UNIVERSIDADE FEDERAL DO RIO GRANDE – FURG
PÓS-GRADUAÇÃO EM OCEANOGRAFIA BIOLÓGICA

BIOMARCADORES DE EXPOSIÇÃO AO ZINCO EM *Amphistegina lessonii* (Amphisteginidae, Foraminifera) DO ARQUIPÉLAGO DE FERNANDO DE NORONHA, PE, BRASIL

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Dissertação apresentada ao Programa de Pós-Graduação em Oceanografia Biológica da Universidade Federal do Rio Grande, como requisito parcial à obtenção do título de MESTRE

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Fevereiro de 2011

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RESUMO

Parâmetros populacionais e bioquímicos de *Amphistegina lessonii* foram utilizados como potenciais biomarcadores para previsão de alterações ambientais nas comunidades coralíneas de Fernando de Noronha. Foram verificadas, em áreas dentro e fora dos limites do Parque Nacional Marinho de Fernando de Noronha (PNMFN), a densidade e o grau de branqueamento das testas dos indivíduos vivos, bem como realizada análise de biomarcadores (atividade de enzimas antioxidantes e dano oxidativo). Além disso, foi realizado o cultivo *A. lessonii* em laboratório, visando avaliar os efeitos do zinco nos parâmetros bioquímicos mencionados acima. *Amphistegina lessonii* apresentou menores densidades nas áreas afetadas pela saída de esgoto e atividades portuárias. Em indivíduos de áreas localizadas fora dos limites do PNMFN a frequência de branqueamento alcançou 25%, enquanto nas áreas dentro do PNMFN essa frequência foi muito baixa (>1,8%). Os biomarcadores mostraram-se muito sensíveis, de forma que indivíduos coletados em campo aparentemente normais já apresentavam sinais de estresse oxidativo e danos em lipídeos e proteínas. Resultados similares foram encontrados nos indivíduos submetidos a altas (68 e 93 µg Zn/l) concentrações de Zn em laboratório, onde se observou a ativação de componentes do sistema de defesa antioxidante, o qual evita a perda completa das algas endossimbiontes. Como consequência, foi observada uma baixa taxa de mortalidade dos indivíduos expostos ao Zn em laboratório. Esses resultados demonstram que a abordagem dos biomarcadores é muito eficaz para identificar áreas impactadas, sendo uma ferramenta confiável de diagnóstico da saúde dos ambientes recifais.

1. INTRODUÇÃO

Perdas irreversíveis dos recifes de coral e eventos de branqueamento associados aos impactos físicos, químicos e biológicos causados pelas atividades humanas têm aumentado a preocupação em relação à saúde destes ecossistemas, os quais têm mostrado um declínio no mundo todo (Hughes *et al.* 2003; Ferreira e Maida 2006; Baker *et al.* 2008).

De acordo com Leão e Kikuchi (2005), os recifes coralíneos que ocorrem na costa do nordeste brasileiro vêm sofrendo estresse devido às ações humanas, principalmente aquelas associadas ao turismo e à eutrofização de águas vizinhas circundantes. Essa eutrofização é proveniente da liberação de esgoto doméstico não tratado, devido à urbanização sem saneamento básico nas zonas costeiras. No entanto, por ser localizado em oceano aberto, distante aproximadamente 345 km da costa do Rio Grande do Norte, o Arquipélago de Fernando de Noronha sofre pouco com a influência continental. Porém, na área fora do Parque, onde está localizado o porto e a principal saída de esgoto da Ilha de Fernando de Noronha, a comunidade de foraminíferos já está aparentemente afetada (Prazeres *et al.* 2008).

Algumas espécies de foraminíferos que habitam ambientes recifais compartilham com os corais algumas características-chave, pois representantes destes dois grupos são importantes produtores de carbonato de cálcio, dependem fisiologicamente da endossimbiose com algas e vêm sofrendo eventos de branqueamento associado ao aumento da radiação UV e à elevação da temperatura nos oceanos (Hallock *et al.* 2006). Além disso, podem ser utilizados como indicadores sensíveis e confiáveis para verificar a qualidade da água adequada ao suporte do

desenvolvimento da comunidade recifal (Cockey *et al.* 1996). Cooper *et al.* (2009) classificou o uso de populações de foraminíferos para avaliar a qualidade da água como alta-prioridade para programas de monitoramento de curta ou longa duração. No Brasil, Barbosa *et al.* (2006) e Barbosa *et al.* (2009) empregaram foraminíferos bentônicos como bioindicadores de saúde nos ambientes recifais no Arquipélago de Fernando de Noronha e em outras áreas recifais brasileiras. Apesar disso, não foi possível se identificar os tipos e mecanismos de estresse sofridos pelos foraminíferos, uma vez que o trabalho foi realizado utilizando apenas carapaças com alterações mineralógicas de indivíduos mortos presentes no sedimento. Neste sentido, é importante ressaltar que além das avaliações tradicionais utilizadas em trabalhos de monitoramento, um diagnóstico celular pode ser utilizado como uma nova abordagem capaz de distinguir os diferentes fatores de estresse (Downs *et al.* 2005).

Sabe-se que muitos recifes de corais estão constantemente sob influencia de aporte de esgoto doméstico, e outros resíduos de atividades antrópicas, que por ser uma grande fonte de matéria orgânica e de metais essenciais, como zinco e cobre, provoca uma exposição prolongada a elevadas concentrações destes elementos pode induzir toxicidade, causando danos à biota marinha (Ferrier-Pagès *et al.* 2005; Kline *et al*. 2006). No entanto, apesar de muito comuns nos ambientes recifais, o efeito tóxico de metais e compostos orgânicos nos organismos que vivem nestes ambientes ainda é pouco conhecido.

Zinco (Zn) é um micronutriente essencial para o funcionamento do metabolismo, e sua deficiência pode levar ao mal funcionamento de diversas enzimas, incluindo aquelas relacionadas a resposta antioxidante, as quais dependem deste metal

como co-fator. No entanto, este metal pode ser tóxico dependendo de sua concentração e biodisponibilidade no ambiente marinho.

Muitos contaminantes, como os metais, estimulam a produção de espécies reativas de oxigênio (ERO) em organismos marinhos, as quais podem provocar danos oxidativos importantes aos lipídios, proteínas e ácidos nucléicos (Lesser 2006). Além disso, o estresse oxidativo pode ser responsável pela expulsão do endossimbionte de seu hospedeiro, e consequente branqueamento de corais e foraminíferos.

Para neutralizar ou reduzir os efeitos nocivos da produção excessiva de ERO, os organismos possuem um sistema de defesa antioxidante compostos por um sistema enzimático e não-enzimático. Quando a produção de ERO excede a capacidade antioxidante do organismo, podem ocorrer danos celulares e moleculares que também podem ser detectados e utilizados como biomarcadores de efeito desta exposição. Entre estes biomarcadores de efeito do estresse oxidativo estão a peroxidação lipídica e a carbonilação de proteínas, ambos consequências irreversíveis e sua detecção amplamente utilizados para detectar danos causados pelo do estresse oxidativo em organismos marinhos (Oakes e van der Kraak 2003; Dalle-Donne *et al.* 2003). Além disso, a detecção de metalotioneínas (MTs) também pode ser utilizada como biomarcadores específicos de exposição a metais biodisponíveis no ambiente. Porém estas proteínas tem demonstrado possuir um papel antioxidante importante, neutralizando os efeitos das EROs dentro da célula (Amiard *et al.* 2006; Monserrat *et al.* 2007).

A vantagem do uso de biomarcadores é o fato que eles detectam rapidamente o efeito de alterações ambientas (Monserrat *et al.* 2007). Com isso, a análise de biomarcadores de efeitos biológicos se faz necessária, a fim de fornecer subsídios para

a utilização dos foraminíferos como bioindicadores da qualidade do ambiente recifal de Fernando de Noronha, e futuramente de outras áreas recifais brasileiras.

2. OBJETIVOS

2.1. Objetivo Geral

O presente estudo tem como objetivo geral avaliar as condições de saúde do foraminífero com endossimbionte *Amphistegina lessonii* presente nos fragmentos de corais do Arquipélago de Fernando de Noronha através da análise de biomarcadores.

2.2. Objetivos Específicos

Para que o objetivo geral da presente proposta seja atingido, pretende-se:

- Determinar a abundância e o grau de branqueamento das testas dos indivíduos da população de *A. lessonii* em áreas não impactadas e impactadas pelo esgoto doméstico;
- Analisar os parâmetros físico-químicos da água do mar nos locais onde o material biológico foi amostrado;
- Avaliar a capacidade antioxidante contra radicais peróxido, peroxidação lipidica, carbonilação de proteínas e concentração de metalotioneínas em indivíduos de *A. lessonii* expostos aos contaminantes em campo;
- Determinar a abundância e o grau de branqueamento das testas dos indivíduos de *A. lessonii* expostos a diferentes concentrações de Zn em laboratório;

- Avaliar a capacidade antioxidante contra radicais peróxido, peroxidação lipidica, a concentração de metalotioneínas e atividade total das superóxido dismutases em indivíduos de *A. lessonii* expostos a diferentes concentrações de Zn em laboratório.

3. MATERIAL E MÉTODOS

Amostras de fragmentos de corais mortos foram coletadas em setembro de 2009 através de mergulho autônomo, nas estações localizadas no Porto Santo Antônio, Praia da Biboca, Baía dos Porcos, Laje Dois Irmãos e Buracão, em profundidade que variaram de 7 a 15 m. Em cada estação foram coletadas três amostras para análise da densidade populacional de *Amphistegina lessonii*, e para análise de biomarcadores. Os fragmentos coletados foram escovados e sua área aferida com o objetivo de estimar a densidade de indivíduos. O sedimento escovado foi colocado em placas de Petri e mantido em repouso, uma vez que os indivíduos de *A. lessonii* tendem a vir à superfície. Uma vez na superfície, estes organismos foram coletados com o auxílio de uma pinça e observados sob um microscópio estereoscópico, para análise das testas. As testas dos indivíduos adultos de *A. lessonii* coletados em campo foram classificadas em ‘não-branqueadas’, ‘parcialmente branqueadas’ e ‘branqueadas’, e o percentual de testas branqueadas foi calculado. Em seguida, o material biológico obtido foi dividido em duas sub-amostras, sendo uma congelada, mantida e transportada ao laboratório em gelo seco para determinação dos biomarcadores (Anexo I). A outra sub-amostra foi mantida e transportada ao laboratório em pote de plástico contendo água do mar e

selado com Parafilm®. Este material foi utilizado para o desenvolvimento do cultivo em laboratório (Anexo II).

Em cada sítio de amostragem foi feita a determinação *in loco* da salinidade (refratômetro portátil), teor de oxigênio dissolvido (oxímetro portátil), pH (pHmetro portátil) e temperatura (termômetro de mercúrio). Além disso, foram coletadas amostras de água filtrada (0,45 µm) dos sítios de coleta, as quais foram imediatamente acidificadas (1% HNO_3) e acondicionadas em frascos âmbar, para posterior determinação em laboratório das concentrações de matéria orgânica dissolvida (analisador de carbono orgânico) e de metais dissolvidos na água (espectrometria de absorção atômica modo chama).

4. RESULTADOS

4.1. Densidade, grau de branqueamento e análise de biomarcadores em *A. lessonii* nas amostras de campo

No presente estudo, a densidade de *A. lessonii* foi maior e o grau de branqueamento dos indivíduos menor nos locais de coleta considerados menos impactados por atividades antrópicas, e onde o mergulho recreativo é proibido. Nas estações próximas ao Porto de Santo Antônio e Praia da Biboca, onde ocorre aporte de esgoto doméstico, as populações de *A. lessonii* já se encontram visivelmente atingidas pelas alterações na qualidade da água destes locais (Anexo I).

As análises de biomarcadores corroboraram com os resultados obtidos com a análise visual das testas de *A. lessonii*. Nos indivíduos coletados dentro da APA, onde ocorre intensa atividade antrópica e influencia de aporte de contaminantes, observou-se uma menor capacidade antioxidante contra radicais peroxil e maior dano em lipídios e proteínas. No entanto, não foram observadas diferenças significativas na concentração de metalotioneínas entre as estações amostradas (Anexo I).

4.2. Análise dos parâmetros físico-químico da água dos sítios de coleta

Os valores dos parâmetros físico-químicos da água como pH, salinidade, temperatura e oxigênio dissolvido ficaram dentro do esperado para águas oceânicas quentes. No entanto, entre os metais analisados, todos se encontraram acima do permitido pela legislação brasileira para águas marinhas, com exceção do zinco para

duas estações amostradas. O nível de matéria orgânica dissolvida foi alto, principalmente nos locais próximos ao Porto Santo Antônio, Praia da Biboca e Baía dos Porcos (Anexo I).

4.3. Cálculo da CE_{50} e análise de biomarcadores em *A. lessonii* expostos a diferentes concentrações de zinco em laboratório

Entre as concentrações testadas neste estudo, não foi possível calcular a concentração letal para 50% dos organismos testados (CL_{50}). Porém, estas concentrações provocaram alterações visuais como branqueamento e escurecimento das carapaças, e um baixo percentual de mortalidade em *A. lessonii*. Foi possível calcular a concentração capaz de provocar efeito em 50% dos indivíduos em 24 e 48 horas de exposição ao zinco (Anexo II).

Os biomarcadores mostraram que apenas as mais altas concentrações de zinco foram capazes de gerar estresse oxidativo, provocando danos aos lipídeos, induzindo a expressão de metalotioneínas e inibindo a atividade das superóxido dismutases (Anexo II).

5. CONCLUSÕES

- As análises de densidade, grau de branqueamento e biomarcadores em *A. lessonii* mostraram que os locais dentro da Área de Proteção Ambiental de Fernando de Noronha estão sendo afetados por atividades antrópicas.
- Análise das populações de *A. lessonii* mostrou-se uma excelente ferramenta para a avaliação da saúde dos ambientes recifais, podendo ser utilizadas como bioindicadores da qualidade da água no arquipélago.
- Metais essenciais, como o Zn, utilizado nos experimentos laboratoriais mostram que o aumento da concentração e tempo de exposição a este contaminante provoca estresse oxidativo bem como danos celulares em *A. lessonii*.

6. REFERÊNCIAS

Amiard, J.C., Amiard-Triquet, C., Barka, S., Pellerin, J., Rainbow, P.S., 2006, Metallothioneins in aquatic invertebrates: Their role in metal detoxification and their use as biomarkers: Aquatic Toxicology, v. 76, p. 160–202.

Baker, A.C., Glynn, P.W., Riegl, B., 2008, Climate change and coral reef bleaching: An ecological assessment of long-term impacts, recovery trends and future outlook: Estuarine, Coastal and Shelf Science, v. 80, p. 435–471.

Barbosa, C.F., Oliveira-Silva, P., Seoane, J.C.S., Ferreira, B.P., Campello, R.C., Turcq, B.J., Almeida, C.M., Portilho-Ramos, R., Soares-Gomes, A., 2006, Diagnóstico da saúde ambiental de ecossistemas recifais da costa brasileira com a utilização de foraminíferos bentônicos. Projeto FOCO-PROBIO-Tamandaré e Fernando de Noronha (PE), Porto Seguro e Arquipélago dos Abrolhos (BA), Brasil. Relatório Técnico do Ministério do Meio Ambiente - Período de 2004 a 2005, Distrito Federal, Brasil.

Barbosa, C.F., Prazeres, M.F., Ferreira, B.P., Seoane, J.C.S., 2009, Foraminiferal assemblage and reef check census in coral reef health monitoring of East Brazilian margin. Marine Micropaleontology, v. 73, p. 62–69.

Cockey, E., Hallock, P., Lidz, B.H., 1996, Decadal-scale changes in benthic foraminiferal assemblages off Key Largo, Florida: Coral Reefs, v. 15, p. 237–248.

Cooper, T.F., Gilmour, J.P., Fabricius, K.E., 2009, Bioindicators of changes in water quality on coral reefs: review and recommendations for monitoring programmes: Coral Reefs, v. 28, p. 589–606.

Dalle-Donne, I., Rossi, R., Giustarini, D., Milzani, A., Colombo, R., 2003, Protein carbonyl groups as biomarkers of oxidative stress: Clinica Chimica Acta, v. 329, p. 23–38.

Downs, C.A., Fauth, J.E, Robinson, C.E., Curry, R., Lanzendorf, B., Halas, J.C., Halas, J., Woodley, C.M., 2005, Cellular diagnostics and coral health: Declining coral health in the Florida Keys: Marine Pollution Bulletin, v. 51, p. 558–569.

Ferreira, B.P., Maida, M., 2006, Monitoramento dos recifes de coral do Brasil – Situação Atual e Perspectivas: Série Biodiversidade 18, Instituto do Meio Ambiente e dos Recursos Naturais Renováveis, Ministério do Meio Ambiente. Brasília, 250 p.

Ferrier-Pagés, C., Houlbrèque, F., Wyse, E., Richard, C., Allemand, D., Boisson, F., 2005, Bioaccumulation of zinc in the scleractinian coral *Stylophora pistillata*. Coral Reefs, v. 24, p. 636–645.

