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RESPOSTA DE BIOMARCADORES EM *AMPHISTEGINA* spp. (AMPHISTEGINIDAE, FORAMINIFERA) EXPOSTOS AO COBRE E ACIDIFICAÇÃO MARINHA

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RIO GRANDE

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Este trabalho é dedicado à memória do meu querido pai, Nelson Marques.

E ao meu querido primo, Klismann, que veio ao mundo a passeio para a todos conquistar.

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Sumário

RESUMO

A contaminação por metais é uma ameaça à qualidade dos recifes de coral. Além disso, impactos globais como a acidificação marinha (AM) podem prejudicar a calcificação em vários organismos que ocorrem nesses ecossistemas, como corais e foraminíferos. Neste contexto, os foraminíferos do gênero *Amphistegina* vem sendo utilizados como bioindicadores da saúde de ambientes recifais. O objetivo do presente estudo foi avaliar o efeito da exposição ao cobre (Cu) e à AM sobre a atividade de enzimas envolvidas na calcificação em foraminíferos e o branqueamento em *Amphistegina* spp. Indivíduos do gênero *Amphistegina* foram coletados no Parque Municipal Marinho do Recife de Fora (Porto Seguro, BA), aclimatados no mesocosmo marinho do Projeto Coral Vivo (Arraial d'Ajuda, Porto Seguro, BA) e depois mantidos sob condição controle (1,0 µg/L Cu dissolvido) ou expostos (10 e 25 dias) a concentrações ambientalmente relevantes de Cu dissolvido (1,6; 2,3 e 3,2 µg/L), combinadas com diferentes níveis de pH da água do mar (8,1; 7,8; 7,5 e 7,2). Após exposição, foram avaliadas as atividades da Ca^{2+}-ATPase e Mg^{2+}-ATPase, bem como a frequência de branqueamento. A exposição combinada ao Cu e AM por 10 dias alterou a atividade da Ca^{2+}-ATPase. Por sua vez, a atividade da Mg^{2+}-ATPase foi reduzida em foraminíferos expostos ao Cu, sendo que essa inibição aumentou com o incremento da AM. A frequência de branqueamento foi maior no pH mais baixo, com um evidente efeito interativo do Cu. Os efeitos interativos da AM e da exposição ao Cu podem ser explicados pela maior disponibilidade de íons livres de Cu em condições mais ácidas, aumentando assim a competição destes íons com o Ca^{2+} e o Mg^{2+} pelos sítios de ligação no organismo, alterando a atividade da Ca^{2+}- e Mg^{2+}-ATPase. Por ter sido realizado em um sistema de mesocosmo, o presente estudo incorpora a complexidade ecológica do ambiente, e fornece resultados realistas do ponto de vista fisiológico e ecotoxicológico. Os resultados indicam que a calcificação e fotossíntese em *Amphistegina* spp. são influenciadas pela exposição ao Cu e a AM.Portanto, sugerem a utilização de foraminíferos como bioindicadores e dos biomarcadores analisados no presente estudo são importantes ferramentas para detectar e monitorar os impactos ecológicos da contaminação da água do mar com Cu, especialmente em um cenário de AM.

Palavras-chave: acidificação dos oceanos; cobre; biomarcadores; foraminíferos; mudanças climáticas globais; recifes de coral.

ABSTRACT

Coral reefs can be threatened by exposure to copper (Cu) and ocean acidification. *Amphistegina* spp. is the most common symbiont-bearing foraminifer in Brazilian reefs. In the present study, specimens of *Amphistegina* spp. were kept in a marine mesocosm under control condition (1.0 µg/L Cu) or exposed to environmentally relevant concentrations of Cu (1.6; 2.3 and 3.2 µg/L) combined with different levels of seawater pH (8.1, 7.8, 7.5, and 7.2). After exposure (10 and 25 days), foraminifers were evaluated to assess the response of biomarkers related to calcification (Ca^{2+}-ATPase and Mg^{2+}-ATPase activity) and visible bleaching. The combination of Cu exposure and seawater acidification inhibited Ca^{2+}-ATPase activity at more extreme values; at lower Cu concentrations and higher pH, responses were more varied. Mg^{2+}-ATPase activity increased at pH 7.8 compared to the pH 8.1 treatment except in the highest Cu exposure; treatments at pH of 7.2 and 7.5 showed enzyme inhibition that was magnified by increasing Cu exposure. After 25 days of exposure, enzyme activities were recovered to the initial levels. Incidences of bleaching were higher at the lowest pH treatment, with the evidence of an additive effect of Cu. The effects of sea water acidification could be explained considering a higher availability of free Cu ions at lowering water pH. This condition would increase the Cu competition with Ca^{2+} and/or Mg^{2+} for the binding sites at the organism, thus inhibiting Ca^{2+}- and Mg^{2+}-ATPase activities. Our results were generated in a mesocosm system, which incorporated ecological complexity to provide more ecologically relevant data. In summary, both calcification and photosynthesis in *Amphistegina* spp. could be affected by Cu and ocean acidification exposure. Also, they support of foraminifers as bioindicators and biomarkers related to calcification as tools to detect and monitor the possible ecological impacts of sea water contamination with Cu, especially in a scenario of ocean acidification.

Keywords: copper; climate change; ocean acidification; biomarker; bioindicator; foraminifer; coral reefs; marine mesocosm.

1. INTRODUÇÃO

Os recifes de coral estão entre os ecossistemas mais ricos e biologicamente diversos do mundo, além de serem fundamentais para a subsistência de milhões de pessoas (Costanza et al., 1997; Veron et al., 2009). Apesar disso, a saúde desses ecossistemas tem sido afetada nas últimas décadas. Dentre as causas das alterações observadas estão os impactos locais, como eutrofização e poluição química, bem como os impactos globais, como aumento da temperatura e acidificação dos oceanos (Hallock et al., 2004). No Brasil, estima-se que aproximadamente 50% dos recifes estejam ameaçados pela ação combinada dos impactos locais e das mudanças climáticas (Rodríguez-Ramírez et al., 2008).

Dentre os impactos locais que ameaçam os recifes de coral, destaca-se a contaminação por metais (van Dam et al., 2011). Muitos metais, como o cobre (Cu), são essenciais para o funcionamento de vários processos celulares, porém são tóxicos em altas concentrações. O Cu é um poluente comum no ambiente marinho, sendo que suas principais fontes são a descarga de esgoto doméstico, efluentes industriais e tintas anti-incrustantes (Turner, 2010).

Dentre os impactos globais com maior potencial de ameaça aos recifes de coral, destaca-se a acidificação marinha (AM). Esse processo é decorrente, principalmente, do aumento na concentração de dióxido de carbono (CO_2) atmosférico (Kleypas et al., 2006). Uma vez que o oceano absorve cerca de ¼ do dióxido de carbono (CO_2) atmosférico, o aumento na concentração atmosférica desse gás leva a uma maior absorção pelos oceanos (equação 1) e ao favorecimento da produção de ácido carbônico (H_2CO_3), com consequente redução do pH e da disponibilidade de íons carbonato (CO_3^{2-}) nos oceanos (equação 2).

(1) $CO_2(atm) \leftrightarrow CO_2(aq)$.

(2) $CO_2(aq) + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^{2-}$.

Desde a era pré-industrial houve um aumento de aproximadamente 38% das concentrações atmosféricas de CO_2 associado às atividades humanas, e um consequente decréscimo de 0,1 unidade de pH da água do mar (IPCC, 2007). Para o ano de 2100, as previsões alertam para um decréscimo de aproximadamente 0,4 unidades de pH da água do mar, o que pode afetar significativamente muitas formas de vida. Os organismos calcificadores constituem um dos grupos de organismos que potencialmente serão os mais afetados por esse processo. A acidificação dos oceanos, ao alterar a disponibilidade de íons carbonato (CO_3^{2-}), pode afetar a produção do carbonato de cálcio ($CaCO_3$) utilizado para a construção de conchas e esqueletos, além de aumentar as taxas de dissolução dessas estruturas (Andersson e Gledhill, 2013).

