bleaching and protein carbonilation in sites where high metal concentrations were found (Prazeres et al.

2012). However, no studies regarding the metal effect on Ca^{2+}-ATPase activity in LBF are reported to date. Considering the role of Ca^{2+}-ATPase in Ca^{2+} transport and shell formation, inhibition of the enzyme activity by metals present in the surrounding water may directly or indirectly influence foraminiferal health and response to other stressors. Since LBF are abundant residents in coral reefs (Hallock et al. 2006) and important producers of carbonate sediments (Langer et al. 1997), evaluation of their response to short-term metal exposure may be useful for ecotoxicological investigations and monitoring.

In light of the above, the goal of the present study was to determine the holobiont Ca^{2+}-ATPase activity in normal-appearing adults of *A. lessonii* at different sites with different levels of metals, and also in those from clean sites exposed to zinc under laboratory conditions.

Material and Methods

Field collection

Sampling sites were located at Fernando de Noronha Archipelago, located 360 km off the northeast coast of Brazil. The archipelago is divided into two areas: an Environmental Protected Area (EPA) designed for sustainable use with permanent human occupation, and the Fernando de Noronha National Marine Park (FNNMP), which is protected from any form of exploitation. Prazeres et al. (2012) observed high concentrations of dissolved Pb, Zn and Cu in two sites located outside the FNNMP.

Sample collection was carried out as described in Prazeres et al. (2012). Five sites located on the leeward side (NW shore) of Fernando de Noronha Island at depths ranging 7-20 m were selected: Santo Antonio Harbor (PSA), Biboca Beach (BIB), Porcos Bay (POR), Dois Irmãos Shoal (LDI), and Buracão (BUR). The two first sites are located inside the EPA, while the others are inside the FNNMP. *Amphistegina lessonii* present on reef cobble were picked and placed on dry ice for further analysis of Ca^{2+}-ATPase activity. Additionally, water samples were collected in duplicate for analysis of dissolved organic carbon (DOC) and dissolved metal (Zn, Cu, Pb, and Cd) concentrations.

Experimental procedures

Live individuals of *A. lessonii* were collected at the BUR site in the FNNMP, and acclimated (3-4 weeks) in a culture chamber with fixed temperature (27±1°C) and photoperiod (12 h light/dark cycle). White fluorescent source providing PAR was used to allow the endosymbiont photosynthesis. Individuals were maintained in synthetic seawater (salinity 36 ppt) with addition of nutrients, as described in Hallock et al. (1986).

Five replicates per treatment (n = 6-8 healthy adults per replicate) were randomly assigned to the 48-h exposure test. Foraminifers were exposed to different Zn concentrations (average ± standard error): 9.53 ± 0.61 (control), 25.20 ± 0.34, 42.01 ± 1.80, 67.67 ± 1.86, 93.37 ± 9.93 µg dissolved Zn/l. At the end of experiment, live foraminifers were collected and frozen (-80ºC) for further enzyme activity analysis.

Ca^{2+}-ATPase activity analysis

Foraminifers from field and laboratory samples were homogenized in a buffer solution containing 500 mM sucrose, 1 mM $_{DL}$-dithiothreitol, 150 mM KCl, 20 mM Tris Base, and 0.1 mM phenylmethylsulfony, with pH adjusted to 7.6. Homogenates were then centrifuged at 10,000×*g* for 20 min at 4°C. Protein content in the homogenate supernatant was determined using the Quant-iT Protein Assay (Invitrogen, USA). Ca^{2+}-ATPase activity was determined spectrophotometrically following the method of Fiske and Subbarow (1925). According to this method, the inorganic phosphate (Pi) released from the ATP consumption during calcium pumping is measured. Pi concentration in the reaction mixture was quantified by spectrophotometry (620 nm) using a reagent kit. Pi concentration was calculated using a standard curve built with monobasic phosphate.