Hallock, P., Williams, D.E., Fisher, E.M., Toler, S.K., 2006, Bleaching in foraminifera with algal symbionts: Implications for reef monitoring and risk assessment: Anuário do Instituto de Geociências, v. 26, p. 108–128.

Hughes, T.P., Baird, A.H., Bellwood, D.R., Card, M., Connolly, S.R., Folke, C., Grosberg, R., Hoegh-Guldberg, O., Jackson, J.B.C., Kleypas, J., Lough, J.M., Marshall, P., Nystro, M., Palumbi, S.R., Pandolfi. J.M., Rosen, B., Roughgarden, J., 2003, Climate change, human impacts, and the resilience of coral reefs: Science, v. 301, p. 929–933.

Kline, D.I., Kuntz, N.M., Breitbart, M., Knowlton, N., Rohwer, F., 2006. Role of elevated organic carbon levels and microbial activity in coral mortality: Marine Ecology Progress Series, v. 314, p. 119–125.

Leão, Z.M.A.N., Kikuchi, R.K.P., 2005. A relic coral fauna threatened by global changes and human activities, Eastern Brazil: Marine Pollution Bulletin, v. 51, p. 599–611.

Lesser, M.P., 2006, Oxidative stress in marine environments: Biochemistry and physiological ecology: Annual Review in Physiology, v. 68, p. 253–278.

Monserrat, J.M., Martinez, P.E., Geracitano, L.A., Amado, L.L., Martins, C.M.G., Pinho, G.L.L., Chaves, I.S., Ferreira-Cravo, M., Ventura-Lima, J., Bianchini, A., 2007, Pollution biomarkers in estuarine animals: Critical review and new perspectives: Comparative Biochemistry and Physiology Part C, v. 146, p. 221–234.

Oakes, K.D., van der Kraak, G.J., 2003, Utility of the TBARS assay in detecting oxidative stress in white sucker (*Catostomus commersoni*) populations exposed to pulp mill effluent: Aquatic Toxicology, v. 63, p. 447–463.

Prazeres, M.F, Oliveira-Silva, P., Lamana, T., Barbosa, C.F., 2008, Avaliação de saúde das comunidades coralíneas de Fernando de Noronha com base em foraminíferos bentônicos associados à fragmentos de coral. III Congresso Brasileiro de Oceanografia, Fortaleza, Ceará. 20 a 24 de maio de 2008.

ANEXO I

Assessment of water quality in coral communities from Fernando de Noronha, Brazil: biomarkers analysis in *Amphistegina lessonii*

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Artigo redigido de acordo com as normas para publicação no perídico *Journal of Foraminiferal Research*, para o qual já foi submetido.

LRH: PRAZERES AND OTHERS

RRH: BIOMARKES IN *AMPHISTEGINA LESSONII*

ASSESSMENT OF WATER QUALITY IN CORAL COMMUNITIES FROM FERNANDO DE NORONHA, BRAZIL: BIOMARKERS ANALYSIS IN *AMPHISTEGINA LESSONII*

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ABSTRACT

The study of symbiont-bearing foraminifers from reef environments provides a low-cost assessment to evaluate water quality. More recently, several cellular biomarkers have been used as a new approach to elucidate different mechanisms of stress response. Our goal was to combine these approaches, focusing on populations of the symbiont-bearing foraminifer *Amphistegina lessonii* from Fernando de Noronha Archipelago (Northeastern Brazil). Reef-rubble samples containing *A. lessonii* were collected at five sampling sites in the leeward side of the main island. Three sites were located inside and two outside the Fernando de Noronha National Marine Park (FNNMP) area. Foraminifers were evaluated regarding their density, degree of bleaching, and their antioxidant capacity against peroxyl radicals (ACAP), lipid peroxidation (LPO), protein carbonylation (PC) and metallothionein-like proteins (MTs) content. Densities of *A. lessonii* were lower at sites located outside the FNNMP, compared with those located inside the FNNMP, which reached a maximum average of 159 ± 29 individuals/100 cm^2. In individuals from sites located outside FNNMP, bleaching frequency reached 25%. In contrast, it was very low at FNNMP sites (< 1.8%). ACAP was higher (2-fold) in individuals collected at the FNNMP sites, which showed lower LPO and PC levels. MTs concentration did not differ significantly among sites. Zinc, copper and lead concentrations were very high at station located outside FNNMP, above the Brazilian Water Quality Criteria for marine water. Dissolved organic carbon concentration was high at all sampling sites. Redundancy analysis revealed a strong correlation between biomarkers and water quality among sampling sites. These results show that the biomarker approach would be effective in

identifying impacted areas and underlying causes, providing a reliable diagnostic to environment health.

INTRODUCTION

Coral reefs are currently a focus of public and scientific debate because of their vulnerability to global disturbances and local anthropogenic impacts (Hughes and others, 2003; Pandolfi and others, 2003; Baker and other, 2008; Uthicke and others, 2010). The causes of reef decline range from global impacts such as unusually high sea water temperatures and ocean acidification, to local stressors, such as coastal development, chemical pollution, eutrophication, increasing of sedimentation, and overexploitation of reef resources. Since the 1980s, coral reef decline and bleaching events have been shown to be related to both local and global changes, which have had increasingly devastating and widespread effects worldwide (Hallock and others, 1992; Baker and others, 2008; Uthicke and others, 2010).

To address reef decline, assessment and monitoring programs have emerged worldwide to discover the causes and consequences of the loss of coral reefs, and to attempt to manage impacts and minimize reef decline. Such programs seek to minimize exploitation pressure and impacts of other human activities, as well assure the future of people whose lives depends directly upon the resources provided by these ecosystems. Furthermore, a great importance has been given to the study of marine organisms capable of being used as indicators of water quality, which is a very important factor in maintaining the growth and regeneration of coral population (Hallock and others, 2003; Linton and Warner, 2003).

Reef-dwelling larger foraminifers share some important characteristics with reef-building corals, such as their dependence upon algal endosymbionts for growth and calcification, and their adaptation to nutrient-poor, warm, shallow-water environments (Hallock, 1996). *Amphistegina* is the dominant diatom symbiont-bearing foraminiferal genus found abundantly on coral reefs and tropical carbonate shelves worldwide (Hallock, 1999; Langer and Hottinger, 2000). It is commonly found living on coralline and filamentous algae on reef substrate, as well as on some macrophytes (Baker and others, 2009). In the South Atlantic Ocean, the most common symbiont-bearing species is *Amphistegina lessonii,* except for some areas of Archipelago of Abrolhos, which are dominated by *Archaias angulatus* (Oliveira-Silva, 2008). These foraminiferal genera are sensitive to water quality and *Amphistegina* spp. bleach in response to excess solar energy, particularly higher-energy wavelengths (Talge and Hallock, 2003; Williams and Hallock, 2004; Hallock and other, 2006a,b). Also, when local environmental conditions change to favor organisms using autotrophic or heterotrophic nutritional modes over organisms using mixotrophic (algal symbiotic) modes, *Amphistegina* populations decline (Hallock, 1996). Hallock and other (2003, 2006a) observed that these protists respond to environmental conditions within days to weeks and provide a low-cost tool to quickly distinguish between local environmental conditions (e.g., water quality) and photo-oxidative stress.

Cooper and others (2009) classified the use of foraminiferal populations to assess the water quality on coral reef environments as high priority for short and long-term monitoring programs. Uthicke and others (2010) proposed that FORAM Index developed by Hallock and others (2003) is indeed an effective bioindicator for the assessment of turbidity/light regimes and organic enrichment of sediments on coral

reefs of the Great Barrier Reefs. In Brazil, the pioneer studies of Barbosa and others (2006, 2009) generated important information on the conditions of Brazilian coral reefs using benthic foraminifer and the FORAM Index, which allowed mapping places considered healthy and those under some kind of stress. Nevertheless, it was not possible to identify from these information the types and mechanisms of stress suffered by the foraminiferal community, because the work was done using only shell, mostly from dead individuals present in the sediment.

Cellular diagnostic system can provide an important addition to the traditional assessments used in monitoring and assessment. This relatively new approach can distinguishing different stressor factors, because they stimulate a specific cellular response within affected organisms (Downs and others, 2005). Oxidative stress is a particularly important component of the stress responses in marine organisms exposed to a variety of insults as a result of changes in environmental conditions such as exposure to thermal, photic or pollution stresses (Lesser, 2006). Therefore, a better understanding of cellular mechanisms that regulate physiological responses in populations of symbiont-bearing foraminifers is important for their use as environmental bioindicators.

STUDY RATIONALE AND OBJECTIVES

Exposure to pollutants such as organic matter and metals can induce the production of reactive oxygen species (ROS), causing important oxidative damage to biomolecules, such as proteins, lipids and nucleic acids (Lesser, 2006). The biomarkers that we chose to investigate in foraminifers to detect different types of damage were:

(1) antioxidant capacity against peroxyl-radicals (ACAP) for an overall evaluation of the oxidative stress response capability; (2) lipid peroxidation (LPO) for detection of oxidative damage to lipids; (3) protein carbonylation (PC) to detect oxidative damage to proteins; and (4) metallothioneins-like proteins, commonly used as an indicator of metal exposure. The advantage of using the cellular biomarkers is the fact that they can quickly detect the pollutant effect, especially in unicellular species such as symbiont-bearing foraminifers, before other populations and the whole coral community is affected.

We selected Fernando de Noronha was selected because it is under influence of the South Equatorial Current (Maida and Ferreira, 1997). Thus being free of chemical contamination from continental waters. In addition, some areas of Fernando de Noronha are protected by a National Marine Park, while the others have significant contribution of wastewater containing dissolved organic matter and metals that can modify biochemical and physiological processes of marine biota. Therefore, the analysis of biochemical and physiological process using biomarkers of biological effects is an essential next step in using foraminifers as bioindicators of water quality, which we can apply to the Fernando de Noronha Archipelago and eventually other Brazilian coral reef areas, and the others can apply to reef worldwide.

Thus, the aim of our study was to evaluate populations of the symbiont-bearing foraminifer *Amphistegina lessonii* both by using established visual assessment methods and by adapting cellular diagnostic methods for use on these protists. Our approach has the potential to substantially expand the applicability of these foraminifers as tools to assess the water quality of coral communities worldwide.

STUDY SITE

Fernando de Noronha Archipelago (4°S 32°W) is located 360 km off the northeast coast of Brazil. It comprises 21 islands, islets and rocks (Ferreira and Maida, 2006). The archipelago was formed by volcanic activity and consists mainly of highly alkaline volcanic and subvolcanic rocks (Almeida, 2000). However, many sedimentary deposits can be found as a result of Pleistocene and Holocene process of accumulation of marine carbonates and associated shoal formation (MMA, 2005).

Fernando de Noronha has a tropical oceanic climate with two distinct seasons, marked by the intensity of rainfall, which is higher from March to May and lower from August to January. The archipelago is strongly influenced by the northern branch of the South Equatorial Current (SEC), and average water temperature is 24°C year round. The water is clear all year long, making the Archipelago a very suitable place for diving (Maida and Ferreira, 1997).

The Archipelago has been a protected area since 1988. It was designated a World Heritage Site by UNESCO in 2001, mostly because its highly productive waters provide feeding ground for important marine vertebrates including cetaceans, sharks, and turtles as they migrate to the Eastern Atlantic coast of Africa. The islands and smaller structures are also critical roosting and nesting habitats for a diversity of marine avifauna. About 50% of Fernando de Noronha Island itself, plus all of the smaller neighboring islands, constitute the Fernando de Noronha National Marine Park (FNNMP). All natural resources are protected from any form of exploitation, although educational, recreational, and scientific use is allowed.

The remaining archipelago area is an Environmental Protected Area (EPA) designed for sustainable use, and has permanent human occupation (Maida and Ferreira, 1997). Potencial human impacts include fishing, agricultural and livestock grazing, harbor activities, as well deficient sanitarian infrastructure and disordered tourism occur (MMA, 2005). Moreover, the solid waste treatment station responsible for the main domestic sewage discharge is located on this part of the island between Santo Antonio Harbor and Biboca beach.

The Archipelago has nine species of hermatypic corals (Ferreira and Maia, 2006). Although these include species that are major reef builders on the continental coast (e.g., *Siderastrea stellata*, *Montastrea cavernosa*, *Mussismilia hispida* and *M. hartii*), there are no structural coral reef formations at the island (Maida and Ferreira, 1997), though coral communities flourish along the rocky shores (Castro and Pires, 2001). In some areas, coral and coralline algal communities are well developed, with colonies growing over the rocky substrate in densities higher than those found in near shore coral reefs of Brazil (Ferreira and Maida, 2006). There are two kinds of bioconstructions by corals bordering the shores of the islands (Leão and Dominguez, 2000). On the leeward shore, an incipient fringing reef can be found, built primarily by coralline algae growing on rocky substrates. In the windward side, exposed to the open ocean, fringing reefs occur but are rare and minimally developed (Hazin and Castro, 2006).

From the biological point of view, the two management zones are not isolated since the activities developed at EPA may impact direct or indirectly the FNNMP area. The archipelago is extremely important both to maintenance of local human communities and to South Atlantic marine vertebrate populations for rest, reproduction

and feeding sites of migratory species of several species (MMA, 2005). These are one of the characteristics which make effective management and monitoring of the FNNMP a priority for the conservation of marine organisms (Hazin and Castro, 2006).

METHODS

SAMPLES COLLECTION

Sampling surveys were carried out in September 2009, during dry season. Samples of cobble-sized pieces of reef rubble or volcanic rocks were haphazardly hand collected by SCUBA divers at five sites located on the leeward side (northwest shore) of Fernando de Noronha Island, at depths ranging from 7 to 20 m. Sampling sites were located at Santo Antonio Harbor (PSA), Biboca beach (BIB), Porcos Bay (POR), Dois Irmãos Shoal (LDI) and Buracão (BUR). The two former sites are located in the EPA, while the other sites are located inside FNNMP (Fig. 1).

Methods are similar to those suggested by Hallock and others (2006a). At each site, five pieces of cobbles were placed into labeled plastic bags and brought to the surface. On the vessel, cobbles were scrubbed into an individual bucket containing seawater using a small brush, to remove attached filamentous algae, sediments and micro-fauna. The resulting sediment concentrate was kept from direct sunlight while transporting to the shore-based laboratory. Bottom area of each cobble was estimated by tracing each piece onto graph paper and determining the area within the traces.

Duplicate water samples were also collected for analysis of dissolved organic carbon (DOC) and dissolved metals that are commonly found in sewage discharge (Zn,

Cu, Pb and Cd). Water samples were filtered (0.45 µm mesh filter) and stored in acidified amber glass bottles, acidified with 1% HNO_3 and kept in the dark at 4°C until laboratorial analysis. Salinity, pH, temperature and dissolved oxygen were also measured.

LABORATORY PROCEDURES

Live examination of A. lessonii

In the laboratory, samples were allowed to settle until the suspended material could be easily rinsed away. The sediment-residue from each sample was transferred to 150 mm Petri dishes and kept undisturbed for 24 h in the shade under no extremes of temperature and light conditions. After this period, samples were examined under a stereomicroscope, and the living adult individuals were counted and analyzed for degree of bleaching, according to Hallock and others (2006a). Adult individuals were identified by estimated the size of *A. lessonii* individuals. These individuals were then classified as normal-appearing, partly-bleached or bleached adults. Following visual classification, the living normal-appearing adults were separated in cryovials and immediately placed on dry ice for later cellular biomarkers analyses, as described below. Additionally, partial-bleached adult individuals were isolated and frozen for analysis of antioxidant capacity against peroxyl-radicals. All the biomarkers analyses were conducted using pre-frozen individuals. Because of the $CaCO_3$ shells of the foraminifers, an ultra-sound sonication procedure was used to maintain the enzyme activities and cellular content integrity.

Determination of total antioxidant capacity against peroxyl radicals (ACAP)

The ACAP assay was performed to measure the biological resistance to various kinds of oxyradicals, to predict their adverse effects on the physiological condition of the organisms (Regoli, 2000). ACAP was measured using a modified protocol described in Amado and others (2009). Briefly, each foraminifer sample was homogenized in a Tris-HCl (100 mM) buffer containing EDTA (2 mM) and $MgCl_2$ (5 mM), and then centrifuged at 10,000 ×*g* for 20 min at 4°C. The resulting supernatant was adjusted to a concentration of 0.75 mg of protein/ml and used for the ACAP measurement. Protein content in the supernatant was determined using the by Quant-iT Protein Assay (Invitrogen, USA). In a white 96-well microplate, 10 µl of supernatant from each sample was added in six wells, together with 127.5 µl of reaction buffer containing 30 mM HEPES (pH 7.2), KCl (200 mM) and $MgCl_2$ (1 mM). In three of the six wells of each sample, 7.5 µl of 2'-Azobis (2-methylpropionamidine) dihydrochloride (ABAP; 1 mM) was added. In other three wells, the same volume of ultrapure water was added (blank reaction). Finally, the fluorescent probe 2′,7′ dichlorofluoresceindiacetate (H_2DCF-DA) was added to all wells at a final concentration of 40 µM. Fluorescence was read (excitation: 488 ηm; emission: 525 ηm) in a microplate reader (Victor 2 – Perkin Elmer) for 30 min, with readings every 5 min at 37 °C. Results were expressed as the difference in fluorescence units (FU) × min area in the same sample with and without ABAP addition and standardized to the ROS area without ABAP (background area).