Experimentos que simulam os cenários de pH previstos para o próximo século relatam uma redução da calcificação em corais (Movilla et al., 2012), cocolitoforídeos (Delille et al., 2005), moluscos (Duarte et al., 2014) e foraminíferos (Fujita et al., 2011; McIntyre-Wressnig et al., 2011; Keul et al., 2013; Khanna et al., 2013; Reymond et al., 2013). As consequências incluem impactos negativos não só nos táxons diretamente afetados e na teia trófica associada a eles, mas em todos os organismos que dependem do habitat formado a partir dos esqueletos de organismos calcificadores.

Os foraminíferos são protistas abundantes no ambiente marinho e caracterizados pela presença de pseudópodes granuloreticulares e uma testa (concha), que pode ser formada por material orgânico, aglutinado, ou calcário. Esse grupo conta com aproximadamente 5.000 espécies "modernas" e 40.000 espécies fósseis, sendo amplamente utilizado como indicadores paleoceanográficos. Os foraminíferos são um importante componente da meiofauna bentônica, e também possuem representantes planctônicos (Pawlowski, 2012). Esses organismos tem um papel importante na

reciclagem de carbono orgânico e na produção mundial de carbonato de cálcio. Além disso, eles vem sendo amplamente utilizados como bioindicadores de poluição estuarina e marinha (Hallock et al., 2003; Barbosa et al., 2009; Martinez-Colón et al., 2009).

O grupo conhecido como *Larger Benthic Foraminifers* (LBF) é um dos principais produtores de sedimento carbonático no ambiente recifal. Estes foraminíferos compartilham características-chave com os corais escleractínios zooxantelados. Por exemplo, são importantes produtores de carbonato de cálcio, dependem fisiologicamente da endossimbiose com microalgas e sofrem eventos de branqueamento devido a diversos tipos de impactos ambientais (Hallock et al., 2006). Este grupo evoluiu de forma a se adaptar a ambientes oligotróficos, o que favoreceu a simbiose com uma diversidade de microalgas (Lee, 2006), bem como estratégias que beneficiaram a reprodução assexual e maturação tardia (Hallock, 1985), o que propiciou assim atingir maiores tamanhos. *Amphistegina* (d'Orbigny, 1826) é o gênero de LBF com diatomáceas endossimbiontes que mais ocorre nos recifes e plataformas carbonáticas tropicais (Langer e Hottinger, 2000). Além disso, possuem ciclo de vida curto (3 a 12 meses), respondem rapidamente a mudanças ambientais e são de fácil coleta e manipulação. Por isso, o monitoramento de populações de *Amphistegina* spp. (Figura 1) se tornou uma ferramenta eficiente e de baixo custo para avaliação da qualidade ambiental em recifes de coral (Hallock, 2006; Prazeres et al., 2012a; Ross e Hallock, 2014). Além disso, várias espécies pertencentes ao grupo dos LBF se mostraram sensíveis aos efeitos das mudanças climáticas (Kuroyanagi et al., 2009; Uthicke et al., 2012; van Dam et al., 2012), e são consideradas verdadeiras sinalizadoras de mudanças climáticas globais (Hallock, 2000).

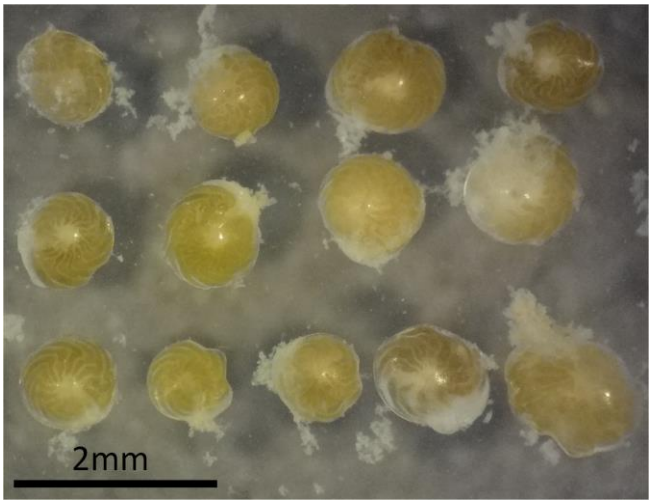

Figura 1- Fotomicrografia de foraminíferos do gênero *Amphistegina*. Fonte: MSc. Douglas Abrantes (Coral Vivo)

Uma vez que a conservação e o gerenciamento dos recifes de coral são de interesse de agências governamentais, de cientistas de diversas áreas e, principalmente, das populações que dependem direta ou indiretamente desses ambientes, é de extrema importância estabelecer estratégias de monitoramento e identificar bioindicadores confiáveis da qualidade ambiental desse ecossistema. Considerando que os LBF são reconhecidamente indicadores da qualidade da água necessária para o suporte do ecossistema recifal (Cooper et al., 2009), seu uso como modelo biológico/bioindicador é apropriado e necessário.

Os efeitos de distúrbios ambientais em uma espécie bioindicadora podem ser detectados em vários níveis da organização biológica, desde compartimentos celulares até indivíduos, populações e assembleias (Walker et al., 1997). Do ponto de vista ecológico, as respostas mais informativas quanto à presença de contaminantes ou outras alterações ambientais, se manifestam na estrutura e função dos ecossistemas (Kelly e Harwell, 1989). Entretanto, a determinação de tais respostas envolve alto custo e demanda muito tempo, além que estas são de difícil interpretação, Assim, quando uma alteração significativa é evidenciada, o ecossistema pode já estar severamente

comprometido (Zagatto e Bertoletti, 2006). Uma vez que toda resposta biológica se manifesta primariamente em nível bioquímico/celular, a resposta de biomarcadores bioquímicos apresentam a vantagem de servir como avisos prévios da degradação ambiental (Depledge et al., 1995; Downs et al., 2005).

ATPases, como a Ca^{2+}-, Mg^{2+}- e $Na^{+}K^{+}$-ATPase, constituem um grupo de enzimas responsáveis pelo transporte ativo de íons através da membrana biológica e que tem sido consideradas como biomarcadores de toxicidade. Em moluscos bivalves, a atividade de ATPases pode ser inibida por vários metais como cromo (Cr), prata (Ag) e Cu (Burlando et al., 2004; Vijayavel et al., 2007; Jorge et al., 2013). A Ca^{2+}-ATPase é a principal enzima responsável pelo transporte ativo de cálcio (Ca^{2+}) e a manutenção do pH alcalino no sítio de calcificação em corais (Al-Horani et al., 2003). Modelos bioquímicos sugerem que a Ca^{2+}-ATPase também atua no transporte de Ca^{2+} e na elevação do pH no sítio de calcificação de foraminíferos (Zeebe & Sanyal, 2002; Erez, 2003; Nooijer et al., 2014). Já foi demonstrado que a atividade da (Ca^{+2}, Mg^{+2})-ATPase pode ser inibida em corais expostos ao Cu (Marangoni et al., 2014), assim como em foraminíferos coletados em regiões com concentrações relativamente altas deste metal (Prazeres et al., 2012b). Estudos sugerem que a Mg^{2+}-ATPase seja responsável por regular o conteúdo intracelular de Mg^{2+} em foraminíferos, sendo essa uma etapa essencial no processo de calcificação nesses protistas (Bentov e Erez, 2006). Desta forma, fica claro que apesar de ser imprescindível para a manutenção da homeostasia iônica em organismos calcificadores, como corais e foraminíferos, a atividade da Ca^{2+} e da Mg^{2+}-ATPase pode ser inibida pela exposição a metais, incluindo o Cu, o que as torna potenciais biomarcadores para detecção de impactos ambientais causados pela contaminação de recifes de coral com esses elementos químicos.