Statistical analysis

Ca^{2+}-ATPase activity was measured in triplicate. Results are expressed as mean ± 1 standard error. Differences among mean values of the experimental groups were detected by one-way analysis of variance (ANOVA) followed by the Tukey's (HSD). Data were checked for homogeneity of variance and normality prior to analysis. In all cases, the significance level adopted was 95% ($\alpha = 0.05$). Negative exponentially-weighted fitting was performed to evaluate a possible correlation between Zn concentration and enzyme activity. A redundancy analysis (RDA) was also performed to illustrate differences in Ca^{2+}-ATPase activity among sampling sites and their dependence on the environmental variables analyzed. All environmental and biomarkers data were *z*-transformed prior to RDA.

Results

Field study

Ca^{2+}-ATPase activity was lower in *A. lessonii* from PSA and BIB sites than in those collected at the sites located inside the FNNMP, such as the LDI site. For example, Ca^{2+}-ATPase activity was 7.4 mmol Pi/mg protein/min in foraminifers collected at the LDI site, while those from the BIB site showed 10-fold lower

activity (0.7 mmol Pi/mg protein/min) ($p<0.01$; Fig. 1). In general, individuals collected inside the FNNMP (POR, LDI and BUR) showed a higher specific Ca^{2+}-ATPase activity, although no significant difference was observed from those collected at sites located outside the FNNMP (PSA and BIB). Redundancy analysis (RDA) revealed a strong and negative correlation between the Cu, Cd and Zn concentration in seawater and the Ca^{2+}-ATPase activity, indicating that higher concentrations of these metals where associated with lower specific enzyme activities .

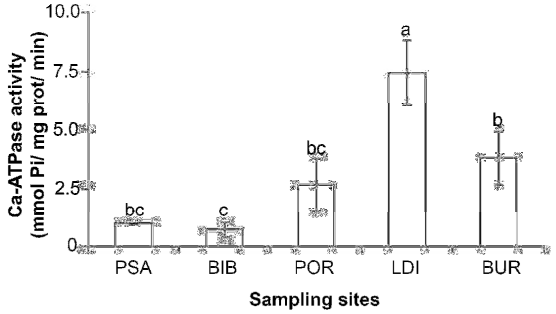

Figure 1: Ca-ATPase activity in normal-appearing holobionts of *Amphistegina lessonii* collected off Fernando de Noronha Island. Data are expressed as mean±SE. Different letters indicate significant differences among sites ($p<0.05$).

Zinc exposure in laboratory

Ca^{2+}-ATPase activity was significantly different in control individuals and those exposed to 42 and 93 µg Zn/l ($p<0.01$; Fig. 2). When exposed to 42 µg Zn/l, *A. lessonii* exhibited an increased enzyme activity (1.5 mmol Pi/mg protein/min). However, an inhibition of enzyme activity was observed in foraminifers exposed to 93 µg Zn/l (0.1 mmol Pi/mg protein/min). Fitting analysis revealed a clear pattern of enhanced enzyme activity and further reduction with the increasing Zn concentration ($r= -0.24$, $p=0.5$; Fig. 3).

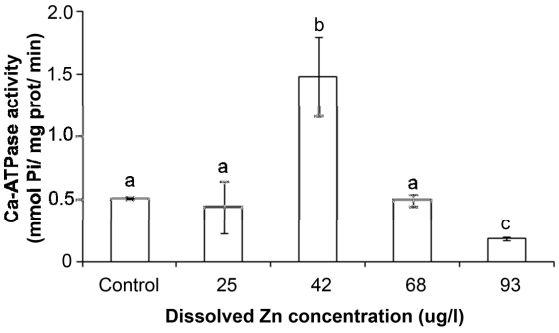

Figure 2: Ca-ATPase activity in normal-appearing holobionts of *Amphistegina lessonii* collected off Fernando de Noronha Island and experimentally exposed to Zn. Data are expressed as mean±SE. Different letters indicate significant differences among sites ($p<0.05$).