The relative difference between ROS area with and without ABAP was considered as a measurement of the antioxidant capacity. Substantial area difference indicates low antioxidant capacity, since high fluorescence levels were obtained after adding ABAP, meaning low competence to neutralize peroxyl radicals (Amado and others, 2009).

Lipid peroxidation (LPO) determination

The 2-thiobarbituric acid reactive substances (TBARS) assay quantifies oxidative stress by measuring the peroxidative damage to lipids. In the present study, it was detected following the fluorescence method described by Oakes and van der Kraak (2003). Briefly, foraminifers were homogenized (1:10; w/v) in 45 µl of 1.15% KCl solution containing 35 µM butylated hydroxytoluene (BHT). Firstly, 10 µl of the 10% homogenate was added to a reaction mixture containing 20 µl of stock BHT solution (67 µM), 150 µl of 20% acetic acid (pH 3.5), 150 µl of 0.8% 2-thiobarbituric acid (TBA), 20 µl 8.1% sodium dodecyl sulfate (SDS) and 50 µl of ultrapure water into glass tubes, in duplicate. The mixture was heated at 95°C in water bath for 30 min. After cooling, 1 ml of ultrapure water and 500 µl n-butanol were added with thorough vortexing. After this, samples were centrifuged at 2,000 ×*g* for 10 min at room temperature. After centrifugation, 150 µl of the immiscible organic layer was removed and added to a white 96-well microplate. Fluorescence (excitacion: 515 ηm; emission: 530 ηm) was measured using a microplate reader (Victor 2 – Perkin Elmer).Concentration of lipid peroxides was expressed as ηmol TBARS/mg

foraminifer, which was calculated from an standard curve built using hydrolyzed tetramethoxypropane (TMP).

Determination of protein carbonyl groups (PC)

Protein carbonyl groups were quantified by one-dimensional electrophoresis and Western blotting immunoassay. The detection of protein carbonyls involve derivatization of the carbonyl group with 2,4-dinitrophenylhydrazine (DNPH) group, which leads to the formation of a stable 2,4-dinitrophenyl (DNP) hydrazone product (Dalle-Donne and others, 2003). Prior to derivatization, protein content was standardized at 0.2 mg/ml of homogenate in order to equalize the samples for loading onto SDS-PAGE and Western blotting assay. In the derivatization step proteins reacted with DNPH in a solution containing 12% SDS and DNPH/TFA stock solution – 20mM DNPH in 20% (v/v) TFA (trifluoroacetic acid). In addition, three positive control-samples were used containing 1, 2 and 4 mM of H_2O_2 to induce protein damage/oxidation. Following incubation for 15 min at room temperature, the reaction mixture was neutralized with 2M Tris-Base containing 30% glyceraldehyde. For each sample, DNPH-derivatized proteins were separated by 1D SDS-PAGE (polyacrylamide 12%) and electroblotted to PVDF membranes, and immunoassayed for carbonyl content with anti-DNP antibody (Invitrogen, USA). Bands were visualized using a chromogenic immunodetection kit (Invitrogen, USA). The density of bands were analyzed for each station after scanning the PVDF membrane.

Metallothionein-like proteins (MTs) content

A high sensitive spectrophotometric technique was used to quantify the metallothionein concentration in *A. lessonii*. This method is a modification of the protocol first described by Viarengo and others (1997). Briefly, each foraminifer sample was homogenized (1:25; w/v) in a buffer solution (pH 8.6) containing 500 mM sucrose, 20 mM of Tris-Base, 100 Mm phenylmethylsulphonylfluoride (PMSF) as antiproteolytic agents, and 0.01% β-mercaptoethanol as a reducing agent. The homogenate was centrifuged at 30,000 × *g* for 45 min to obtain a supernatant containing metallothioneins.

The supernatant was then treated with cold (-20°C) absolute ethanol and chloroform, and centrifuged at 20,000 × *g* for 30 min at 4°C. At the collected supernatant was added 37% HCl and absolute cold ethanol. Samples were maintained at -20°C for 1 h and centrifuged again at 20,000 × *g* for 30 min. The metallothionein-containing pellet was then washed with 87% ethanol and 1% chloroform in homogenizing buffer, and centrifuged at under the previously described conditions. The pellet was resuspended in 150 µl 0.25 M NaCl with subsequent addition of 150 µl 1N HCl containing 4 mM EDTA. A volume of 100 µl of each sample was added to 1.4 ml of a buffer solution (pH 8) containing 200 mM Na-phosphate, 2 M NaCl and 2mM of DTNB (5,5-dithiobis-2-nitrobenzoic acid). Finally, 350 µl of each sample was transferred to a transparent 96-well microplate, and its absorbance measured at 405 ηm. Metallothionein was estimated using reducing glutathione (GSH) as a reference standard.

Water samples analysis

Samples from each station were analyzed for concentrations of dissolved organic carbon (DOC) and dissolved metals. For DOC analysis, each sample was acidified using pure HCl, until reaching pH of 2.5. DOC concentration was measured using a total carbon analyzer (TOC-VCPH, Shimadzu, Japan) that uses the combustion catalytic method for efficiently analyzing carbon compounds. The concentrations of dissolved Cu, Cd, Zn and Pb were determined using a method described in Nadella and others (2009). This methodology removes the salt ions presented in high concentration in seawater while concentrating several elements, in preparation for analysis by atomic absorption spectrophotometry (AAS 932, GBC, Australia). For each metal analyzed, a standard curve was built using the manufacturer's standards (1g/l) for each metal analyzed.

Data analyses

A. lessonii density was calculated dividing the total number of living specimens (juveniles plus adults) by the combined bottom area of all cobbles from that sample, calculated in cm^2. Density was expressed as number of living individuals per 100 cm^2. Bleaching in adult individuals was calculated as the number of partly bleached adults divided by the sum of normal-appearing adults and partly bleached adults, as described in Hallock and others (2006a). In addition, linear correlation analysis was performed to verify if density was related to percentage of partly bleached adult individuals. Biomarkers assays were performed in triplicate and results are expressed as mean ± 1 Standard Error (SE). All data from density and biomarker assays were checked for

homogeneity of variance and normality prior to one-way Analysis of Variance (ANOVA) and post-hoc Tukey's (HSD) test to determine differences between two or more groups. In all cases, significance level adopted was 95% (α=0.05). A redundancy analysis (RDA) was conducted to illustrate the differences in biomarkers expression between sampling sites and their dependence on the environmental variables. All environmental and biomarkers data were *z*-transformed prior to RDA.

RESULTS

DENSITY AND BLEACHING IN *A. LESSONII*

Densities of living individuals of *A. lessonii* varied among the sampling sites. Higher densities were found at those sites located inside the National Park area, i.e., BUR and LDI, with densities of 159±29 and 104±11 individuals/100 cm^2, respectively. The lowest density was found at PSA site, with only 38±3 individuals/100 cm^2 (Fig. 2). These differences were statistically significant, particularly between among stations located inside and outside the Park area (P=0.01).

Bleaching was observed mainly at those stations located near the sewage disposal outfall, outside the Park area. At BIB, incidences of bleaching exceeded 25%, the highest degree of partial bleaching observed, followed by PSA (17%) and POR (15%). The lowest partial bleaching in adults was observed at LDI (Fig. 2).

Linear regression analysis showed that density and partial bleaching in adults individuals is negatively correlative, i.e., increasing density of *A. lessonii* on reef

rubble in Fernando de Noronha is related to decreasing degree of bleaching (r^2 = 0.69; P=0.0001; Fig. 3).

BIOMARKERS ANALYSES

Results from ACAP analysis indicated that individuals of *A. lessonii* from PSA and BIB sites have less capability to cope with ROS formation, since ROS concentrations were significantly higher in individuals from those sites than in LDI and BUR (P<0.01; Fig. 4). Although POR is located inside the FNNMP, *A. lessonii* populations there apparently also have lower competence against ROS formation. Concentration of peroxyl radicals was lower in individuals from the LDI site, with an area of $4.1\times10^8\pm1.9\times10^6$ FU × min, which was significantly lower (P<0.01) than that of individuals from BUR site, which showed a mean area of $5.5\times10^8\pm2.4\times10^7$ FU × min (Fig. 4).

Partially-bleached individuals from stations outside FNNMP showed the same pattern as normal-appearing individuals. However, ROS area from these foraminifers was in general much lower, meaning a more competent antioxidant system against peroxyl radicals formation (Fig. 4). In individuals from LDI site, ROS area was 20-fold lower in bleached individuals compared to the normal-appearing individuals, with a value of $6.2\times10^6\pm5.6\times10^5$ FU × min, but only 2-fold lower in bleached foraminifers from the PSA site ($3.7\times10^8\pm3.6\times10^6$ FU × min).

Regarding LPO (TBARS) values were significantly different between sites located inside and outside the Park area (P<0.01). TBARS formation was attenuated with distance from the sewage disposal. TBARS values in individuals from the POR

site were 53% lower than on those from the BIB site, which showed the highest mean value (0.005±0.001 ηmol TBARS/mg foraminifer). The lowest value was found in individuals from the BUR site, with a mean value of 0.001±0.0002 ηmol TBARS/mg foraminifer (Fig. 5).

The Western blot immune detection of carbonyl groups assay showed increased oxidative damage of proteins in those foraminifers living at the BIB site, which not only showed an higher density (pixels) of bands but also an enhanced frequency of immune-reactive bands ($P<0.01$; Fig. 6). Moreover, most of carbonylation detected by the methodology employed were at high-weight proteins, ranging from 190 to 60 kDa. Individual *A. lessonii* from the BUR site showed no oxidized protein through the Western blot immune detection assay.

Metallothioneins-like proteins concentration did not differ significantly between individuals from sites located inside and outside FNNMP ($P=0.07$). Nevertheless, a tendency of decreasing MTs concentration was observed towards the FNNMP area. MTs concentration was higher in individuals from the BIB site (1.54±0.04 μmol/ g), followed by those from BUR site (1.45±0.01 μmol/ g). The lowest mean value was found in individuals from the LDI site (1.07±0.20 μmol/ g; Fig. 7).Individuals from the PSA and POR sites showed intermediate values (1.34 and 1.44 μmol/ g, respectively).

RDA indicated that dissolved metal concentration in the water only explained a small amount of the observed variance in the distribution of *A. lessonii* among sampling sites. In contrast, DOC alone explained nearly six times as much of the variance. The redundancy analysis separated sampling sites from each other with respect to the biomarkers and wate parameters that most contributed to *A. lessonii*

response. Density of individuals was negatively correlated to all others biomarkers and percentage of bleaching as well as water parameters, except for Cd concentration in the water, where it revealed to have no correlation (Fig. 8). In addition, the percentage of bleaching was strongly correlated with DOC concentration in the water, while antioxidant capacity was strongly correlated with copper, cadmium and lead present in water samples from PSA site, which showed the higher metal concentrations (Fig. 8 and Table 1).

WATER PHYSICOCHEMICAL PARAMETERS

Temperature, pH, salinity and dissolved oxygen values were considered normal for equatorial oceanic areas, with sea surface temperatures consistently 26 to 27 ºC, pH around 8, salinities ~37 and dissolved oxygen ranging from 5.5 to 5.9 mg O_2/l (Table 1). In contrast, DOC concentrations at BIB and POR sites, and dissolved metals concentrations such as Pb and Cu at all sampling sites were considered very high for an oceanic archipelago (Table 1). Pb and Cu concentrations are above the Water Quality Criteria from the U.S. Environmental Protection Agency (US-EPA) and the Brazilian National Council for Environment (CONAMA) regulation for marine waters.

DISCUSSION

The degradation of coral reefs caused by human activities and the frequent foraminiferal and coral bleaching events worldwide have generated interest in the adaptive value and stability of algal symbioses in these oligotrophic ecosystems.

Symbiont-bearing foraminifers in particular have been used as bioindicators of water quality for some years. Population density estimation and visual assessment of selected symbiont-bearing species are particularly used to distinguish local and global stressors. In the present study, we present the first effort using living symbiont-bearing foraminifer that combines the traditional visual assessment with measurements of cellular response to oxidative stress.

We found that in areas under human influence, *A. lessonii* not only showed visual alterations such as spotted, broken, deformed and bleached shells, but also physiological and biochemical responses such as lower antioxidant capacity against peroxyl radicals, lipid and protein damages. These responses were likely caused by the breakdown of the algal-symbiont photosystem II induced by changes in water quality (e.g., increase in dissolved organic matter) and possibly also photo-oxidative processes (Downs and others, 2002; Douglas, 2003).

Bleached *A. lessonii* individuals also were reported by Rossi (1999) at sites from Fernando de Noronha near those from the present study. This author also described the main factors that might cause changes in symbiont-bearing population, such as urban runoff, boat fuel, and anti-fouling paints. This phenomenon was also reported for *Amphistegina* spp. sampled by Barbosa and others (2006). However this last work considered only dead shells, which is problematic, since dead shells could have been classified as bleached individuals.

The process involved in the oxidative stress response observed in the present study seem to be similar to that described for hermatypic corals, which induces the expulsion of the algal-endosymbiont as a mechanism of get free from the damaged algal cells (Douglas, 2003). Moreover, bleaching in *A. lessonii* might be caused by

damage to a specific protein that maintains the association with their diatom-symbiont, the CSSA (Common Symbiont Surface Antigen) glycoprotein (Lee, 2006). Any injury to the CSSA protein can cause the disruption of symbiont's protective mechanisms. As a result, the foraminifer triggers the defense response by digesting what it recognizes as foreign cells, leading to bleaching. Factors such as high concentrations of metals such as copper, which were found to exceed the Water Quality Criteria in the PSA site (14.5 μg/l), have been demonstrated to interact and alter algal responses to host signaling compounds that regulate symbiotic algae in hermatypic coral (Grant and others, 2003).

ACAP was higher in partly-bleached *A. lessonii* compared to the normal-appearing individuals that bear unimpaired-endosymbionts. This novel finding lead to the conclusion that bleached foraminifers could be more capable to cope with oxidative stress. Once the antioxidant capacity threshold increases, as the ROS formation decreases with the digestion of the dysfunctional endosymbiotic-algae, foraminifers may be able to recover from the oxidative stress and damage.

Even though located inside the FNNMP, *A. lessonii* from the POR site showed low antioxidant capacity and some bleaching (~ 15%), but no damage to lipid or proteins was detected. At this sampling site, DOC and Zn concentration were higher than expected for that area, since it is located inside the FNNMP and at a distance from any source of pollution. However, this sampling site is located in an enclosed bay, with low water circulation rate. Therefore, the *A. lessonii* population response observed in the present study could be not associated with human impact, but perhaps with water eutrophication driven by guano accumulation from sea birds colonies located at Porcos' Bay, where sets the POR sampling site (Filho and others, 2009).

Amphistegina lessonii individuals from sampling sites located outside the FNNMP (PSA and BIB), which were not able to cope with oxidative stress and were apparently healthy, showed oxidative damage to lipid and proteins, the latter being observed at higher levels in *A. lessonii* from the POR site. Damage observed indicates that although apparently healthy with uniform golden-brown color, the decline in water quality (e.g., increasing of DOC concentration) may have led to an inefficient response against the oxidative stress response. As a consequence damage to membrane lipids and proteins were observed. The higher lipid peroxidation observed in *A. lessonii* from sampling sites could indicate a pre-bleaching stage. Talge and Hallock (2003) observed that partly-bleached individuals from the Florida Keys showed disintegration of the membranes possibly resulted from the degradation of the endoplasm the symbionts are located.

A negative correlation between density and degree of bleaching was also observed by Hallock and others (2006a) in reefs from the Florida Keys when sampling during summer of 1994-1999. However, density of individuals at those reefs is much higher than in our study site. These authors also stated that low density of individuals could be caused by the higher degree of bleaching. In the present study, this tendency was observed in sampling sites outside the FNNMP, where bleaching could suppress reproduction as indicated by low numbers of juveniles in the populations. Moreover, as also reported by Williams and others (1997) in Florida reefs, the years with the highest incidences of bleaching were also the years with the lowest population densities and lowest proportions of juveniles. The possible reason for these events could be explained by the higher amount of energy provided by the endosymbiont-algae to their hosts. Once bleached, they lose their appropriate carbon-supply in this

nutrient-limited environment (Lee, 2006), leading to a sub-nutritional state of the foraminifers. Furthermore, foraminifers may also expend a high amount of energy to maintain homeostasis during conditions associated with bleaching and oxidative stress response, as has been observed in corals (Downs and others, 2002).