A maioria dos estudos que envolvem contaminação por metais em foraminíferos, avaliam os efeitos destes processos de forma isolada (Le Cadre & Debenay, 2006; Kuroyanagi et al., 2009). Sabe-se que muitos metais bivalentes, como o Cu, tendem a se tornar mais tóxicos em condições mais ácidas (Nikinmaa, 2013). Além disso, os efeitos deletérios da exposição crônica a contaminantes químicos pode aumentar a vulnerabilidade dos organismos aos efeitos das mudanças climáticas (Negri et al., 2011; van Dam et al., 2012). Sendo assim, um cenário de contaminação aquática por metais combinado à AM deve ser melhor compreendida. Cabe ressaltar que até o momento não existem estudos na literatura que relatem os efeitos bioquímicos e fisiológicos da interação desses fatores em organismos recifais.

Com base no exposto acima, o conhecimento dos efeitos bioquímicos e fisiológicos da exposição ao Cu e à AM, de forma isolada e combinada, bem como a identificação de biomarcadores de impactos locais e globais em foraminíferos, é de fundamental importância para avaliação e monitoramento da qualidade ambiental em recifes de coral.

2. OBJETIVOS

2.1 Objetivo Geral

Avaliar a resposta de biomarcadores bioquímicos e fisiológicos em foraminíferos do gênero *Amphistegina* frente a cenários de acidificação dos oceanos e contaminação aquática por cobre e.

2.2 Objetivos Específicos

- avaliar as atividades da Ca^{2+}-ATPase e da Mg^{2+}-ATPase corporal em foraminíferos *Amphistegina* spp. expostos a concentrações ambientalmente relevantes de cobre por 10 e 25 dias em um mesocosmo marinho;

- avaliar as atividades da Ca^{2+}-ATPase e da Mg^{2+}-ATPase corporal em foraminíferos *Amphistegina* spp. expostos a diferentes níveis de acidificação da água do mar por 10 e 25 dias em um mesocosmo marinho;

- determinar as atividades da Ca^{2+}-ATPase e da Mg^{2+}-ATPase corporal em foraminíferos *Amphistegina* spp. expostos a diferentes combinações de concentrações de cobre e níveis de acidificação da água do mar por 10 e 25 dias em um mesocosmo marinho;

- determinar a frequência de branqueamento e mortalidade nos foraminíferos expostos às condições experimentais descritas acima.

3. MATERIAL E MÉTODOS

3.1 Abordagem experimental

3.1.1 Mesocosmo marinho

O experimento de variação de pH e exposição ao cobre foi realizado no mesocosmo marinho do Projeto Coral Vivo (Arraial d'Ajuda, BA), utilizando-se aquários adaptados para ensaios ecotoxicológicos, como descrito por Marangoni et al. (2014). Resumidamente, o Mesocosmo Marinho do Projeto Coral Vivo é um sistema experimental aberto que troca água permanentemente com uma franja recifal (Recife do Araçaípe, Arraial d'Ajuda, BA) localizada a 500 m da base onde o sistema encontra-se instalado. A água captada é bombeada para cisternas subterrâneas onde recebem tratamentos para redução de pH através de injeção de CO_2 (Figura 2). Soluções estoque de Cu, preparadas em reservatórios de 1.000 L, foram diluídas 10 vezes com água bombeada das cisternas. A água com tratamento de pH (AM) e/ou contaminação por Cu chega aos aquários através de bombas peristálticas. O volume total de água no aquário é

renovado 3 vezes/h (150 ml/min), sendo que o descarte da água de rejeito é feito após passagem por filtros de carvão ativado.

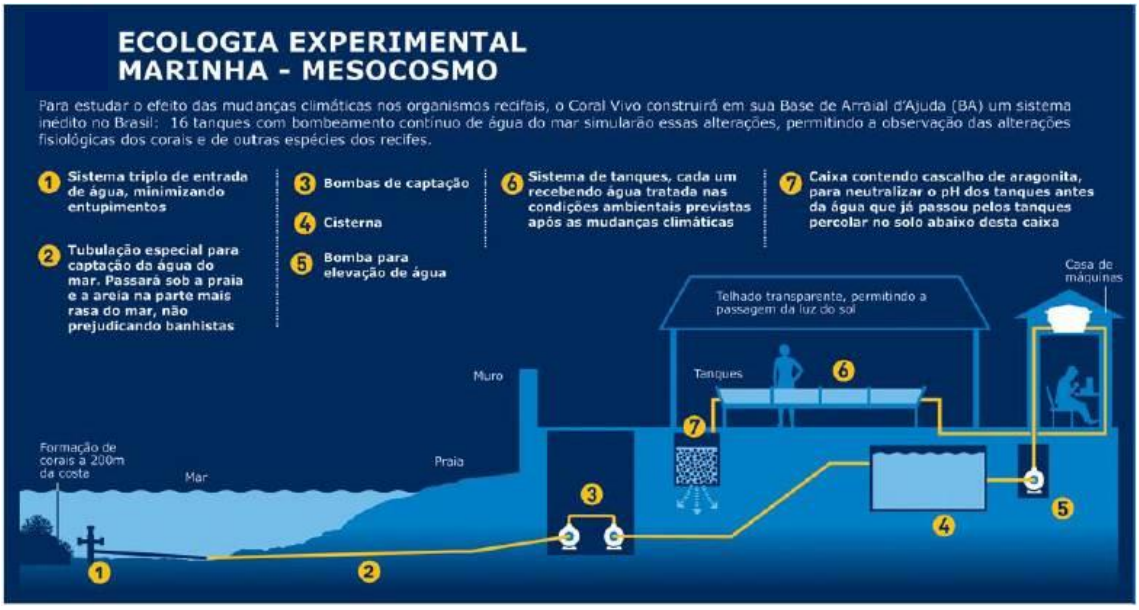

Figura 2- Esquema geral de funcionamento do mesocosmo marinho antes da implantação da estrutura para ensaios ecotoxicológicos (aquários e caixas d'água para preparação de meio contaminado por cobre). Fonte: Projeto Coral Vivo.

Os aquários permitem testar 4 condições de pH da água do mar e 4 concentrações de cobre, totalizando 16 combinações de tratamentos realizados em triplicata. As condições e variações diárias naturais do ambiente (temperatura, turbidez, salinidade, radiação, taxa de nutrientes, fitoplâncton e zooplancton) são mantidas pelo sistema, sendo que a iluminação natural é atenuada pelo uso de Sombrite 70%, visando simular a irradiação máxima equivalente a 2,5 m de profundidade no Recife de Fora (Porto Seguro, BA).

3.1.2 Exposição ao cobre (Cu)

As soluções estoque de Cu eram preparadas diariamente nos reservatórios com 1.000 L, a partir de uma solução padrão de $CuCl_2$ (1 g/L Cu). Os reservatórios recebiam água do mar bombeada do recife adjacente e as soluções eram preparadas 24 h antes do

seu uso para permitir o equilíbrio e especiação do metal na água do mar. Para obter um gradiente de concentração de Cu, foram adicionados 10, 30 e 50 ml da solução padrão de Cu em diferentes reservatórios, com o intuito de obter nos aquários, após diluição com água bombeada das cisternas, as concentrações nominais de 1, 3 e 5 µg/L Cu acima da concentração encontrada naturalmente no local de captação de água (~1,04 µg/L Cu), respectivamente.