Discussion

Field and laboratory results demonstrate a clear influence of dissolved metal concentration on Ca^{2+}-ATPase activity. *Amphistegina lessonii* collected at the LDI site (inside the FNNMP) showed a higher Ca^{2+}-ATPase activity, where the lowest concentrations of dissolved metals were found in the water. Individuals collected at the BIB site (outside the FNNMP) showed a strong negative correlation between enzyme activity and dissolved metal concentrations in the water, especially Cu and Cd. Both metals are known to inhibit Ca^{2+}-ATPase activity in mussels (Viarengo et al. 1996) and crabs (Burke et al. 2009). Regarding benthic foraminifers, no previous data on the effect of these metals on Ca^{2+}-ATPase activity are available. However, it has been demonstrated that these metals are likely causing bleaching, lipid peroxidation and protein carbonilation in adult holobionts (Prazeres et al. 2012). These damages can also interfere with enzyme activity, since they can alter membrane permeability. Therefore, the reduced Ca^{2+}-ATPase activity observed in the present study may be also related to the metal-induced oxidative damage.

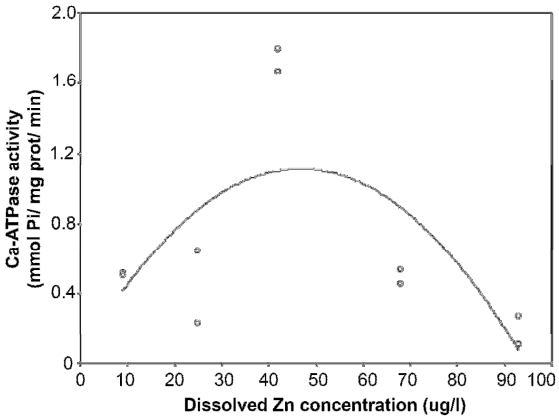

Figure 3: Negative fitting between Ca-ATPase activity in normal-appearing holobionts of *Amphistegina lessonii* and dissolved Zn concentration in the medium.

In laboratory-exposed *A. lessonii*, Prazeres et al. (2011) showed that exposure to increasing Zn concentrations induces a lower antioxidant capacity and enhances lipid peroxidation, resulting in bleaching and increased metal toxicity. Sandeman (2008) suggested that lipid peroxidation may be one of the sources of Ca^{2+} for calcification in corals. Nevertheless, *A. lessonii* exposed to sub-lethal Zn concentrations showed enhanced Ca^{2+}-ATPase activity at 42 µg Zn/l. The higher lipid peroxidation

observed in *A. lessonii* exposed to Zn (Prazeres et al. 2011) might have caused a leakage of intracellular Ca^{2+}, causing the observed rise in Ca^{2+}-ATPase activity at 42 µg Zn/l to pump out the excess of Ca^{2+}. In turn, a reduced enzyme activity was observed at 93 µg Zn/l likely due to a direct metal effect on the enzyme. It is suggested that Zn ions are capable of forming a complex with ATP, binding to Ca^{2+}-ATPase, inhibiting the enzyme activity (Dias and Coelho 2007). Also, Zn and Ca appear to compete for the same ion channel (Santore et al. 2002). Therefore, Zn can exert a toxic effect by inhibiting the Ca uptake.

Taken altogether, findings reported here are direct evidence of the negative influence of metals on Ca^{2+} homeostasis. It is important to note that any disturbance on Ca^{2+}-ATPase activity may have severe consequences on biomineralization and calcite deposition processes in foraminifers, since these animals depend on the enzyme activity to enhance intracellular pH for calcification (Erez 2003). As a consequence, foraminifers will produce fragile tests, which would be more vulnerable to increasing threats of global changes and ocean acidification.

Acknowledgement

We are thankful to the Brazilian Ministry of Environment (MMA), the Brazilian Environmental Agency (IBAMA/MMA), and the Chico Mendes Institute for Protection of Biodiversity (ICMBio/MMA) provided authorization to conduct the study and support for fieldwork. International Development Research Centre (IDRC, Ottawa, Canada) provided financial support. MFP is a MSc fellow from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brasília, Brazil).

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