The MTs are classically considered as biomarkers for detecting exposure to trace metals (Viarengo and others, 1999). In the present study, MTs did not show any significant difference in *A. lessonii* individuals among sampling sites. Also, RDA did not show any clear correlation between metals and MT concentration. Indeed, although metal concentrations observed at PSA and BIB sites were above the acceptable concentration by environmental regulations from USA and Brazil, the high DOC concentration observed in these sampling sites could be interacting with these metals, reducing their toxicity and bioavailability to epiphytic foraminifers (Bresler and Yanko, 1995; Martinez-Colon and other, 2009). Moreover, MT synthesis is not only induced by non-essential metals such as Cd and Pb, but also can be stimulated to maintain the intracellular balance of essential metals like Cu and Zn (Amiard and others, 2006). Therefore, our results could be expressing a background MT concentration found in *A. lessonii* from Fernando de Noronha sampling sites.

RDA analysis showed a positive correlation for the sampling sites located outside the FNNMP (PSA and BIB) and also the POR site, where the most human impact-activities occur. This analysis indicates that DOC and copper concentrations could be the main factors influencing the percentage of bleaching and lipid peroxidation in *A. lessonii* individuals from these sampling sites. Also, it is possible to observe that sampling sites located inside the FNNMP showed a positive correlation with density, corroborating with the ANOVA results, which indicated that density was

indeed higher in those stations. Furthermore, a negative relation between density and all biomarker parameters analyzed in the present study was found. In addition, redundancy analysis indicated a marked change in cellular oxidative stress condition related to water quality parameters, especially enhanced concentration of DOC, Zn and Cu.

These findings suggested that although apparently healthy, *A. lessonii* individuals showed signals of physiological disruption, such as lipid peroxidation and protein carbonylation. The poorer water quality may lead to a decline in abundance of symbiont-bearing taxa, since they have been shown in the present study in a cellular scale to be very sensitive at the cellular scale to changes in water quality. Consequently, there would be a shift from symbiont-bearing individuals to smaller, herbivorous and detritivorous foraminifers that lack algal symbionts (Cockey and others, 1996). In coral reefs, this shifting record is being used worldwide as a primary indicator of water quality (e.g., Hallock and others, 2003; Uthicke and Nobes, 2008; Barbosa and others, 2009).

Although bleaching is commonly related to above-optimum water temperatures in corals, Williams and Hallock (2004) showed that *Amphistegina* are extremely sensitive to photo-oxidative stress induced by short, high energy wavelength of light (blue to ultraviolet), which is consistent with seasonal differences in bleaching incidences found in the Florida reef tract and latitudinal differences found off the west coast of Australia (Hallock and others, 2006b). These may not be the factors influencing *A. lessonii* population differences in Fernando de Noronha. Because of its oceanic influence, pH, salinity and water temperature are very stable in this region. Incidences of bleaching are quite low and consistent with chronic levels now observed

in the Florida reefs. The significant difference in the antioxidant capacity and lipid peroxidation levels observed in foraminifers from Fernando de Noronha Island are likely related to local factors rather than large climate changes. High levels of essential (Zn and Cu) and non-essential (Pb) metals, and of DOC at sampling sites located outside the FNNMP may be an alert of the impact generated by human activities occurring in the Archipelago. These activities will likely increase associated with increasing tourism and local population growth. Though we are able to ascertain that the local stressor induces an oxidative stress in *A. lessonii* populations, the precise identification of the stressor remains allusive.

CONCLUSIONS AND PERSPECTIVES

We have provided evidence that differences in *A. lessonii* population densities and bleaching incidences between the FNNMP and the EPA of Fernando de Noronha could be associated with local factors, at least for sampling sites located on the north side of Fernando de Noronha island. Main factors are elevated concentrations of DOC and of essential and non-essential metals. Other organic compounds and nutrients not measured in the present study and their potential involvement in the observed biological effects cannot be ruled out. Further development of health assessment diagnostic methods using cellular biomarkers for other chemical compounds, such as hydrocarbons, and also laboratory toxicological studies, will certainly provide more corroborating evidence and help to identify the putative stressor in symbiont-bearing foraminifers.

The cellular diagnostic approach (biomarkers) potentially allows the fate of coral reefs to be forecasted in light of local and perhaps global stress events. In this regard, good predictive markers for bleaching/mortality are those that are usually directly associated with cellular damage, such as antioxidant capacity, lipid peroxidation and protein carbonyl. In fact, these biomarkers have been demonstrated in the present study as good prognostic indicators for symbiont-bearing foraminifers. Further development of cellular biomarkers for other species of reef-dwellers foraminifers, such as the heterotrophic and stress-tolerant taxa, could also greatly enhance the ability of resource managers to make ecological forecasts.

ACKNOWLEDGEMENTS

We are thankful to Diego Maio for design the Fernando de Noronha map. Prof. Pamela Hallock Muller for greatly improved the English and for insightful comments on the manuscript. Prof. Virginia M. Tavano for critically reviewing the manuscript. The Brazilian Ministry of Environment (MMA), the Brazilian Environmental Agency (IBAMA/MMA) and the Chico Mendes Institute for Protection of Biodiversity (ICMBio/MMA) for providing authorization to conduct the study and support for field work. International Development Research Centre (IDRC, Ottawa, Canada) for financial support. MFP is a MSc fellow from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brasília, Brazil). AB is a fellow from the Brazilian CNPq (Proc. # 304430/2009-9).

REFERENCES

ALMEIDA, F.F.M., 2000, Arquipélago de Fernando de Noronha – Registro de monte vulcânico do Atlântico Sul, *in* Schobbenhaus, C. and others (eds.), Sítios Geológicos e Paleontológicos do Brasil. DNPM/CPRM - Comissão Brasileira de Sítios Geológicos e Paleobiológicos (SIGEP), Brasília, p. 361–368.

AMADO, L. L., GARCIA, M.L., RAMOS, P.B., FREITAS, R.F., ZAFALON, B., FERREIRA, J.L.R., YUNES, J.S., and MONSERRAT, J.M., 2009, A method to measure total antioxidant capacity against peroxyl radicals in aquatic organisms: Application to evaluate microcystins toxicity: Science of the Total Environment, v. 407, p. 2115–2123.

AMIARD, J.C., AMIARD-TRIQUET, C., BARKA, S., PELLERIN, J., and RAINBOW, P.S., 2006, Metallothioneins in aquatic invertebrates: Their role in metal detoxification and their use as biomarkers: Aquatic Toxicology, v. 76, p. 160–202.

BAKER, A.C., GLYNN, P.W., and RIEGL, B., 2008, Climate change and coral reef bleaching: An ecological assessment of long-term impacts, recovery trends and future outlook: Estuarine, Coastal and Shelf Science, v. 80, p. 435–471.

BAKER, R.D., HALLOCK, P., MOSES, E.F., WILLIAMS, D.E., and RAMIREZ, A., 2009, Larger foraminifers of the Florida reef tract, USA: Distribution patterns on reef-rubble habitats: Journal of Foraminiferal Research, v. 39, p. 267–277.

BARBOSA, C.F., OLIVEIRA-SILVA, P., SEOANE, J.C.S., FERREIRA, B.P., CAMPELLO, R.C., TURCQ, B.J., ALMEIDA, C.M., PORTILHO-RAMOS, R., and SOARES-GOMES, A., 2006, Diagnóstico da saúde ambiental de ecossistemas recifais da costa brasileira com a utilização de foraminíferos bentônicos. Projeto FOCO-PROBIO-

Tamandaré e Fernando de Noronha (PE), Porto Seguro e Arquipélago dos Abrolhos (BA), Brasil. Relatório Técnico do Ministério do Meio Ambiente - Período de 2004 a 2005, Distrito Federal, Brasil.

———, PRAZERES, M.F., FERREIRA, B.P., and SEOANE, J.C.S., 2009, Foraminiferal assemblage and reef check census in coral reef health monitoring of East Brazilian margin. Marine Micropaleontology, v. 73, p. 62–69.

BRESLER, V., and YANKO, V., 1995, Chemical ecology: a new approach to the study of living benthic epiphytic foraminifera: Journal of Foraminiferal Research, v. 25, p. 267–279.

CASTRO, C.B., and PIRES, D.O., 2001, Brazilian coral reefs: what we already know and what is still missing: Bulletin of Marine Science, v. 69, p. 357–371.

COCKEY, E., HALLOCK, P., and LIDZ, B.H., 1996, Decadal-scale changes in benthic foraminiferal assemblages off Key Largo, Florida: Coral Reefs, v. 15, p. 237–248.

COOPER, T.F., GILMOUR, J.P., and FABRICIUS, K.E., 2009, Bioindicators of changes in water quality on coral reefs: review and recommendations for monitoring programmes: Coral Reefs, v. 28, p. 589–606.

DALLE-DONNE, I., ROSSI, R., GIUSTARINI, D., MILZANI, A., and COLOMBO, R., 2003, Protein carbonyl groups as biomarkers of oxidative stress: Clinica Chimica Acta, v. 329, p. 23–38.

DOUGLAS, A.E., 2003, Coral bleaching – how and why?: Marine Pollution Bulletin, v. 46, p. 385–392.

DOWNS, C.A., FAUTH, J.E., HALAS, J.C., DUSTAN, P., BEMISS, J., and WOODLEY, C.M., 2002, Oxidative stress and seasonal coral bleaching: Free Radical Biology and Medicine, v. 33, p. 533–543.

———, ———, ROBINSON, C.E., CURRY, R., LANZENDORF, B., HALAS, J.C., HALAS, J., and WOODLEY, C.M., 2005, Cellular diagnostics and coral health: Declining coral health in the Florida Keys: Marine Pollution Bulletin, v. 51, p. 558–569.

FERREIRA, B.P., and MAIDA, M., 2006, Monitoramento dos recifes de coral do Brasil – Situação Atual e Perspectivas: Série Biodiversidade 18, Instituto do Meio Ambiente e dos Recursos Naturais Renováveis, Ministério do Meio Ambiente. Brasília, 250 p.

FILHO, R.C.B., SOUZA, A.E.A.B., FREITAS, G.L., NUNES, M.F.C., SOUZA, E.A., and FILHO, D.C., 2009, A garça-vaqueira (*Bulbucus ibis* Linnaeus, 1958) e o atobá-de-pé-vermelho (*Sula sula* Linnaeus, 1766) no Arquipélago de Fernando de Noronha: uma abordagem ecológica comparativa: Ornithologia, v. 3, p. 101–114.

GRANT, A.J., GRAHAM, K., FRANKLAND, S., and HINDE, R., 2003, Effect of copper on algal-host interactions in the symbiotic coral *Plesiastrea versipora*: Plant Physiology and Biochemistry, v. 41, p. 383–390.

HALLOCK, P., 1996, *Amphistegina* (Foraminiferida) densities as a practical, reliable, low-cost indicator of coral reef vitality, *in* Crosby, M.P., Gibson, G.R., and Potts, K.W. (eds.), A Coral Reef Symposium on Practical, Reliable, Low Cost Monitoring Methods for Assessing the Biota and Habitat Conditions of Coral Reefs, January 26-27, 1995. Office of Ocean and Coastal Resource Management,

National Oceanic and Atmospheric Administration, Silver Spring, MD, USA, p. 37–43.

———**,** 1999, Symbiont-bearing foraminifera, *in* Sen Gupta, B.K. (ed.), Modern Foraminifera. Dordrecht, Kluwer Academic Publishers, Netherlands, p. 123–139.

———, TALGE, H.K., SMITH, K.M., and COCKEY, E.M., 1992, Bleaching in a Reef-dwelling foraminifer, *Amphistegina gibbosa:* Proceedings of the 7th International Coral Reef Symposium, p. 42–47.

———, LIDZ, B.H., COCKEY-BURKHARD, E.M., and DONNELY, K.B., 2003, Foraminifera as bioindicators in coral reef and monitoring: The FORAM Index: Environmental Monitoring and Assessment, v. 81, p. 221–238.

———, WILLIAMS, D.E., FISHER, and E.M., TOLER, S.K., 2006a, Bleaching in foraminifera with algal symbionts: Implications for reef monitoring and risk assessment: Anuário do Instituto de Geociências, v. 26, p. 108–128.

———, ———, TOLER, S.K., FISHER, E.M., and TALGE, H.K., 2006b, Bleaching in reef-dwelling foraminifers: implications for reef decline: Proceedings of the 10th Coral Reef Symposium, p. 729–737.

HAZIN, M.C., and CASTRO, C.B., 2006, Parque Nacional Marinho de Fernando de Noronha, *in* Prates, A.P.L. (ed.), Atlas dos Recifes de Coral nas Unidades de Conservação Brasileiras. 2ª Edição, Ministério do Meio Ambiente, Brasília, p. 68–75.

HUGHES, T.P., BAIRD, A.H., BELLWOOD, D.R., CARD, M., CONNOLLY, S.R., FOLKE, C., GROSBERG, R., HOEGH-GULDBERG, O., JACKSON, J.B.C., KLEYPAS, J., LOUGH, J.M., MARSHALL, P., NYSTRO, M., PALUMBI, S.R., PANDOLFI. J.M.,

ROSEN, B., and ROUGHGARDEN, J., 2003, Climate change, human impacts, and the resilience of coral reefs: Science, v. 301, p. 929–933.

LANGER, M.R., and HOTTINGER, L., 2000, Biogeography of selected ‘‘larger’’ foraminifera:Micropaleontology, v. 46, p. 105–126.

LEÃO, Z.M.A.N., and DOMINGUEZ, J.M.L., 2000, Tropical coast of Brazil: Marine Pollution Bulletin, v. 46, p. 112–122.

LEE, J.J., 2006, Algal symbiosis in larger foraminifera: Symbiosis, v. 42, p. 63–75.

LESSER, M.P., 2006, Oxidative stress in marine environments: Biochemistry and physiological ecology: Annual Review in Physiology, v. 68, p. 253–278.

LINTON, D.M., and WARNER, G.F., 2003, Biological indicators in the Caribbean coastal zone and their role in integrated coastal management: Ocean and Coastal Management, v. 46, p. 261–276.

MAIDA, M., and FERREIRA, B.P., 1997, Coral reefs of Brazil: An overview: Proceeding of the 8th International Coral Reef Symposium, v. 1, p. 263–274.

MARTÍNEZ-COLON, M., HALLOCK, P., and GREEN-RUÍZ, C., 2009, Strategies for using shallow-water benthic foraminifers as bioindicators of potentially toxic elements: A review: Journal of Foraminiferal Research, v. 39, p. 278–299.

MMA – MINISTÉRIO DO MEIO AMBIENTE, 2005, Plano de Manejo – APA Fernando de Noronha, Rocas, São Pedro e São Paulo, Brasília, 1223 p.

NADELLA, S.D., FITZPATRICK, J.L., FRANKLIN, N., BUCKING, C., SMITH, S., and WOOD, C.M., 2009, Toxicity of dissolved Cu, Zn, Ni and Cd to developing embryos of the blue mussel (*Mytilus trossolus*) and the protective effect of dissolved organic carbon: Comparative Biochemistry and Physiology, Part C, v. 149, p. 340–348.

OAKES, K.D., and KRAAK, G.J.V.D., 2003, Utility of the TBARS assay in detecting oxidative stress in white sucker (*Catostomus commersoni*) populations exposed to pulp mill effluent: Aquatic Toxicology, v. 63, p. 447–463.

OLIVEIRA-SILVA, P., 2008, Diagnóstico ambiental dos ecossistemas recifais de Abrolhos e Porto Seguro, BA, com ênfase em foraminíferos e indicadores geoquímicos: PhD thesis, Universidade Federal Fluminense, 165 p.

PANDOLFI, J.M., BRADBURY, R.H., SALA, E., HUGHES, T.P., BJORNDAL, K.A., COOKE, R.G., MCARDLE, D., MCCLENACHAN, L., NEWMAN, M.J.H., PAREDES, G., WARNER, R.R., and JACKSON, J.B.C, 2003. Global trajectories of the long-term decline of coral reef ecosystems: Science, v. 301, p. 955–957.

REGOLI, F., 2000, Total oxyradical scavenging capacity (TOSC) in polluted and translocated mussels: a predictive biomarker of oxidative stress: Aquatic Toxicology, v. 50, p. 351–361.

ROSSI, A., 1999, Foraminíferos quaternários do Arquipélago de Fernando de Noronha: Taxonomia, ecologia, distribuição batimétrica e faciológica: Master Dissertation, Universidade do Vale do Rio dos Sinos, 141 p.

TALGE, H.K., and HALLOCK, P., 2003, Ultra structural responses in field-bleached and experimentally stressed *Amphistegina gibbosa* (Class Foraminifera): Journal of Eukaryotic Microbiology, v. 50, p. 324–333.

UTHICKE, S., and NOBES, K., 2008, Benthic Foraminifera as ecological indicators for water quality on the Great Barrier Reef: Estuarine, Coastal and Shelf Science, v. 78, p. 763–773.

———, THOMPSON, A., and SCHAFFELKE, B., 2010, Effectiveness of benthic foraminiferal and coral assemblages as water quality indicators on inshore reefs of the Great Barrier Reef, Australia: Coral Reefs, v. 29, p. 209–225.