3.1.3 Exposição à acidificação da água do mar (AM)

Reatores de gás carbônico (CO_2), dispostos em três das quatro cisternas subterrâneas, acidificavam a água bombeada do recife. A medida do pH da água do mar e o seu consequente ajuste para obter os tratamentos experimentais desejados, foram realizados continuamente. Um sistema computadorizado (ReefAngel), acoplado a sensores de pH, auxiliava no registro e no controle dos tratamentos. As demais variáveis abióticas (salinidade, temperatura e incidência luminosa) eram medidas diariamente. Os tratamentos adotados no experimento incluíram o cenário atual (pH ~8.16), bem como aqueles previstos por Caldeira e Whicket (2005), com valores de 0,3 [correspondente à previsão SRES B1 (IPCC, 2007)], 0,6 [SRES A2 (IPCC, 2007)] e 0,9 [cenário com emissões atmosféricas > 5000 pg C até 2300] unidades de pH abaixo do pH atual da água do mar no local do estudo (pH ~8.16).

3.2 Coleta de material biológico

Fragmentos de esqueleto de coral foram coletados no Parque Municipal Marinho do Recife de Fora (Porto Seguro, BA), por meio de mergulho autônomo. Os fragmentos foram armazenados em sacos plásticos do tipo Ziploc com água do local e mantidos à sombra até a chegada à base do Projeto Coral Vivo (Arraial d'Ajuda, Porto Seguro, BA), onde foram escovados em baldes com água do mar para dissociar sedimento, algas

e meiofauna. Após decantação do material em suspensão, a água foi descartada e o sedimento residual distribuído em placas de Petri de 150 mm contendo água do mar.

Indivíduos adultos (>0,6 mm) e aparentemente saudáveis (sem sinais visuais de branqueamento ou parasitismo) do gênero *Amphistegina* foram triados com auxílio de microscópio estereoscópico e acondicionados em placas de Petri de 80 mm contendo água do mesocosmo não tratada (controle). Amostras (n = 4; 7 indivíduos em cada amostra) de *Amphistegina* foram coletadas e congeladas em nitrogênio líquido para posterior análise de biomarcadores, representando assim as condições dos indivíduos no ambiente.

3.3 Manutenção, aclimatação dos organismos e coletas durante o experimento

Em cada aquário, foi colocada uma placa de Petri de 80 mm contendo cerca de 30 indivíduos de *Amphistegina* spp. As placas foram cobertas com uma tela de meiofauna (malha de 63 µm), para permitir a troca de água e prevenir a fuga dor organismos, e receberam uma camada adicional de sombrite, para atenuar os níveis de irradiação (Vogel & Uthicke, 2012).

Após 12 dias de aclimatação, foram coletadas amostras (n = 6; 7 indivíduos em cada amostra) de *Amphistegina*, as quais foram congeladas em nitrogênio líquido para posterior análise dos biomarcadores, representando assim as condições dos indivíduos após o período de aclimatação. Após 10 e 25 dias de experimento, a porcentagem de indivíduos mortos ou com alterações visuais foi verificada, e um *pool* de 7 indivíduos foi coletado de cada aquário. As amostras foram condicionadas em tubos criogênicos (2 ml) e congeladas em nitrogênio líquido para posterior análise dos biomarcadores, conforme descrito abaixo.

3.4 Biomarcadores bioquímicos (atividades enzimáticas)

As amostras foram homogeneizadas por ultrassom em tampão de sacarose e centrifugadas (10.000 g; 4°C; 20 min). O sobrenadante foi coletado e imediatamente utilizado para as análises de biomarcadores. As atividades da Ca^{2+}-ATPase e da Mg^{2+}-ATPase foram determinadas pelo método da liberação do fosfato inorgânico (Pi), como descrito por Vijayavel et al. (2007), com algumas modificações. Brevemente, para estimar a atividade da Ca^{2+}-ATPase , 7 µl do homogeneizado foi incubado em meio de reação contendo Tris-HCl (20 mM), NaCl (189 mM), $MgCL_2$ (5 mM), $CaCl_2$ (5 mM), ATP (3 mM) e ouabaína (1 mM). Para a Mg^{2+}-ATPase, 7 µl do homogeneizado foi incubado em meio de reação contendo imidazol (50 mM), NaCl (189 mM), $MgCl_2$ (5 mM), EGTA (0,2 mM), ATP (3 mM) e ouabaína (1 mM). A quantidade de Pi liberado pela enzima analisada foi estimada pelo método colorimétrico descrito por Fiske e Subarrow (1925), utilizando o kit comercial Fosfato (Doles, Goiânia, GO). A detecção colorimétrica foi feita em leitora de microplacas (ELX 808, Biotek, Vermont, EUA) a 630 nm. O conteúdo de proteínas no homogeneizado foi determinado utilizando-se o kit fluorimétrico *Quant-it Protein Assay* (Invitrogen, USA). Os resultados foram expressos em mM Pi/mg proteína/min.

3.5 Biomarcadores fisiológicos (branqueamento e mortalidade)

Em cada tempo experimental (10 e 25 dias), os organismos foram avaliados quanto a ocorrência de branqueamento visível, de acordo com Hallock et al. (2006). A mortalidade foi determinada pela ausência de atividade dos pseudópodes ou presença de testa completamente branca. A porcentagem de alterações visuais (branqueamento e/ou presença de manchas marrons) e mortalidade foram obtidas pela divisão do número de foraminíferos afetados pelo número total de foraminíferos testados na respectiva placa.

3.6 Análise da concentração de cobre (Cu) na água

Para análise da concentração de Cu, a cada 7 dias foram coletadas amostras da água do mar utilizada no experimento. De cada tratamento, foram coletados 10 ml de água filtrada (filtro de 0,45 µm de poro) para a determinação da concentração de Cu dissolvido. As amostras foram acondicionadas em tubos tipo Falcon de 15 ml, acidificadas ($HN0_3$ 1%) e mantidas sob refrigeração até análise. Em laboratório, as amostras foram dessalinizadas, seguindo os procedimentos descritos por Nadella et al. (2009), e a concentração de Cu dissolvido medida em espectrofotômetro de absorção atômica acoplado a forno de grafite (Perkin-Elmer, Waltham, MA, EUA).

3.7 Análises estatísticas

Os dados foram expressos como média ± erro padrão. Os dados dos biomarcadores dos foraminíferos coletados em campo foram comparados com aqueles obtidos para os foraminíferos após aclimatação no Mesocosmo Marinho utilizando-se o teste *t* de Student. Para avaliar as respostas dos biomarcadores aos tratamentos experimentais, os dados foram submetidos à análise de variância (ANOVA) fatorial de duas vias (concentração de Cu e nível de AM) para cada tempo experimental (10 e 25 dias). Para os termos onde foram encontradas diferenças significativas ($p<0,05$), as médias foram comparadas utilizando-se o teste *a posteriori* de Student-Newman-Keuls (SNK). Os dados foram previamente transformados matematicamente utilizando a função raiz quadrada para que os pressupostos da ANOVA (normalidade dos dados e homogeneidade das variâncias) pudessem ser atendidos. As análises foram realizadas na linguagem de programação R (R Development Core Team, 2014), com auxílio do pacote GAD (Sandrini-Neto e Camargo, 2014).

4. SÍNTESE DOS RESULTADOS

4.1 Concentração de cobre (Cu) na água

A concentração média (± erro padrão) de Cu dissolvido na água do mar captada pelo mesocosmo (Praia de Araçaípe, Arraial d'Ajuda, BA) foi de 1,04 ± 0,13 µg/L. As concentrações de Cu dissolvido nos meios experimentais foram de 1,65 ± 0,12, 2,32 ± 0,04 e 3,23 ± 0,01 µg/L para as concentrações nominais testadas de 1, 3 e 5 µg/L, respectivamente. Dessa forma, obteve-se um gradiente de concentração de Cu dissolvido próximo ao esperado, e com valores de concentrações médias passíveis de ocorrência frequente em ambientes costeiros.

4.2 Tratamentos de acidificação da água do mar (AM)

Os valores médios de pH da água foram de 8,19 ± 0,007; 7,84 ± 0,015; 7,50 ± 0,040 e 7,26 ± 0,016. Dessa forma, os tratamentos de AM alcançados no mesocosmo corresponderam ao pH da água do mar no local de estudo (controle; C), 0,35 unidades de pH abaixo do controle (C-0,3), 0,6 unidades de pH abaixo do controle (C-0,6) e 0,93 unidades de pH abaixo do controle (C-0,9). A variabilidade observada nos dados de pH da água nos tratamentos experimentais é, em parte, intrínseca ao sistema automático de controle utilizado, que monitora continuamente a variação natural do pH da água do mar ao longo de todo o dia, e ao longo de toda a duração do experimento. Problemas técnicos (troca de reatores de CO_2 e troca/manutenção de sensores de pH) também contribuíram para a variabilidade observada nas medições de pH da água.

4.3 Aclimatação dos organismos

Não houve diferença significativa ($p>0.05$) na atividade da Ca^{2+}-ATPase e da Mg^{2+}-ATPase corporal entre os foraminíferos de referência de campo e aqueles aclimatados

ao mesocosmo, indicando assim que o tempo e as condições de aclimatação dos organismos-teste ao mesocosmo foram adequados.

4.4 Atividade da Ca^{2+}-ATPase corporal

Aos 10 dias de experimento, foi observado um efeito significativo da interação entre a AM e a contaminação pelo Cu na atividade da Ca^{2+}-ATPase. Sem adição do metal, a AM não causou efeito significativo na atividade da Ca^{2+}-ATPase. Nos organismos expostos a 2,3 µg/L Cu, houve uma redução da atividade da Ca^{2+}-ATPase dependente do nível de AM. Nos organismos expostos a 1,6 µg/L e 3,2 µg/L Cu, houve uma tendência de aumento da atividade enzimática nos tratamentos de pH mais ácidos, ou seja, 7,2 e 7,5, respectivamente. Aos 25 dias de experimento, os resultados foram semelhantes em todos os tratamentos de pH, à exceção dos organismos expostos a 2,3 µg/L Cu.

4.5 Atividade da Mg^{2+}-ATPase corporal

Após 10 dias de experimento, a interação entre a AM e a exposição ao Cu causou um efeito significativo na atividade da Mg^{2+}-ATPase, porém este efeito foi menos marcado do que aquele observado para a atividade da Ca^{2+}-ATPase, uma vez que o padrão de resposta ao pH da água foi semelhante em todas as condições de contaminação por Cu. De forma geral, foi observada uma compensação da atividade enzimática no pH médio de 7,8 e uma inibição da atividade da enzima no pH médio mais ácido (7,2), em todas as condições de exposição ao Cu. Aos 25 dias de experimento, os organismos se recuperaram dos efeitos causados pela AM, à exceção daqueles expostos a 1,6 µg/L Cu, os quais continuaram a manter uma situação de inibição da atividade da Mg^{2+}-ATPase na condição mais ácida (pH médio de 7,2).

Ao avaliar os dados agrupados por tratamento de pH da água, observa-se que não houve efeito significativo da exposição ao Cu nos organismos mantidos na água do mar com pH normal (controle). Porém, na água do mar com pH médio de 7,5 e 7,8, os foraminíferos expostos ao Cu mantiveram uma atividade enzimática menor que aqueles mantidos sob condição controle, com uma recuperação dos níveis iniciais após 25 dias de experimento, evidenciando assim um efeito interativo da exposição ao Cu e a AM.

4.6 Branqueamento e mortalidade

Aos 25 dias de experimento, foi observada uma alteração significativa na frequência de branqueamento em função da AM. Na ausência de adição de Cu na água do mar, não houve efeito da exposição à AM. No entanto, os organismos expostos às concentrações de Cu testadas apresentaram uma maior frequência de branqueamento quando mantidos na água com pH 7,2 por 25 dias, evidenciando assim um marcado efeito interativo da exposição ao Cu. Por sua vez, a mortalidade apresentou uma tendência de maiores valores entre os organismos mantidos no pH mais baixo, mas foi sempre inferior a 10% em todos os tratamentos experimentais.

5. CONCLUSÕES

- A exposição às concentrações de cobre testadas não afetou a atividade das enzimas envolvidas no processo de calcificação (Ca^{2+}-ATPase e da Mg^{2+}-ATPase), a frequência de branqueamento e a mortalidade dos organismos mantidos em água do mar com pH natural (controle).
- A curto prazo (10 dias), a atividade da Ca^{2+}-ATPase foi alterada pela interação entre a exposição ao cobre e à acidificação da água do mar, enquanto a atividade da Mg^{2+}-ATPase foi sensível à acidificação da água do mar e a um efeito interativo da exposição ao cobre.

- A longo prazo (25 dias), a interação da exposição ao cobre e à acidificação marinha aumentou a frequência de branqueamento, revelando ser este um biomarcador com resposta mais tardia aos estressores analisados, quando comparada àquela observada para os biomarcadores bioquímicos analisados.
- A maior toxicidade do cobre em condições ácidas afeta a atividade da Ca^{2+}-ATPase e da Mg^{2+}-ATPase, e consequentemente pode causar distúrbios ionoregulatórios, bem como distúrbios fisiológicos decorrentes do processo de branqueamento.
- Apesar da aparente recuperação da atividade das enzimas analisadas, a exposição ao cobre e à acidificação marinha, de forma combinada, tem um grande potencial de prejudicar a calcificação desses organismos, assim como aumentar a susceptibilidade dos foraminíferos ao branqueamento.
- O uso de foraminíferos do gênero *Amphistegina* como indicadores de distúrbios envolvendo cenários de acidificação dos oceanos e contaminação por metais em ambientes recifais se mostra apropriado.
- Os biomarcadores bioquímicos e fisiológicos utilizados se mostraram importantes ferramentas para monitorar os efeitos biológicos a curto e médio prazo da acidificação dos oceanos e da contaminação ambiental por metais.

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7. ANEXO – Manuscrito submetido ao periódico *Global Change Biology*

Combined effect of copper exposure and ocean acidification on the responses of biomarkers in the symbiont-bearing foraminifer *Amphistegina* spp. (Amphisteginidae, Foraminifera)

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Combined effect of copper exposure and ocean acidification on the responses of biomarkers in the symbiont-bearing foraminifer *Amphistegina* spp. (Amphisteginidae, Foraminifera)

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Keywords: copper; climate change; ocean acidification; biomarker; bioindicator; foraminifer; coral reefs; marine mesocosm.