VIARENGO, A., PONZANO, E., DONDERO, F., and FABBRI, R., 1997, A simple spectrophotometric method for metallothionein evaluation in marine organisms: an application to Mediterranean and Antarctic molluscs: Marine Environmental Research, v. 44, p. 69–84.

———, BURLANDO, B., CAVALETTO, M., MARCHI, B., PONZANO, E., and BLASCO, J., 1999. Role of metallothionein against oxidative stress in the mussel *Mytilus galloprovincialis*. American Journal of Physiology, v. 277, p. 1612–1619.

WILLIAMS, D.E., HALLOCK, P., TALGE, H.K., HARNEY, J.N., and MCRAE, G., 1997, Response of *Amphistegina gibbosa* populations in the Florida Keys (U.S.A.) to a multi-year stress event (1991-1996): Journal of Foraminiferal Research, v. 27, p. 264–269.

——— and ———, 2004, Bleaching in *Amphistegina gibbosa* d'Orbigny (Class Foraminifera): observations from laboratory visible and ultraviolet light experiments: Marine Biology, v. 145, p.641–649.

TABLE 1. Physicochemical parameters of the water collected at the Fernando de Noronha sampling sites. Mean concentration of metals and DOC are expressed in µg/l and mg C/l, respectively. DO = dissolved oxygen (mg O_2/l). ST = surface water temperature (ºC).

Sampling site	**Zn**	**Cu**	**Cd**	**Pb**	**DOC**	**pH**	**Salinity**	**DO**	**ST**
PSA	113	14.5	16	172	7.25	8.1	37	5.5	26
BIB	113	12.0	19	254	8.10	8.0	36	5.9	27.1
POR	88	12.5	9	162	8.05	8.0	37	5.7	26.7
LDI	79	11.5	13	137	5.80	8.2	37	5.5	26.9
BUR	118	10.0	20	49	3.45	8.0	37	5.9	26

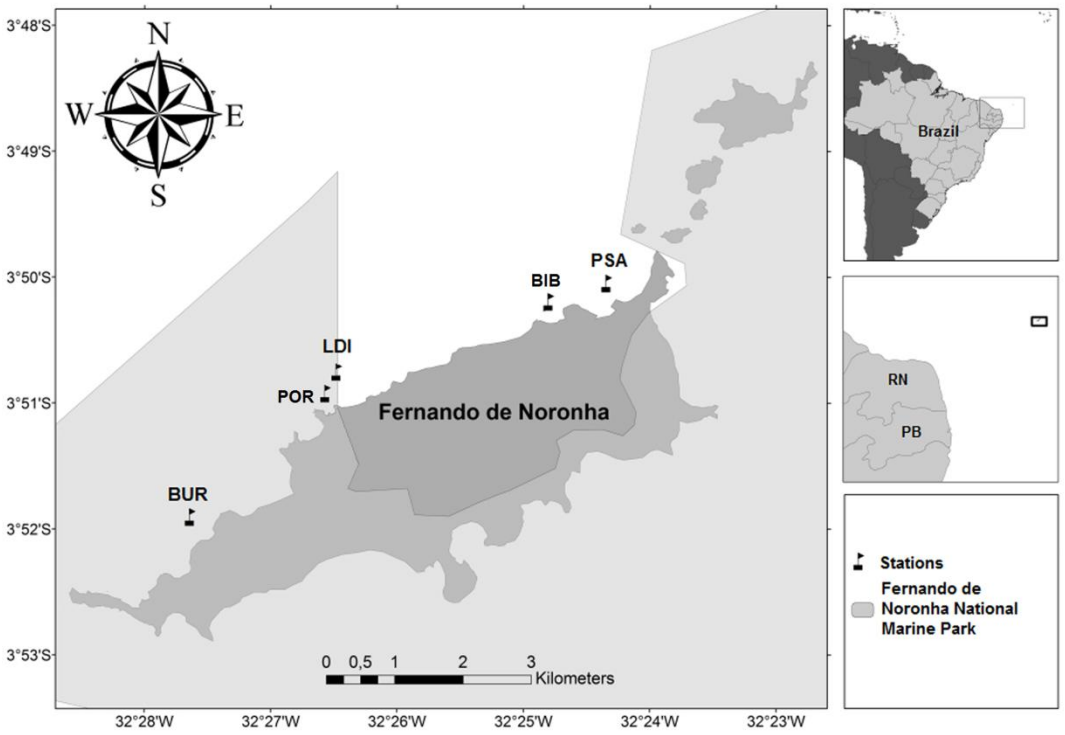

FIGURE 1. Sampling sites at the North shore of the Fernando de Noronha Island (Northeastern Brazil): Santo Antonio Harbor (PSA), Biboca beach (BIB), Porcos Bay (POR), Dois Irmãos Shoal (LDI) and Buracão (BUR). PSA and BIB sites are located outside the Fernando de Noronha National Marine Park.

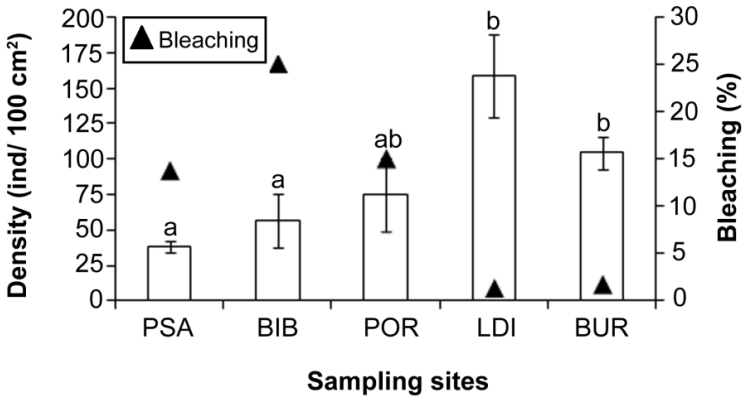

FIGURE 2. Density (individuals/100 cm^2) and degree (%) of bleaching in *Amphistegina lessonii* from the sampling sites at the Fernando de Noronha Island (Northeastern Brazil). Data are expressed as mean ± SE. Triangle (▲) represents the percentage of bleaching. Different letters indicate significant differences among sites ($P < 0.05$).

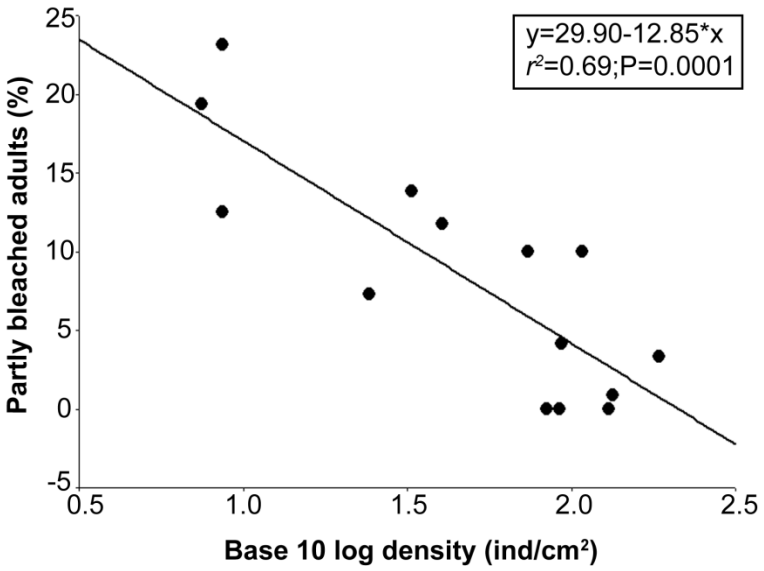

FIGURE 3. Correlation between the percentage of partly bleached adults and the base 10 log of abundance of *Amphistegina lessonii* collected at the different sampling sites in the Fernando de Noronha Island (Northeastern Brazil).

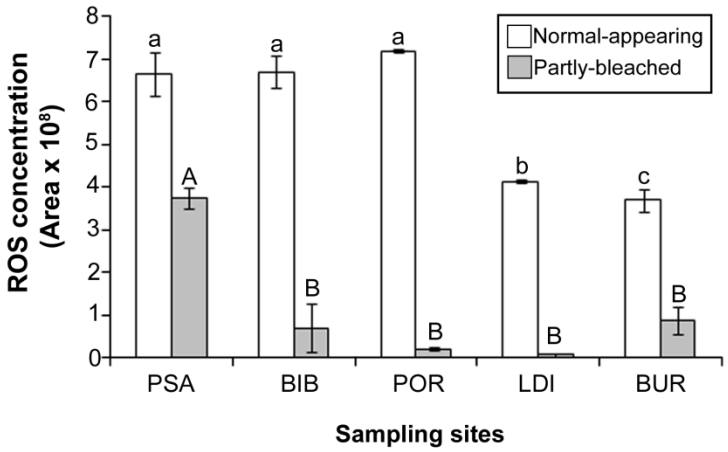

FIGURE 4. Antioxidant capacity against peroxyl radicals expressed as ROS concentration in normal-appearing (white bars) and partly bleached (gray bars) *Amphistegina lessonii* collected at the different sampling sites in the Fernando de Noronha Island (Southeastern Brazil). Data are expressed as mean ± SE. Different letters indicate significant differences among sites ($P < 0.05$).

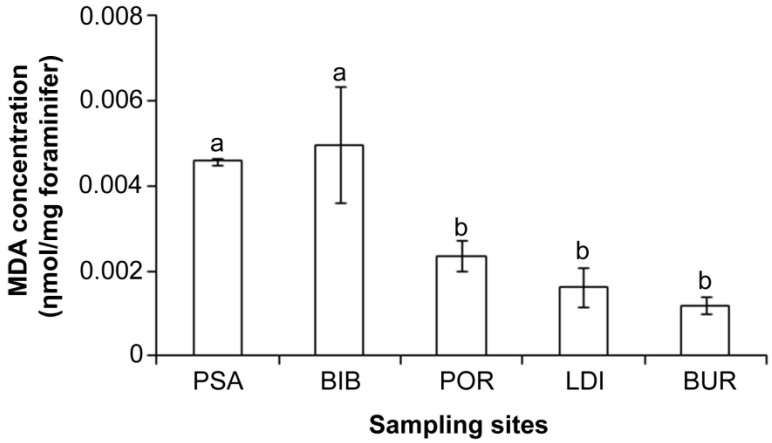

FIGURE 5. Lipid peroxidation expressed as MDA concentration in *Amphistegina lessonii* collected at the different sampling sites in the Fernando de Noronha Island (Southeastern Brazil). Data are expressed as mean ± SE. Different letters indicate significant differences among sites ($P < 0.05$).

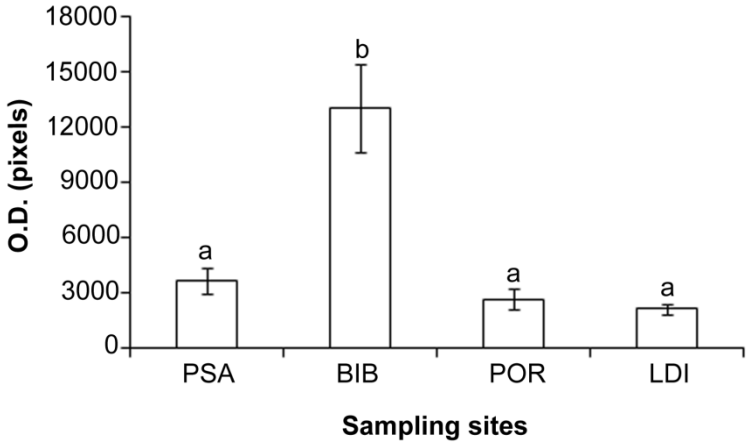

FIGURE 6. Protein oxidation expressed as optical density area (O.D.) in *Amphistegina lessonii* collected at the different sampling sites in the Fernando de Noronha Island (Northeastern Brazil). In foraminifers from the BUR site, no protein carbonylation was detected using the Western blotting immune assay. Data are expressed as mean ± SE. Different letters indicate significant differences among sites ($P < 0.05$).

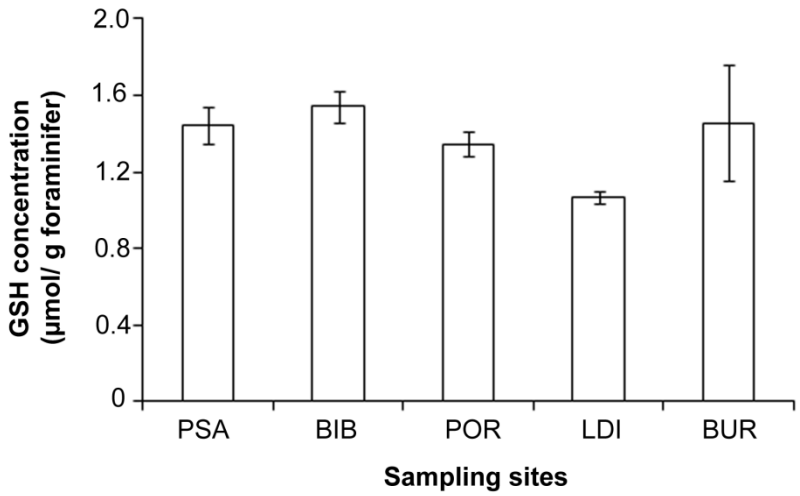

FIGURE 7. Metallothionein-like proteins concentration expressed as GSH concentration in *Amphistegina lessonii* collected at the different sampling sites in the Fernando de Noronha Island (Northeastern Brazil). Data are expressed as mean ± SE. No significant difference was observed among sites.

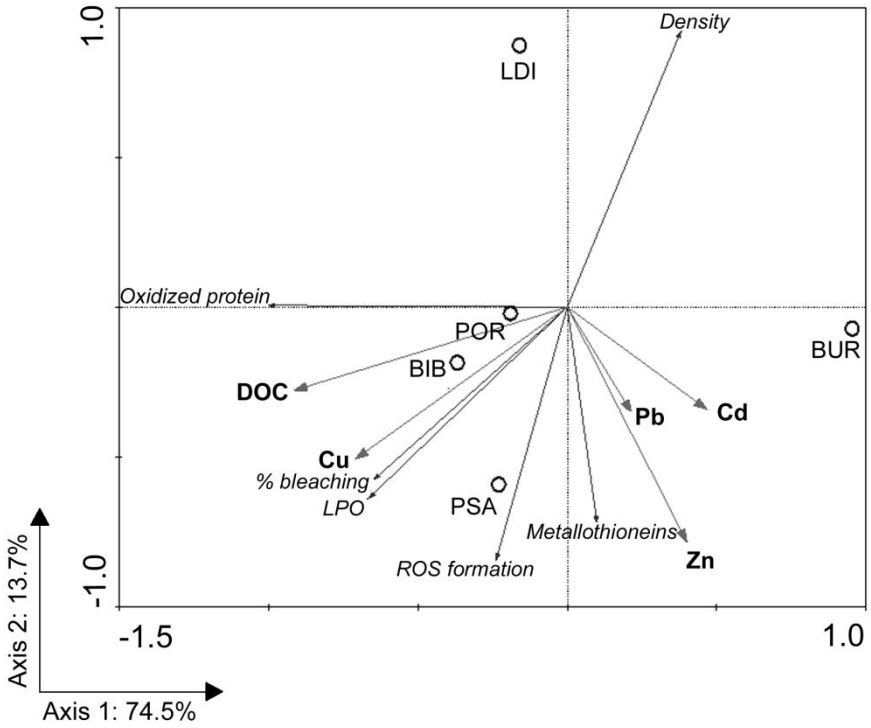

FIGURE 8. Redundancy analysis triplot for biomarkers data in *Amphistegina lessonii* and physicochemical parameters (DOC and dissolved metal concentrations) of water collected at the different sampling sites in the Fernando de Noronha Island (Northeastern Brazil).

ANEXO II

Biomarkers response to zinc exposure in the symbiont-bearing foraminifer *Amphistegina lessonii* (Amphisteginidae, Foraminifera)

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Artigo redigido de acordo com as normas para publicação no perídico *Journal of Experimental Marine Biology and Ecology*, para o qual já foi submetido.