Type of paper: Original Research – Primary Research Article

Abstract

Coral reefs can be threatened by exposure to copper (Cu) and ocean acidification. *Amphistegina* spp. is the most common symbiont-bearing foraminifer in Brazilian reefs. In the present study, specimens of *Amphistegina* spp. were kept in a marine mesocosm under control condition (1.0 µg/L Cu) or exposed to environmentally relevant concentrations of Cu (1.6; 2.3 and 3.2 µg/L) combined with different levels of seawater pH (8.1, 7.8, 7.5, and 7.2). After exposure (10 and 25 days), foraminifers were evaluated to assess the response of biomarkers related to calcification (Ca^{2+}-ATPase and Mg^{2+}-ATPase activity) and visible bleaching. The combination of Cu exposure and seawater acidification inhibited Ca^{2+}-ATPase activity at more extreme values; at lower Cu concentrations and higher pH, responses were more varied. Mg^{2+}-ATPase activity increased at pH 7.8 compared to the pH 8.1 treatment except in the highest Cu exposure; treatments at pH of 7.2 and 7.5 showed enzyme inhibition that was magnified by increasing Cu exposure. After 25 days of exposure, enzyme activities were recovered to the initial levels. Incidences of bleaching were higher at the lowest pH treatment, with the evidence of an additive effect of Cu. The effects of sea water acidification could be explained considering a higher availability of free Cu ions at lowering water pH. This condition would increase the Cu competition with Ca^{2+} and/or Mg^{2+} for the binding sites at the organism, thus inhibiting Ca^{2+}- and Mg^{2+}-ATPase activities. Our results were generated in a mesocosm system, which incorporated ecological complexity to provide more ecologically relevant data. In summary, both calcification and photosynthesis in *Amphistegina* spp. could be affected by Cu and ocean acidification exposure. Also, they support of foraminifers as bioindicators and biomarkers related to calcification as tools to detect and monitor the possible ecological impacts of sea water contamination with Cu, especially in a scenario of ocean acidification.

Keywords: copper; climate change; ocean acidification; biomarker; bioindicator; foraminifer; coral reefs; marine mesocosm.

Introduction

Coral reefs are among the most biologically diverse ecosystems in the world, and are essential to the livelihoods of millions of people (Costanza et al., 1997; Veron et al., 2009). Nevertheless, the environmental quality of these ecosystems has markedly declined in recent decades. Among the main causes of this decline, are local impacts such as eutrophication and chemical pollution, as well as global impacts such as rising sea temperature and ocean acidification (Hallock et al., 2004; Fabricius, 2005; Veron et al., 2009). In Brazil, (Rodriguez-Ramírez et al., 2008) estimated that approximately 50% of reefs are threatened by the combined action of local impacts and global climate change.

Among the local impacts threatening coral reefs, metal contamination can be an important factor (van Dam et al., 2011). Many metals, including copper, are essential to the functioning of various cellular processes but are toxic at high concentrations. Copper is a common pollutant in the marine environment and its main sources are related to the discharge of domestic sewage, industrial effluents and antifouling paints (Turner, 2010).

At the same time, ocean acidification is one of the global impacts with great potential to harm coral reefs (Kleypas et al., 2006). Because the ocean absorbs around ¼ of the atmospheric carbon dioxide (CO_2), the rise in atmospheric concentrations of this gas has increased the dissolved CO_2 concentration in the oceans causing a reduction in seawater pH and in carbonate ion (CO_3^{2-}) availability in surface waters of the oceans. Since the pre-industrial era, there has been a 38% increase in atmospheric CO_2 concentrations associated with human activities, and a consequent pH decrease of 0.1 in seawater (IPCC, 2007). Forecasts for the year 2100 predict a decrease of approximately

0.4 pH units, which can significantly affect many life forms, especially calcifying organisms (Kleypas et al., 2006). Lower pH and decreased availability of CO_3^{2-} ions can inhibit the production of calcium carbonate ($CaCO_3$) by organisms, as well as increase the dissolution rates of their skeletons (Andersson and Gladhill, 2013).

Experiments simulating some of the predicted pH scenarios for the next century have indicated a decline of calcification in corals (Movilla et al., 2012), cocolithophorids (Delille et al., 2005), mollusks (Duarte et al., 2014), and foraminifers (Fujita et al., 2011; McIntyre-Wressnig et al., 2011; Keul et al., 2013; Khanna et al., 2013; Reymond et al., 2013). Consequences are negative impacts not only to the directly affected taxa and trophic web associated with them, but in all organisms thatrely on habitats that are formed from the skeletons of calcifying organisms.

Foraminifers are ubiquitous shelled protists in the marine environment. They have an important role in the global production of calcium carbonate ($CaCO_3$) (Langer, 2008). Also, foraminifera have been widely used as bioindicators of estuarine and marine pollution (Hallock et al., 2003; Barbosa et al., 2009; Martinez-Colón et al., 2009). The *Larger Benthic Foraminifers* (LBF) includes important producers of carbonate sediment in reef environments, and are commonly used as bioindicators of coral reef health (Hallock et al., 2003; Hallock, 2012). These foraminifers share key features with symbiont-bearing scleractinian corals. For example, they are major producers of calcium carbonate, physiologically dependent on endosymbiosis with microalgae, and undergo bleaching events in response to photo-oxidative stress (Hallock et al., 2006). *Amphistegina* is a diatom-bearing LBF genus that most frequently occurs in tropical reefs and carbonate platforms (Langer and Hottinger, 2000). They have a relatively short life cycle (compared with corals), respond quickly to environmental changes and are easily collected and manipulated. Therefore, monitoring

populations of *Amphistegina* spp. can be an efficient and cost-effective tool for assessing the environmental quality in coral reefs (Hallock, 2006; Prazeres et al., 2012a; Ross and Hallock, 2014). Moreover, several LBF species are sensitive to the effects of climate change (Kuroyanagi et al., 2009; Uthicke et al., 2012; van Dam et al., 2012), and are deemed reliable biosensors of global climate change (Hallock, 2000). Considering the importance of coral reefs, it is of utmost importance to establish monitoring strategies and identify reliable bioindicators of environmental quality for these ecosystems. The LBF are recognized indicators of the water quality necessary to support the reef ecosystem (Cooper et al., 2009). Therefore, further development of techniques and procedures to more effectively use LBF species as biological models and bioindicators is appropriate and necessary.

The presence of a toxicant or an altered environmental condition (e.g., higher temperature, reduced salinity or acidification) causes cellular or biochemical alterations before leading to reduced biological function, disease, or mortality. Consequently, biochemical biomarkers have the potential of serving as early warning signs of environmental degradation (Depledge et al., 1995; Downs et al., 2005). ATPases, such as Ca^{2+}-, Mg^{2+}- and $Na^{+}K^{+}$-ATPase, are membrane-bound enzymes responsible for active transport of ions that have been considered as sensitive biomarkers of environmental disturbances. In mussels and sea urchins, activity of ATPases may be inhibited by exposure to many metals (Burlando et al., 2004; Vijayavel et al., 2007; Jorge et al., 2013; Tellis et al., 2014).

Ca^{2+}-ATPase is the primary enzyme responsible for active calcium transport and maintenance of alkaline pH in corals (Al-Horani et al., 2003). Modeling suggest that it is also involved in Ca^{2+} transport, as well as elevating the pH at the site of calcification in foraminifers (Zeebe & Sanyal, 2002; Erez, 2003; Nooijer et al., 2014). Marangoni et

al. (2014) found that (Ca^{+2}, Mg^{+2})-ATPase activity may be inhibited in corals exposed to copper, as well as in foraminifers collected in regions with relatively high concentrations of copper (Prazeres et al., 2012b). Recent studies suggest that Mg^{2+}-ATPase is responsible for regulating Mg^{2+} in foraminifers, which is an essential step for biomineralization in these protists (Bentov & Erez, 2006). Therefore, we propose that the activities of these enzymes can be suitable biomarkers for detection of environmental impacts on reef ecosystems influenced by exposure to copper.

A few studies have considered the combined effects of climate change processes and local impacts on coral reef organisms (Negri et al., 2011; Uthicke et al., 2012; van Dam et al., 2012; Reymond et al., 2013). Considering that multiple stressors condition are likely scenarios to occur until the end of the century, experiments to evaluate the response of organisms to multiple stressors in combination are essential to predict the effects of global climate change on biological systems (Wernberg et al., 2012).

The aim of our study was to evaluate the combined effect of copper contamination and ocean acidification on the response of biochemical biomarkers related to calcification in *Amphistegina* spp., a common diatom-bearing foraminifer in Brazil. We anticipate that results from our study will allow the identification of potential early warning biomarkers related to global and local environmental impacts.

Material and Methods

Experimental approach

We tested the effects of copper t at different seawater pH levels in a marine mesocosm (Coral Vivo Project). This mesocosm is an open, flow-through experimental system, that exchanges water continuously with a fringing reef (Araçaípe Reef, Arraial

d'Ajuda, BA), which is located 500 m away from the experimental base. The collected water was pumped into four underground cisterns (5000 L), where CO_2 was injected to reduce pH to achieve the desired level of seawater acidification. Measurements and subsequent adjustments in the pH of seawater in the experimental treatments were continuously performed. A computer system (ReefAngel, Freemont, CA, USA) coupled to pH sensors assisted in the registration and control of treatments. Seawater pH and other major physicochemical parameters (salinity, light incidence and temperature) were daily monitored.

Scenarios simulated in this experiment included the current pH of the seawater pumped from the reef (pH ~8.16) and three levels of acidification. Low acidification (LA), medium acidification (MA) and high acidification (HA) levels corresponded to reductions of 0.3, 0.6 and 0.9 pH units respectively, compared with the current pH of seawater pumped from the reef. These levels of acidification were selected based upon the forecasts reported by Caldeira and Wickett (2005).

Seawater contamination with copper was performed using stock solutions of copper (as $CuCl_2$) prepared daily in 1000 L reservoirs. These stock solutions were 10% diluted with seawater pumped from the cistern, for which the pH was adjusted to the desired level of acidification. Nominal copper concentrations tested were 0 (no copper addition), 1, 3 and 5 μg/L.

Using peristaltic pumps, 48 test aquaria (10 L each) were fed with seawater prepared by mixing the water from the reservoir (copper-contaminated) and cistern (acidified seawater). Total seawater in the test aquaria was renewed at a rate of 3 times/h, and used water was disposed after being treated with activated carbon filters. Test aquaria in the mesocosm received natural sunlight attenuated by shading (Sombrite 70%) to mimic the amount of incident light at the local reefs, and followed the natural

day/night cycles (Santos et al., 2014). The experiments were performed for up to 25 days.

Foraminifera collection and acclimation

Reef rubble samples were hand collected by SCUBA diving at the Recife de Fora Municipal Park (Porto Seguro, BA, northwestern Brazil) in sites between 16° 24' 31,87" S 038° 58' 37,17" W and 16° 25' 6,10" S 038° 59' 27,00" W. Collections were performed under permission of the Brazilian Environmental Agency - SISBIO (permit # 85926584).

At each site, several cobbles were placed into plastic bags, brought to the surface, and transported to the research station at Arraial d'Ajuda. Cobbles were then scrubbed using a small brush into buckets containing seawater to detach the associated algae, sediment and meiofauna. The residual material was divided into several 150-mm Petri dishes containing seawater, and kept undisturbed for 24 h in the shade. Sediments were then sorted for adult (>0.6 mm) *Amphistegina* spp. individuals showing golden-brown color and pseudopodial activity. Samples (n = 4) of foraminiferal pools were randomly collected and stored (-80^{o}C) for biomarkers analyses, as described below.

Remaining foraminifers were acclimated to the test aquaria under control condition for 12 days prior to the experiment. One 80-mm Petri dish containing thirty *Amphistegina* spp. individuals was placed at each of the 48 test aquaria of the marine mesocosm. Dishes were covered with plankton mesh-net to allow water exchange and prevent escape of the specimens. Each plate was wrapped in a layer of shade cloth to reduce the irradiance levels, as suggested by other authors working with photosymbiont-bearing foraminifera species (Schmidt et al., 2011; Vogel & Uthicke, 2012). After 12 days of acclimation period, samples (n = 6) of foraminiferal pools were

randomly collected and stored in -80^{o}C until further analysis of biomarkers, as described below.

Biomarkers analysis

After 10 and 25 days of experiment, samples (6-10 foraminifers per sample) were collected from each test aquarium, transferred to cryogenic tubes and stored at -80°C until further analysis of biomarkers, as described below. Mortality and visual alterations were verified at each sampling time using a stereomicroscope.

Foraminiferal samples were homogenized (1:20 w/v) in a buffer solution (pH 7.5) containing 500 mM sucrose, 1 mM DL dithiothreitol, 150 mM KCl, 20 mM Tris Base, and 0.1 mM phenylmethylsulfonyl and using an ultrasound sonicator (Sonaer Ultrasonics, Farmingdale, NY, USA). Homogenates were centrifuged at 10,000 x g at 4°C for 20 min. The supernatant was collected and immediately used in the assays.

Ca^{2+}-ATPase and Mg^{2+}-ATPase activities were measured based on the amount of inorganic phosphate (Pi) released following the procedures described by Vijayavel et al. (2007), with some modifications. Briefly, Ca^{2+}-ATPase activity was assayed in a reaction medium containing Tris-HCl (20 mM), NaCl (189 mM), $MgCL_2$ (5 mM), $CaCl_2$ (5 mM), ATP (3 mM) and ouabain (1 mM). For Mg^{2+}-ATPase activity, the homogenate was incubated in a reaction medium containing Tris-HCl (20 mM), NaCl (189 mM), $MgCL_2$ (5 mM), EGTA (0,2 mM), ATP (3 mM) and ouabain (1 mM). Pi concentration in the reaction medium was quantified by spectrophotometry (630 nm) using a commercial reagent kit (Fosfato, Doles, Goiânia, GO, Brazil) based on the method described by Fiske and Subarrow (1925). The amount of protein content in the sample homogenate was measured by fluorescence using a commercial reagent kit

(Quant-it Protein Assay, Invitrogen, USA). Results were expressed in mM Pi/mg protein/min.

Bleaching and mortality assessment

For each experimental time, specimens exhibiting either bleaching or mortality were counted. Bleaching was evaluated according to Hallock et al. (2006). Death criterion was the absence of pseudopodial activity or the presence of a completely white test. Percentages of visual alterations (bleaching and presence of dark brown areas) and mortality (death) were obtained by dividing the number of affected foraminifers by the total number of foraminifers examined for that treatment.

Dissolved copper concentration in seawater

For analysis of dissolved copper concentration throughout the experiment, samples of each experimental medium were collected weekly, filtered (0.45 µm-mesh filter),acidified (1% HNO_3), and kept refrigerated until analysis. In the laboratory, samples were desalted according to Nadella et al. (2009), and the dissolved copper concentration determined by graphite furnace atomic absorption spectrometry (Perkin-Elmer, Waltham, MA, USA).

Data analysis

All data were expressed as mean ± standard error. Mean data for biomarkers of foraminifers collected in the field were compared to those of foraminifers acclimated to the test aquarium using the Student's *t* test. Tests of the combined effects of copper exposure and seawater acidification were performed using factorial two-way analysis of variance (ANOVA) for data obtained at each sampling time (10 and 25 days of

exposure). Data were square-root transformed to meet ANOVA assumptions. For those terms that were found to be significant different (P <0.05), mean values were compared using the Student-Newman-Keuls (SNK) test. In addition, relationship between bleaching frequency and biochemical alterations was evaluated by linear regression. All statistical analyses were performed in the R programming language (R Development Core Team, 2014) combined with GAD (Sandrini-Neto and Camargo, 2014) package.