Biomarkers response to zinc exposure in the symbiont-bearing foraminifer *Amphistegina lessonii* (Amphisteginidae, Foraminifera)

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ABSTRACT

The acute effects of zinc (Zn) were evaluated in the symbiont-bearing foraminifer *Amphistegina lessonii* from the Fernando de Noronha Archipelago (Northeastern Brazil). Foraminifers were acutely (48 h) exposed to dissolved Zn concentrations ranging from 9.5 to 93.4 µg Zn/l. Endpoints analyzed included mortality, visual alterations (white spots and dark-brown areas in the test), oxidative stress biomarkers (reactive oxygen species generation, lipid peroxidation and total superoxide dismutase activity) and concentration of metallothionein-like proteins in whole individuals after Zn exposure. No significant mortality was observed during 48-h exposure period to dissolved Zn. However, a significant percentage of individuals showed visual alterations (white spots and/or dark-brown areas in the test) aftr 24 and 48 h of Zn exposure. In fact, a significant positive correlation between this endpoint and dissolved Zn concentrations was observed for both times of exposure. Based on this endpoint, the 24-h and 48-h EC_{50} values in their corresponding 95% confidence intervals for total measure Zn concentrations were calculated as 112.2 (199.5-86.5) and 43.65 (57.3-34.9) µg Zn/l, respectively. Based on this dissolve Zn concentration, they were 100.70 (175.87-75.35) and 38.20 (49.38-29.72) µg Zn/l, respectively. Therefore, a significant increase in Zn toxicity was observed with increasing time of exposure. After 48 h of Zn exposure, whole body antioxidant capacity was lower in normal-appearing individuals than those in initial stage of bleaching. Increases in lipid peroxidation, metallothionein-like protein concentration and total SOD activity was

observed at a greater extent in pale/partly-bleached individuals associated with an increased Zn toxicity measured as visual alterations. These finding suggest that an activation of some components of the antioxidant system occurred in *A. lessonii* to counteract the oxidative stress induced by Zn exposure, and consequently avoid a possible complete loss of the symbiont.

Keywords: *Amphistegina lessonii*; biomarkers; foraminifer; oxidative stress; zinc

INTRODUCTION

Coral reefs are suffering a long-term global decline, yet the causes remain controversial. Reef environments are a common feature of many shallow coastal areas in tropical regions, and are at increasing risk due to water pollution (Reichelt-Brushett and Harrison, 1999). Consequently, reef biota that thrive in near shore environments is often exposed to elevated levels nutrients (e.g., phosphate, nitrate and ammonia) and pollutants (e.g., pesticides, oil, toxins and metals). Increased levels of these contaminants are associated with human activities such as harbor dredging and sewage discharge (Ferrier-Pagès et al., 2005; Kline et al., 2006). They are present in batteries, alloys, fertilizers and antifouling painting.

Little is known about the physiological effects of very common toxic elements, such as contaminant metal and organic compounds, on coral reefs' fauna (e.g., Bielmyer et al., 2010). Exposure to these compounds can induce the generation of reactive oxygen species (ROS), which causes important oxidative damage to proteins,

lipids and nucleic acids, leading to photosynthesis inhibition and coral bleaching (Lesser, 2006).

Zinc (Zn) is a well-known essential micronutrient required for normal metabolism. Its deficiency may lead to malfunction of several enzymes, including those related to the antioxidant responses, which depend on Zn as cofactor. However, Zn can be toxic at high concentration, depending upon its bioavailability. In open oceans, Zinc concentrations in surface waters are often very low, with an average concentration of 5 µg Zn/l (Reichelt-Brushett and Harrison, 1999). However, in sites near urban areas like in Fernando de Noronha Island (Northeastern Brazil) Zn concentration can reach values as high as 118 µg Zn /l (Prazeres et al., 2011). Although essential to the metabolism, little is known about the adverse effects of this metal as an oxidative stressing factor in marine organisms, especially foraminifers.

In foraminifers, excessive metals concentrations are known to cause chamber deformities, and reproductive and cytological disturbances, especially the presence of mercury and copper (Bresler and Yanko, 1995; Nigam et al., 2009). However, toxicological experiments dealing with exposure of symbiont-bearing foraminifers to metals are not available to date. Since they play an important role in coral reefs, providing a reliable bioindicator of water quality suitable on time scales from weeks to months (Hallock et al., 2006), the study on their response to short-term exposure to metals may be useful for ecotoxicological investigations and monitoring (Bresler and Yanko, 1995).

Amphistegina spp. are diatom-bearer foraminifers abundantly found in coral reefs and tropical carbonate shelves worldwide (Langer and Hottinger, 2000). They are commonly living on coralline and filamentous algae on reef substrate, as well as on

some macrophytes (Baker et al., 2009). Foraminifers from the genus *Amphistegina* can exploit a wide range of depths, although *Amphistegina lessonii*, which is the most abundant species found in South Atlantic Ocean, has preference for depths varying from 6 to 20 m, although it can distributed down to 50 m (Hohenegger et al., 1999). This species was used as bioindicator of water quality at the Fernando de Noronha Archipelago (Prazeres et al., 2011), and demonstrated to be a reliable tool for assessing of environmental health for coral reef growth and maintenance. These authors found coral reef areas at the Archipelago affected by sewage disposal of solid wastes and other human activities, where metal concentrations such as Pb, Cu and Zn were above the Water Quality Criteria established by Brazilian Environmental Regulations (CONAMA, 2008). At these sites, populations of *A. lessonii* showed low density, high degree of bleaching, less tolerance for coping with oxidative stress and higher damage to lipids and proteins (Prazeres et al., 2011).

The potential threats to coral reefs in Brazil and worldwide is clear. Therefore, it is important to understand the response of bioindicators organisms such as the symbiont-bearing foraminifers to pollutants, to development of appropriate management strategies. In addition, applications of foraminifers as bioindicators require strong scientific evidences based on both field and laboratory experiments that specifically examine the influence of potentially toxic elements and other pollutants at community, assemblage, population, individual, and gene expression levels (Martínez-Colon et al., 2009).

In light of the above, the goal of the present study was to determine the short-term effects of Zn exposure in the symbiont-bearing foraminifer *A. lessonii*. The oxidative stress response at the cellular level was assessed through measurements of

several biomarkers (antioxidant capacity level, lipid peroxidation, metallothionein-like proteins concentration and total superoxide dismutase activity).

MATERIAL AND METHODS

Collection and acclimation of *A. lessonii*

Adult individuals of *A. lessonii* were collected by SCUBA diving in a reference site inside the Fernando de Noronha National Marine Park (Fernando de Noronha, PE, Northeastern Brazil). Specimens were collected from pieces of dead reef rubble, which is believed to be the preferred habitat of this species (Fujita, 2004; Murray, 2006; Baker et al., 2009). These reef rubbles were immediately scrubbed using a small brush into a bucket containing seawater from the collection site. The collected material was kept in the shade for decantation of the residual sediment without exposure to extremes conditions of light incidence and temperature. The residual sediment was taken to the field-based laboratory and the suspended material was rinsed away, until apparently clean residual sediment was obtained.

In the field-based laboratory, the sediment was split into several aliquots and placed into 150 mm Petri dishes, covered with synthetic seawater at salinity 36 (Coralife®) for separation of the living individuals of *A. lessonii*. The material was left undisturbed for 24 h at relatively low light and room temperature. As *Amphistegina* is negatively geotaxic, it climbed up through the sediment n towards the surface. Sometimes it even climbed up to the dish walls, as described by Hallock et al., (2006). After this period, a high number of living healthy individuals showing golden-brown

color and exhibiting pseudopodial activity were separated using a stereomicroscope and placed into polypropylene pots until further processing in the main laboratory.

Prior to the beginning of the toxicity test, isolated living individuals of *A. lessonii* were maintained in 150 mm glass Petri dishes covered with plastic wrap and acclimated for 3-4 weeks in a culture chamber. They were kept at 27±1°C and photoperiod was set at 12 h light/dark cycle using white fluorescent source, providing p*hotosynthetically active radiation (*PAR) for the endosymbionts photosynthesis. Foraminifers were maintained in synthetic seawater (salinity 36) with addition of nutrients, as described by Hallock et al. (1986).

Acute toxicity test

Healthy individuals of approximately the same diameter (0.6-0.9 mm) were separated and placed into covered glass Petri dishes and kept at the same conditions described for the acclimation period. The diameter of each specimen was measured using a microscope equipped with a micrometer reticule ocular.

For the 48-h toxicity test, *A. lessonii* individuals were exposed to four concentration of Zn according to their ecological relevance and the Ambient Water Quality Criteria established by the U.S. Environmental Protection Agency (90 µg dissolved Zn/l; U.S.EPA, 2009) and Brazilian National Council for Environment (90 µg total Zn/l; CONAMA, 2008). Total and dissolved Zn concentrations were measured by the atomic absorption espectrophometry (AAS 932, GBC. Australia) in non-filtered and filtered (0.45-µm mesh filter) water samples collected at the end of experiment, as previously described (Prazeres et al., 2011). Measured dissolved Zn concentrations

(mean ± standard error) in the experimental media were: 9.53±0.61 (control; without Zn addition), 25.20±0.34, 42.01±1.80, 67.67±1.86, 93.37±9.93 µg Zn/l.

Five replicates (n= 6-8 foraminifers per replicate) were randomly assigned to different Zn concentrations. Experimental media containing Zn was prepared in synthetic seawater (salinity 36) at least 24 h prior to the experiment. All glassware was previously cleaned with diluted nitric acid (1%) and thoroughly rinsed several times with Milli-Q water before use. During the 48-h exposure period, cultures were maintained at 27±1°C, without changing the culture medium. After 48 h of exposure, foraminifers were visually inspected for any changing in color and pseudopodial activity. Death was assumed when individuals showed no pseudopodial activity or a completely white carapace. Percentage of visual alteration was determined dividing the number of affected foraminifers (e.g., white spots and dark brown area or death) by total number of foraminifers exposed. Degree of bleaching was performed according to Hallock et al. (2006). At the end of the exposure period (48-h), living foraminifers were collected an immediately frozen (-80°C) for further analyses of biomarkers as described below.

Total antioxidant capacity against peroxyl radicals (ACAP)

ACAP assay was performed to measure the biological resistance to various kinds of oxyradicals, thus providing useful indications to predict oxyradical-mediated adverse effects on the physiological condition of the organisms (Regoli, 2000). Antioxidant capacity was measured using the fluorescence technique following the method described by Amado et al. (2009). Briefly, foraminifer samples were homogenized (0.75 mg of protein/ml), and placed into a white 96-well microplate with

or without 2'-Azobis (2-methylpropionamidine) dihydrochloride (ABAP), which was used as the peroxyl radical generator, together with the fluorescent probe 2′,7′ dichlorofluoresceindiacetate (H_2DCF-DA). Protein content in the homogenized was determined using the Quant-iT Protein Assay (Invitrogen, USA). Resulting fluorescence in the reaction mixture was measured (excitation: 488 ηm; emission: 525 ηm) using a microplate reader (Victor 2 – Perkin Elmer) every 5 min for up to 45 min. Results were expressed as the difference in fluorescent units × min area for each sample in the presence and the absence of ABAP. Data were normalized to ROS area without ABAP (background area).

The relative difference between ROS area with and without ABAP was considered as a measurement of the antioxidant capacity, with area difference meaning low antioxidant capacity, since high fluorescence levels were obtained after adding ABAP, meaning low competence to neutralize peroxyl radicals (Amado et al., 2009).

Lipid peroxidation (LPO)

The 2-thiobarbituric acid reactive substances (TBARS) assay quantifies the oxidative stress by measuring the peroxidative damage to lipids (LPO). This method was used to quantify LPO in foraminifer samples from the present study. Measurements were made using a fluorescence technique following the protocol described by Oakes and van der Kraak (2003). Homogenates of foraminifer samples (1:20; w/v) were used to perform the TBARS assay. Concentrations of malondialdehyde (MDA) generated in the reaction mixture were measured by fluorescence (excitation: 515nm; emission: 530 nm) in a microplate reader (Victor 2, Perkin Elmer, USA). Lipid peroxides concentration was expressed as nmol MDA/mg

foraminifer. MDA concentration was calculated from a standard curve built with hydrolyzed tetramethoxypropane (TMP).

Metallothionein-like proteins concentration (MTLP)

MTLP content was analyzed in foraminifers homogenized (1:25; w/v) prepared in 20 mM Tris–HCl buffer (pH 8.6) containing 500 mM sucrose, 0.006 mM phenylmethylsulphonyfluoride (PMSF) and 0.01% β-mercaptoethanol. After acidic ethanol/chloroform fractionation of the homogenate, MTLP were quantified by spectrophotometry using GSH as standard (Viarengo et al., 1997). It should be noted that this method quantifies all sulphydryl groups, thus being not specific for metallothioneins detection. However, it is useful for detection and measurement of metallothioneins-like proteins (Bocchetti and Regoli, 2006).

Total superoxide dismutase activity (SOD)

Total superoxide dismutase (SOD) activity was measured in accordance to the method described by McCord and Fridovich (1969). This method is based on the xanthine/xanthine oxidase assay and reduction of cytochrome *c*. The assay was carried out with foraminifer homogenates (1.5 mg/ml of protein) in a reaction buffer (pH 7.8) containing potassium phosphate (50 mM), cytochrome *c* (10 µM), xanthine oxidase (50 µM) and EDTA (100 µM). Reaction was started by adding 50 U of xanthine oxidase in a final volume of 750 ul of reaction mixture. Cytochrome *c* reduction was followed spectrophotometrically (550 nm) for 60 sec. Results were expressed as units of SOD activity per milligram of protein (U/mg of protein), where one unit of SOD is defined as the activity causing 50% inhibition of cytochrome *c* reduction at 25ºC.

Data analysis

The concentration causing toxicity in 50% of the individuals tested (EC_{50}) and their corresponding 95% confidence intervals were calculated for total and dissolved Zn concentrations using Probit analysis. EC_{50} values were determined for 24 and 48 h of Zn exposure. Values obtained were compared using the Chi-square test.

Significant differences in ACAP, LPO, MTLP and total SOD activity mean values among treatments were detected by one-way analysis of variance (ANOVA). Data normality and homogeneity of variances were previously checked. When these ANOVA assumptions were violated, a log transformation was applied to data prior to analysis. If significant changes were detected, differences among treatments were identified using the Tukey's HSD test. In all cases, significance level adopted was 95% (α=0.05).

RESULTS

Acute Zinc toxicity

In control foraminifers (no Zn addition to the water), no mortality was observed during the 48-h exposure period. In Zn-exposure foraminifers, only a few individuals died after 48-h exposure period. Therefore, it was not possible to estimate the median lethal concentration (LC_{50}) based on the Zn concentrations tested.

Despite the lack of mortality, visual alterations were observed in *A. lessonii* individuals exposed to Zn. The most common alterations were the presence of small white spots in the test. Foraminifers showing this kind of visual alteration were

characterized as bleached individuals, even when spots were present in a very low degree. Also, a feature of dark-brown areas was observed, especially in individuals exposed to 68 and 93 µg Zn/l. At 68 µg Zn/l, browner individuals were observed in all replicates after both 24 and 48 h of exposure. However, none of these individuals died during the experimental period. Furthermore, this unusual brown color observed was followed by a return to *A. lessonii* natural 'healthy' color.

Visual alterations observed in *A. lessonii* exposed to Zn (white spots and dark-brown areas) were positively correlated ($p<0.05$) with dissolved Zn concentration. Chi-square results showed significant increase in Zn toxicity ($\chi^2=22.45$; $p=0.0002$) with the increasing time of exposure (Fig. 1). Based on this endpoint, the 24-h and 48-h EC_{50} values and their corresponding 95% confidence intervals for total measured Zn were calculated as 112 (199.5-86.5) and 44 (57.3-34.9) µg/l, respectively. Based on dissolved Zn concentrations, these values were 101 (175.9-75.35) and 38.2 (49.4-29.7) µg/l, respectively.

Biomarkers responses

In normal-appearing *A. lessonii*, ACAP was significantly different in control individuals and those exposed to the higher Zn concentrations tested (68 and 93 µg Zn/l; $p=0.04$). At these Zn concentrations, ROS production was 2-fold higher, reaching an area of 2.6×10^7 and 4.8×10^7, (relative area of 9.82 and 9.22) for 68 and 93 µg Zn/l, respectively. Furthermore, ROS production was significantly higher in normal-appearing individuals than in pale/partly-bleached individuals at these Zn concentrations (Fig. 2).

In normal-appearing foraminifers, LPO did not significantly change after Zn exposure, except when 68 µg Zn/l was tested. In this case, *A. lessonii* individuals showed a 3-fold higher MDA concentration (2.5×10^{-2} ηmol MDA/mg foraminifer; p=0.03) than control ones. In pale/partly-bleached individuals, LPO levels did not follow a clear pattern of Zn concentration dependence. However, LPO was generally higher in these individuals than in the normal-appearing ones (Fig. 3).

In normal-appearing *A. lessonii*, a significant increase (p<0.01) in whole body MTLP (expressed as GSH concentration) concentration was observed in individuals exposed to the highest dissolved Zn concentration tested (93 µg Zn/l) with respect to the control ones. Partly-bleached *A. lessonii* individuals exposed to 68 µg Zn/l showed significantly (p<0.01) higher MTLP content than the normal-appearing ones (Fig. 4).

In normal-appearing individuals, total SOD activity was inversely related to the dissolved Zn concentration, i.e., increasing Zn concentration induced lower enzyme activity. Total SOD activity gradually decreased, reaching ~50% of its activity at 93 µg Zn/l. In pale/partly-bleached foraminifers, total SOD activity was significantly higher at 93 µg Zn/l than at 68 µg Zn/l. Furthermore, these individuals showed significantly higher total SOD activity than the normal-appearing ones when exposed to 93 µg Zn/l (Fig. 5).

DISCUSSION

Although Zn is an essential micronutrient for normal metabolism, it can be toxic when presented in excessive concentrations (Li et al., 2006). Ion the present study, Zn concentrations tested showed no significant acute lethal toxicity in the

symbiont-bearing foraminifer *A. lessonii*. In fact, it is known that many foraminifers species produce extracvellular acid mucopolysacharides that can play a significant role in their ecology. Among other functions these mucopolysacharides are involved in foraminifer anti-chemical defense. They form a diffusion barrier and have numerous negatively charged groups that can bind cationic compounds, including metal ions (Bresler and Yanko, 1995), thus reducing metal accumulation and toxicity. Therefore, the lack of foraminifer mortality observed in the present study after 48 h of exposure to Zn could be associated at least in part to a possible production of extracellular mucopolysaccharides by *A. lessonii*. This finding also suggests that the Ambient Water Quality Criteria for Zn in marine water established by the United States (90 µg dissolved Zn/l; U.S.EPA, 2009) and Brazilian (90 µg total Zn/l; CONAMA, 2008) Environmental Regulations are safe for foraminifers that thrive in coral reef environments, at least when considering lethal effects after a short period of Zn exposure (48 h).

Despite no foraminifer mortality, in the present study after exposure to the Zn concentrations tested, a clear response of *A. lessonii* individuals to metal exposure was expressed as small bleached and browner spots in their tests. Therefore, pale/partly-bleached individuals were observed after Zn exposure. Also, a lower competence against peroxyl radicals and oxidative damage, detected as lipid peroxidation, was observed in all experimental conditions, except in the control group of foraminifers. It is well known that metal exposure induces ROS production, leading to severe cellular injury and death (Lesser, 2006). In turn, antioxidant capacity is an important mechanism to prevent oxidative damage. Therefore, induction of antioxidant enzymes is an important protective mechanism to minimize cell oxidative damage in metal

polluted environments. In fact, the activity of one or more enzymes from the antioxidant defense system is generally increased when organisms are exposed to stressful conditions. Furthermore, elevated enzyme activity levels are generally associated with higher tolerance to stress.

As also observed by Prazeres et al. (2011) in field-sampled individuals, *A. lessonii* tested in laboratory showing a uniform brown color had a lower antioxidant capacity than those in initial bleaching stage. These findings strongly suggest the protective effect of the algae in symbiont-bearing foraminifers, since the toxic effect of Zn seemed to first affect the endosymbiont rather than the host cell. Once in initial stage of the bleaching process, the foraminifers may be able to recover from the oxidative stress caused by the breakdown of the algal-photosystem II (Douglas, 2003). Moreover, the symbiont loss was reported in experiments with corals being exposed to Zn and Cu. In this case, bleaching was also observed as a consequence of the reduction of photosynthesis efficiency (Ferrier-Pagès et al., 2005; Bielmyer et al., 2010). Likewise, symbiont-bearing foraminifers might respond similarly to increasing metal bioavailability, where the symbiont expulsion would be a mechanism of metal detoxification. In fact, it is known that corals are able to accumulate heavy metals to a large extent and thus be more tolerant than their symbiont hosts (Peters et al., 1997).

In the present study, oxidative damage (LPO) was higher in pale/partly-bleached foraminifers as compared to the normal-appearing individuals. This is generally a consequence of the disintegration of endoplasm membranes during the bleaching process (Talge and Hallock, 2003). This result corroborates with observations from Talge and Hallock (1995), where *A. gibbosa* individuals from the Florida Keys showed a gradient of damage from early deterioration of endoplasm in

normal-appearing individuals to complete disintegration of the endoplasm in partly-bleached/bleached specimens. In this case, LPO might be a result of the bleaching process in addition to the toxic effect of Zn itself to *A. lessonii*. Moreover, these results suggest that although apparently normal, foraminifers were under oxidative stress during the short-term (48 h) exposure to Zn.

Considering the role of metallothioneis in the homeostasis of trace metals (Amiard et al., 2006), the physiological pool of MTLP would compensate the increased Zn bioavailability and could also act as an antioxidant agent in pale/partly-bleached foraminifers. Indeed, the levels of these proteins were higher in individuals at the initial bleaching stage. These results suggest that an activation of MTLP synthesis occurred after Zn exposure to counteract the oxidative stress and toxicity induced by the metal, especially in foraminifers exposed to high Zn concentrations tested (68 and 93 µg Zn/l).

Among antioxidant enzymes, SOD activity constitutes the first antioxidant response of the enzymatic defense system (Richier et al., 2005). However, the inhibition of total SOD activity observed in normal-appearing *A. lessonii* with increasing Zn concentration. A similar result was also observed in the dinoflagellate *Gonyaulax polyedra* when acutely (48 h) exposed to Pb (Okamoto and Colepicolo, 1998). On the other hand, this inhibitory response of SOD activity was not observed in the microalgae *Pavlova viridis* exposed to Zn. In this case, no significant change in SOD activity was observed (Li et al., 2006). One reasonable explanation for the observed Zn-induced inhibition of total SOD activity in normal-appearing A. lessonii individuals could be that severe oxidative stress would have caused an enzyme inactivation and degradation. Despite the decrease in the level of this enzymatic

antioxidant agent (total SOD activity), a significant increase in the level of the non-enzymatic defense agent (MTLP) was observed after exposure to the higher Zn concentrations tested, as described above. However, the increased level of the non-enzymatic agent did not compensate the observed decrease in the enzymatic agent. As a result, an imbalance between ROS formation and antioxidant defenses led to a cellular damage (LPO) after *A. lessonii* exposure to 93 µg Zn/l.

After entering an initial stage of bleaching, a 50% increase in total SOD activity was observed in *A. lessonii* exposed to 93 µg Zn/l. After releasing the damaged algae, as previously mentioned, the enzymatic apparatus would be able to recover. In fact, it was shown that oncrease in the levels of both enzymatic and non-enzymatic antioxidant agents may play an important role in preventing the process of complete symbiont loss (Downs et al., 2002). Therefore, it is suggested that the increased total SOD activity acted as a first antioxidant defense and might be responsible for the augment ACAP levels observed in pale/partly-bleached individuals exposed to 68 and 93 µg Zn/l. Despite this augment response against the oxidative stress, they were not able to avoid oxidative damage, since LPO is an irreversible cellular damage and in this case likely caused by the algal-symbiont release.

It is clear from the results reported here that increased Zn concentration in seawater has a direct relationship to the great increase in ROS formation over the 48-h exposure period. It is also evident that ROS formation exceeded the cellular antioxidant capacity, caused the observed oxidative damage (LPO and total SOD activity inhibition) in normal-appearing and pale/partly-bleached individuals. It is important to notice that these biological effects were observed in the symbiont-bearing foraminifer *A. lessonii* exposed to concentrations lower than the acute the Ambient

Water Quality Criteria established by the U.S. Environmental Protection Agency (90 µg dissolved Zn/l; U.S.EPA, 2009) and Brazilian National Council for Environment (90 µg total Zn/l; CONAMA, 2008). Criteria used to protect tropical reef ecosystems were derived mainly using toxicity data from temperate organisms, and more recently from coral species (Bielmyer et al., 2010). However, according to results from the present study, and taking into consideration the use of reef-dwelling foraminifers as bioindicator of water quality in coral reefs environments, they indeed respond fast to the increased metal bioavailability in the seawater at cellular level. Therefore, despite the fact that these sensitive organisms are protected by the present Ambient Water Quality Criteria for Zn established by the American and Brazilian Environmental Regulations in terms of acute lethality, the symbiont-bearing foraminifer *A. lessonii* showed to be in a stressful condition when exposed to 93 µgZn/l, a concentration very close to those ambient water quality criteria.

Nevertheless, further studies on long-term effect of Zn on growth, development and reproduction of symbiont-bearing foraminifers are recommended to derive more adequate criteria to protect the coral reef biota. This statement is based on results from the present study showing that increasing the time of exposure to Zn significantly enhanced the metal toxicity. Furthermore, Zn is known to compete with Ca for the same ion channel (Santore et al., 2002). Therefore, direct effects on chamber addition on calcifying organisms would be expected after long-term Zn exposure.

ACKNOWLEDGEMENTS

We thank the Brazilian Ministry of Environment (MMA); the Brazilian Environmental Agency (IBAMA/MMA) and the Chico Mendes Institute for Protection of Biodiversity (ICMBio/MMA) for providing authorization to conduct the study and support for field work; International Development Research Center (IDCR; Ottawa, Canada) for financial support. MFP is a MSc fellow from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brasilia, Brazil). AB is a fellow from the Brazilian CNPq (Proc. #304430/2009-9) and is supported by the International Canada Research Chair Program from IDRC.

REFERENCES

Amado, L. L., Garcia, M.L., Ramos, P.B., Freitas, R.F., Zafalon, B., Ferreira, J.L.R., Yunes, J.S., Monserrat, J.M., 2009. A method to measure total antioxidant capacity against peroxyl radicals in aquatic organisms: Application to evaluate microcystins toxicity. Sci. Total Environ. 407, 2115–2123.

Amiard, J.C., Amiard-Triquet, C., Barka, S., Pellerin, J., Rainbow, P.S., 2006. Metallothioneins in aquatic invertebrates: Their role in metal detoxification and their use as biomarkers. Aquat. Toxicol. 76, 160–202.

Baker, R.D., Hallock, P., Moses, E.F., Williams, D.E., Ramirez, A., 2009. Larger foraminifers on the Florida reef tract, USA: Distribution patterns on reef-rubble habitats. J. Foramin. Res. 39, 267–277.

Bielmyer, G.K., Grosell, M., Bhagooli, R., Baker, A.C., Langdon, C., Gillette, P., Capo, T.R., 2010. Differential effects of copper on three species of

scleractinian corals and their algal symbionts (*Symbiodinium* spp.). Aquat. Toxicol. 97, 125–133.

Bocchetti, R., Regoli, F., 2006. Seasonal variability of oxidative biomarkers, lysosomal parameters, metallothioneins and peroxisomal enzymes in the Mediterranean mussel *Mytilus galloprovincialis* from Adriatic Sea. Chemosphere 65, 913–921.

Bresler, V., Yanko, V., 1995. Acute toxicity of heavy metals for benthic epiphytic foraminifera *Pararotalia spinigera* (Le Calvez) and influence of seaweed-derived DOC. Environ. Toxicol. Chem. 14, 1687–1695.

CONAMA, 2008. Conselho Nacional do Meio Ambiente. Resoluções do Conama: resoluções vigentes publicadas entre julho de 1984 e novembro de 2008, Brasília, DF.

Douglas, A.E., 2003. Coral bleaching – how and why? Mar. Pollut. Bull. 46, 385-392.

Downs, C.A., Fauth, J.E., Halas, J.C., Dustan, P., Bemiss, J., Woodley, C.M., 2002. Oxidative stress and seasonal coral bleaching. Free Radical Bio. Med. 33, 533–543.

Ferrier-Pagés, C., Houlbrèque, F., Wyse, E., Richard, C., Allemand, D., Boisson, F., 2005. Bioaccumulation of zinc in the scleractinian coral *Stylophora pistillata*. Coral Reefs 24, 636–645.

Fujita, K., 2004. A field colonization experiment on small-scale distributions of algal symbiont-bearing larger foraminifera on reef rubble. J. Foramin. Res. 34, 169–179.

Hallock, P., Forward, L.B., Hansen, H.J., 1986. Influence of environment on test shape of *Amphistegina*. J. Foramin. Res. 16, 224–231.

Hallock, P., Williams, D.E., Fisher, E.M., Toler, S.K., 2006. Bleaching in foraminifera with algal symbionts: Implications for reef monitoring and risk assessment. Anuário do Instituto de Geociências 26, 108–128.

Hohenegger, J., Yordanova, E., Nakano, Y., Tatzreiter, F., 1999. Habitats of larger foraminifera on the upper reef slope of Sesoko Island, Okinawa, Japan. Mar. Micropaleontol. 36, 109–168.

Kline, D.I., Kuntz, N.M., Breitbart, M., Knowlton, N., Rohwer, F., 2006. Role of elevated organic carbon levels and microbial activity in coral mortality. Mar. Ecol. Prog. Ser. 314, 119–125.

Langer, M.R., Hottinger, L., 2000. Biogeography of selected "larger" foraminifera. Micropaleontology 46, 105–126.

Lesser, M.P., 2006. Oxidative stress in marine environments: Biochemistry and physiological ecology. Annu. Rev. Physiol. 68, 253–278.

Li, M., Hu, C., Zhu, Q., Chen, L., Kong, Z., Liu, Z., 2006. Copper and zinc induction of lipid peroxidation and effects on antioxidant enzyme activities in the microalga *Pavlova viridis* (Prymnesiophyceae). Chemosphere 62, 565–572.

Martínez-Colon, M., Hallock, P., Green-Ruíz, C., 2009. Strategies for using shallow-water benthic foraminifers as bioindicators of potentially toxic elements: A review. J. Foramin. Res. 39, 278–299.

McCord, J.M., Fridovich, I., 1969. Superoxide dismutase an enzymatic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244, 6049–6055.

Murray, J., 2006. Ecology and applications of benthic foraminifera. Cambridge University Press, United Kingdom.

Nigam, R., Linshy, V.N., Kurtarkar, S.R., Saraswat, R., 2009. Effects of sudden stress due to heavy metal mercury on benthic foraminifer *Rosalina leei*: Laboratory culture experiment. Mar. Pollut. Bull. 59, 362–368.

Oakes, K.D., Kraak, G.J.V.D., 2003. Utility of the TBARS assay in detecting oxidative stress in white sucker (*Catostomus commersoni*) populations exposed to pulp mill effluent. Aquat. Toxicol. 63, 447–463.

Okamoto, O.K., Colepicolo, P., 1998. Response of superoxide dismutase to pollutant metal stress in the marine dinoflagellate Gonyaulax polyedra. Comp. Biochem. Phys. C 119, 67–73.

Peters, E.C., Gassmann, N.J., Firman, J.C., Richmond, R.H., Power, E.A., 1997. Ecotoxicology of tropical marine ecosystems. Environ. Toxicol. Chem. 16, 12–40.

Prazeres, M.F., Martins, S.E., Bianchini, A., 2011. Assessment of water quality in coral communities from Fernando de Noronha, Brazil: Biomarkers analysis in *Amphistegina lessonii*. J. Foramin. Res., submitted.

Regoli, F., 2000. Total oxyradical scavenging capacity (TOSC) in polluted and translocated mussels: a predictive biomarker of oxidative stress. Aquat. Toxicol. 50, 351–361.

Reichelt-Brushett, A.J., Harrison, P.L., 1999. The effect of copper, zinc and cadmium on fertilization success of gametes from scleractinian reef corals. Mar. Pollut. Bull. 38, 182–187.

Richier, S., Furla, P., Plantivaux, P., Merle, P-L., Allemand, D., 2005. Symbiosis-induced adaptation to oxidative stress. J. Exp. Biol. 208, 277–285.

Santore, R.C., Mathew, R., Paquin, P.R., DiToro, D., 2002. Application of the biotic ligand model to predicting zinc toxicity to rainbowtrout, fathead minnow, and *Daphnia magna*. Comp. Biochem. Phys. C 133, 271–285.

U.S. EPA, 2009. National Recommended Water Quality Criteria. Available online at http://www.epa.gov/ost/criteria/wqctable/

Talge, H.K., Hallock, P., 1995. Cytological examination of symbiont loss in a benthic foraminifera, *Amphistegina gibbosa*. Mar. Micropaleontol. 26, 107–113.

Talge, H.K., Hallock, P., 2003. Ultrastructural responses in field-bleached and experimentally stressed *Amphistegina gibbosa* (Class Foraminifera). J. Eukaryot. Microbiol. 50, 324–333.

Viarengo, A., Ponzano, E., Dondero, F., Fabbri, R., 1997. A simple spectrophotometric method for metallothionein evaluation in marine organisms: an application to Mediterranean and Antarctic mollusks. Mar. Environ Res. 44, 69–84.

LEGEND TO FIGURES

Figure 1. Percentage of individuals showing visual alterations (white spots and dark-brown areas) after exposure (24 and 48 h) of the symbiont-bearing foraminifer *Amphistegina lessonii* from the Fernando de Noronha Archipelago (Northeastern Brazil) to different concentrations of dissolved Zn.

Figure 2. Levels of reactive oxygen species (ROS) generation in normal appearing (white bars) and pale/partly bleached (gray bars) individuals of the symbiont-bearing foraminifer *Amphistegina lessonii* from the Fernando de Noronha Archipelago (Northeastern Brazil) exposed (48 h) to different concentrations of dissolved Zn. Data are expressed as mean ± standard error (n = 5).Different small and capital letters indicate significantly different ($p < 0.05$) mean values among experimental groups of normal appearing and pale/partly bleached foraminifers, respectively. * indicates significantly different mean values between normal appearing and pale/partly bleached foraminifers for each Zn concentration.

Figure 3. Levels of lipid peroxidation (MDA concentration) in normal appearing (white bars) and pale/partly bleached (gray bars) individuals of the symbiont-bearing foraminifer *Amphistegina lessonii* from the Fernando de Noronha Archipelago (Northeastern Brazil) exposed (48 h) to different concentrations of dissolved Zn. Data are expressed as mean ± standard error (n = 5). Different small and capital letters indicate significantly different ($p < 0.05$) mean values among experimental groups of normal appearing and pale/partly bleached foraminifers, respectively. * indicates

significantly different mean values between normal appearing and pale/partly bleached foraminifers for each Zn concentration.

Figure 4. Levels of metalothionein-like proteins (MTLP) expressed as glutathione (GSH) concentration in normal appearing (white bars) and pale/partly bleached (gray bars) individuals of the symbiont-bearing foraminifer *Amphistegina lessonii* from the Fernando de Noronha Archipelago (Northeastern Brazil) exposed (48 h) to different concentrations of dissolved Zn. Data are expressed as mean ± standard error (n = 5). Different small letters indicate significantly different ($p < 0.05$) mean values among experimental groups of normal appearing foraminifers. * indicates significantly different mean values between normal appearing and pale/partly bleached foraminifers exposed to 68 μg dissolved Zn/l.

Figure 5. Total superoxide dismutase (SOD) activity in normal appearing (white bars) and pale/partly bleached (gray bars) individuals of the symbiont-bearing foraminifer *Amphistegina lessonii* from the Fernando de Noronha Archipelago (Northeastern Brazil) exposed (48 h) to different concentrations of dissolved Zn. Data are expressed as mean ± standard error (n = 5). Different small and capital letters indicate significantly different ($p < 0.05$) mean values among experimental groups of normal appearing and pale/partly bleached foraminifers, respectively. * indicates significantly different mean values between normal appearing and pale/partly bleached foraminifers for each Zn concentration.

Figure 1

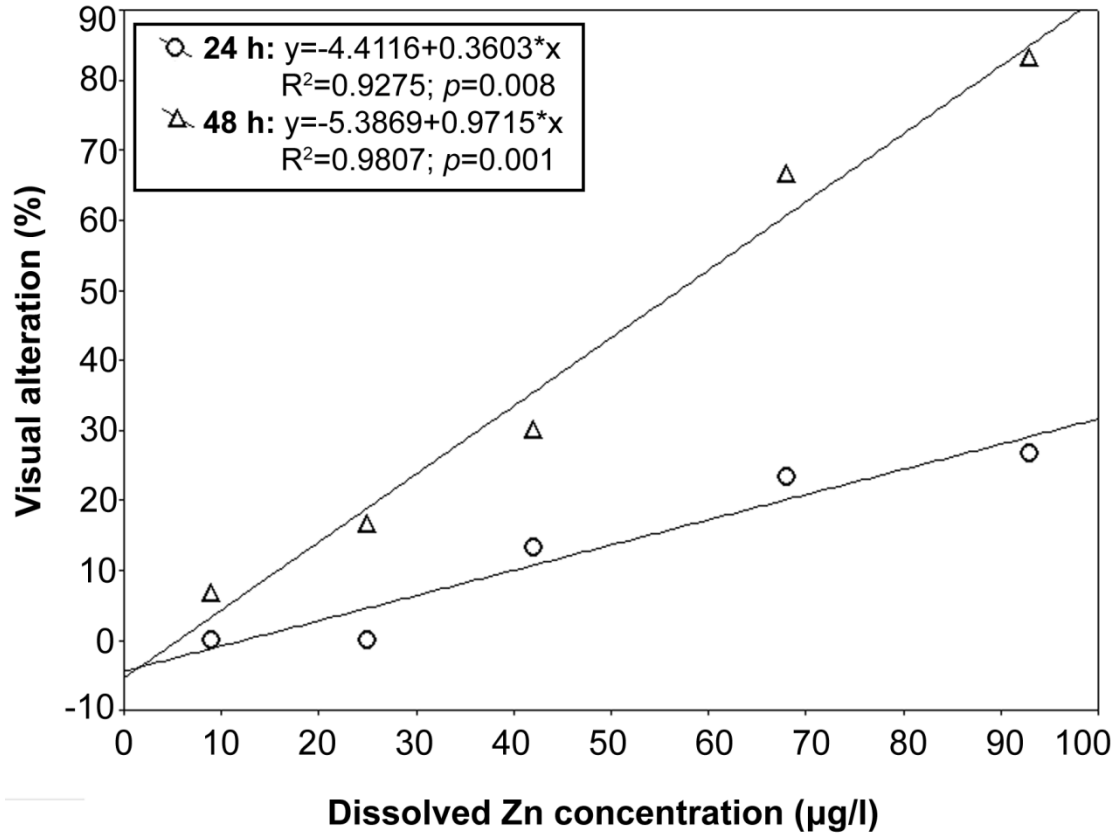

24 h: y=-4.4116+0.3603*x
R²=0.9275; p=0.008
48 h: y=-5.3869+0.9715*x
R²=0.9807; p=0.001
Visual alteration (%)
Dissolved Zn concentration (µg/l)
90
80
70
60
50
40
30
20
10
0
-10
0
10
20
30
40
50
60
70
80
90
100

Figure 2

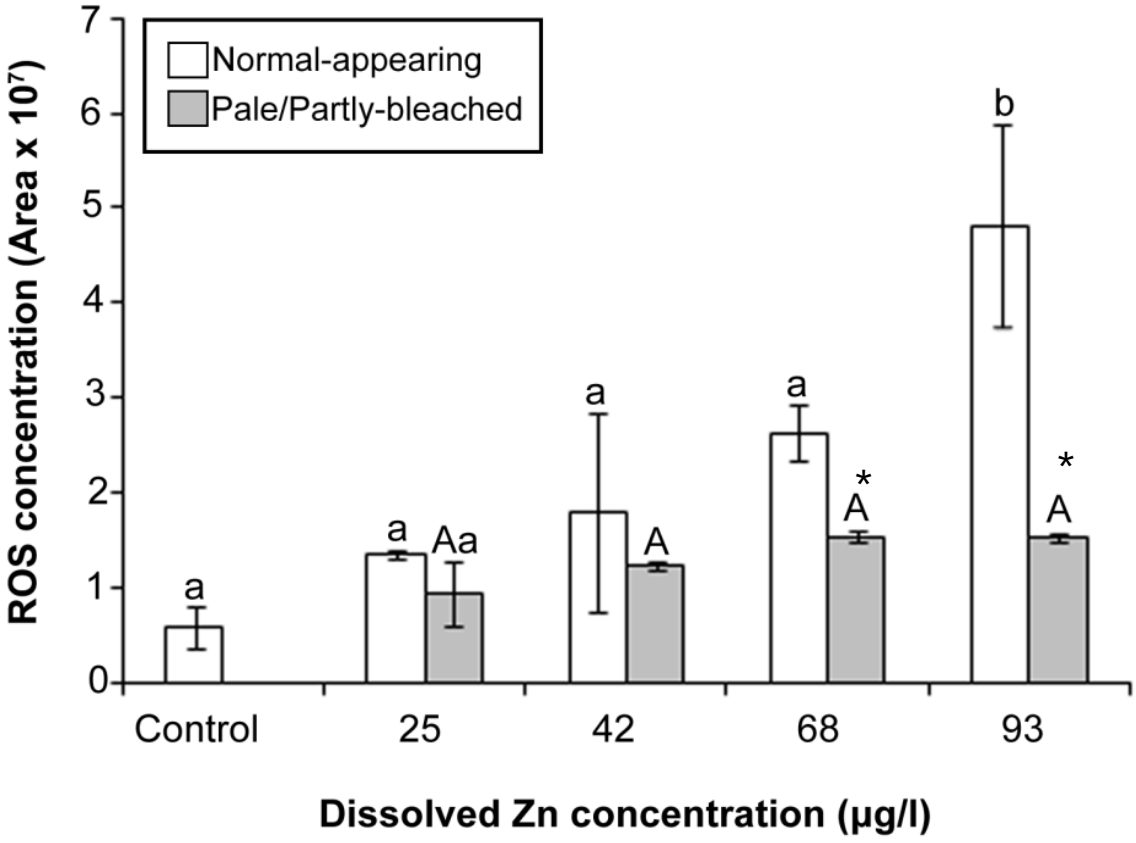

Normal-appearing
Pale/Partly-bleached
ROS concentration (Area x 10⁷)
0
1
2
3
4
5
6
7
a
a
Aa
a
A
a
*
A
b
*
A
Control
25
42
68
93
Dissolved Zn concentration (µg/l)

Figure 3

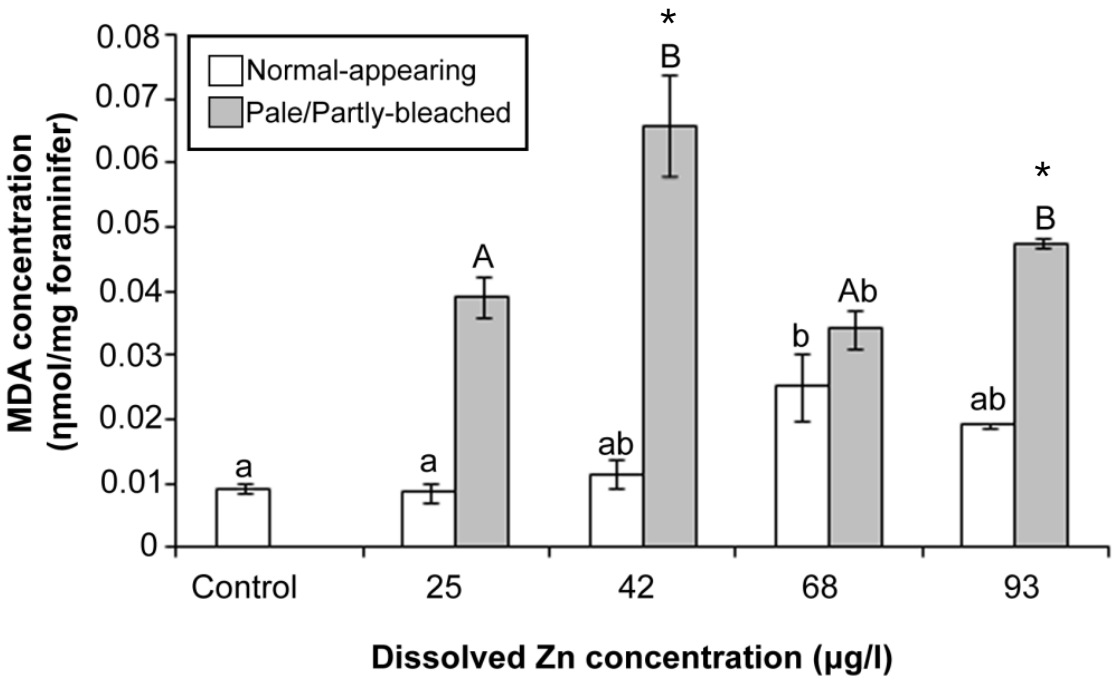

MDA concentration (ηmol/mg foraminifer)
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
Normal-appearing
Pale/Partly-bleached
a
a
A
ab
*
B
b
Ab
ab
*
B
Control
25
42
68
93
Dissolved Zn concentration (µg/l)

Figure 4

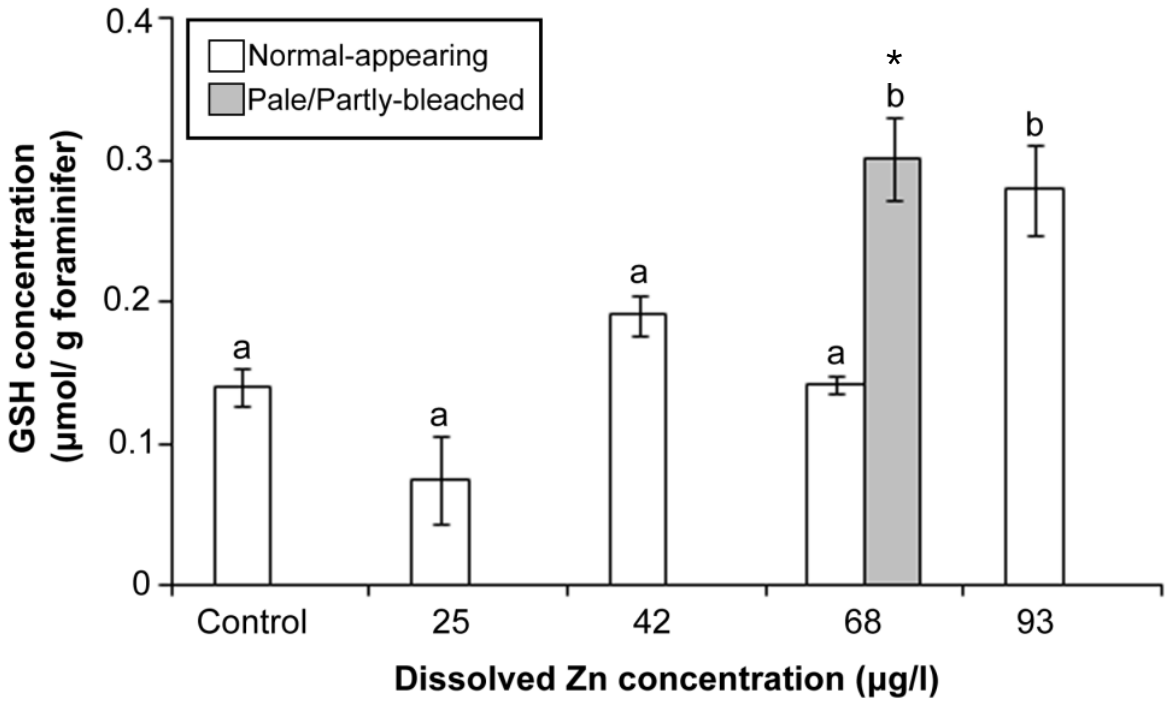
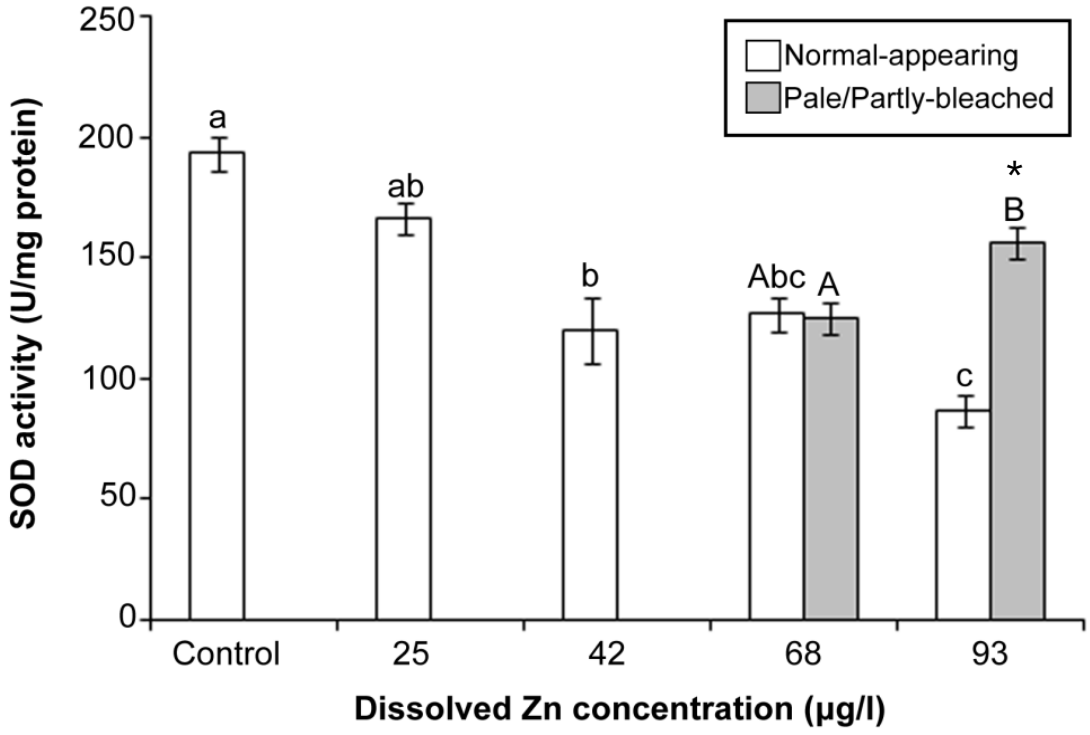

Normal-appearing
Pale/Partly-bleached
GSH concentration (µmol/ g foraminifer)
0.4
0.3
0.2
0.1
0
a
a
a
*
b
b
Control
25
42
68
93
Dissolved Zn concentration (µg/l)

Figure 5


Normal-appearing
Pale/Partly-bleached
SOD activity (U/mg protein)
250
200
150
100
50
0
a
ab
b
Abc
A
c
*
B
Control
25
42
68
93
Dissolved Zn concentration (µg/l)