Results

Physicochemical parameters of seawater

Mean temperature (26 ± 0.8°C) and salinity (35.5 ± 1.25) of the seawater in the marine mesocosm were similar to those found in the adjacent reef area. Furthermore, those parameters did not change significantly during the acclimation and the experimental period. The mean pH throughout the experiment in the different treatments corresponded to 8.1 (control), 7.8, 7.5 and 7.2 (Fig. 1). Measured dissolved copper concentrations in the experimental media were 1.0 ± 0.13 (control), 1.6 ± 0.12, 2.3 ± 0.04 and 3.2 ± 0.01 µg/L Cu.

Response of biochemical biomarkers

No significant difference was observed in Ca^{2+}-ATPase and Mg^{2+}-ATPase activity between field reference and marine mesocosm acclimated foraminifers (Fig. 2).

Data on Ca^{2+}-ATPase activity and the results of statistical analyses are shown in Figure 3 and Tables 1-2, respectively. Ca^{2+}-ATPase activity was significantly influenced by combinations of copper exposure and seawater acidification after 10 and 25 days of treatment (Table 1). Without copper addition (control condition), seawater

acidification had no marked effect on Ca^{2+}-ATPase activity after 25 days, though at 10 days there were significant differences between pH treatments. In foraminifers exposed to 2.3 µg/L Cu, a pH-dependent inhibition of the Ca^{2+}-ATPase activity was observed at both sampling times (10 and 25 days). Nevertheless, after 25 days there was significant enhancement of Ca^{2+}-ATPase activity in the pH 7.8 treatment, with inhibition in lower pH treatments. In foraminifers exposed to 1.6 µg/L Cu at pH 7.2 and 3.2 µg/L Cu at pH 7.5 for 10 days, increased Ca^{2+}-ATPase activity was observed. Inhibition of Ca^{2+}-ATPase activity was observed in foraminifers exposed to 1.6 µg/L Cu at pH 7.5 for 10 days, and in foraminifers exposed to 2.3 µg/L Cu at pH 7.2. After 25 days of treatment, all foraminifers exposed to pH 7.2 showed a relatively low Ca^{2+}-ATPase activity (Fig 3), roughly half that observed in foraminifers from the field reference sample and from those acclimated to the control conditions in the marine mesocosm (Fig. 2).

Data on Mg^{2+}-ATPase activity and the respective results for statistical analyses are shown in Figure 4 and Tables 3-4. Unlike Ca^{2+}-ATPase, Mg^{2+}-ATPase activity response followed similar patterns in all copper treatments, evidencing the low significance of crossed effects between stressors. Seawater pH had a significant effect on the enzyme activity in foraminifers exposed for 10 days. An increase in Mg^{2+}-ATPase activity was observed at pH 7.8 in all treatments except the 3.2 µg/L Cu concentration. At pH 7.8, Mg^{2+}-ATPase activity was highest at 1.0 µg/L Cu and similarly somewhat lower in the 1.6 and 2.3 µg/L Cu treatments. Activity declined back to control levels at pH 7.5, further declining at pH 7.2. At that highest Cu concentration, inhibition was directly proportional to decline in pH (Fig. 4A). After 25 days in experimental conditions, Mg^{2+}-ATPase activity in all treatments was similar to that observed after acclimation to the control conditions in the marine mesocosm, except for

foraminifers exposed to 1.6 µg/L Cu which exhibited Mg^{2+}-ATPase inhibition at pH t of 7.2 and 7.8.

Foraminifera bleaching and mortality

Percentages of specimens exhibiting some bleaching by treatment are shown in Figure 5, with results for statistical analyses shown in Tables 5-6. After 10 days, only the specimens in the lowest pH (7.2) and the highest Cu concentration (3.2 µg/L) exhibited higher percentages of bleaching than the control treatment. After 25 days, reduced pH with no or minimal (1.6 µg/L) Cu addition resulted in no significant increase in bleaching. However, bleaching increased with both reduced pH and increased Cu concentrations at the two higher Cu treatments, indicating an additive effect of copper exposure. Regression analysis indicated a significant negative relationship ($R = -0.32$; p value = 0.02) between Mg^{2+}-ATPase activity after 10 days of exposure and bleaching percentage after 25 days of experiment.

Mortality was low (<3%) until the first sampling time (10 days) and not related to any experimental treatment. After 25 days of exposure, mortality had a tendency of increase in foraminifers kept at the lowest pH tested (7.2). However, it was <10% in all treatments.

Discussion

In the present study, we have tested copper concentrations that are lower than the maximum allowed by Brazilian environmental regulations (5 µg/L Cu - CONAMA, 2005). Under normal conditions of seawater temperature and pH, copper concentrations tested did not cause a significant effect on the biochemical biomarkers analyzed, nor on bleaching or mortality. However, some Brazilian reefs are subjected to chronic contamination with copper and show low densities and high rate of bleaching in

Amphistegina lessonii (Prazeres et al., 2012a). Indeed, copper concentrations reported by these authors (13 ± 1.3 µg/L Cu), in Fernando de Noronha (PE), were four times higher than the highest concentration tested in the present study (3.2 µg/L Cu).

Previous studies evaluating the effects of copper on foraminifers reported the occurrence of deformed tests (Geslin et al., 2002; Frontalini and Coccioni, 2008), reduced growth and cytological abnormalities (Le Cadre and Debenay, 2006). These abnormalities include proliferation of lipid vesicles and cytological damage, suggesting that copper has the potential to affect membranes, and consequently impair the functioning of membrane-bound enzymes. Test' abnormalities and reduced growth may be due to metal-induced disturbances in biochemical processes related to calcification. In the present study, no test' abnormalities or bleaching associated with copper exposure were observed in foraminifers kept under control pH (8.1). This lack of effect can be explained by considering the low copper concentrations tested, which were even lower than those found in field study performed by Prazeres et al. (2012a). Moreover, the action of metallothioneins in the homeostasis of trace metals has been suggested to protect foraminifers against metal toxicity (Le Cadre and Debenay, 2006; Prazeres et al., 2011; 2012a), as has the involvement of mucopolysacharides in foraminiferal anti-chemical defense (Bresler and Yanko, 1995).

Potential impacts of ocean acidification are a subject of extensive current discussion. Several studies considering the responses of calcifying organisms have been performed in the past ten years (Jokiel et al., 2008; Wernberg et al., 2012), and a considerable attention has been given to foraminifers. Reymond et al. (2013) described growth inhibition in imperforate foraminifers with dinoflagellate symbionts exposed to pH 7.6. Likewise, Kuroyanagi et al. (2009), McIntyre-Wressnig et al. (2011), and

Hikami (2011) found reduced calcification and/or growth in high-Mg-calcite species kept at low pH.

However, when responses of perforate foraminifers to acidified conditions are considered, results vary and sometimes seem contradictory. For symbiont-barren hyaline species cultured in high pCO_2 environment, Dissard et al. (2010) found reduced shell weight in *Ammonia tepida*. Also, Khanna et al. (2013) found signs of test dissolution and deformation features. On the other hand, Vogel and Uthicke (2012) did not find any negative effect of changing pH on growth of diatom-bearing species. Similarly, McIntyre-Wressnig et al. (2013) found no effect on test growth in *Amphistegina gibbosa*, but did detect signs of shell dissolution. However, Fujita et al (2011) reported higher net calcification at intermediate (7.9-7.8) and reduced pH (~7.7).

Since heterotroph species seems to be more often affected than symbiont-bearing species by changes in seawater pH, a compensatory effect of the symbiosis seems to occur. Higher CO_2 concentration in seawater can favor symbiont densities and production (Reymond et al., 2013). In fact, CO_2 is the preferred form of dissolved inorganic carbon taken up by symbionts (ter Kuile et al., 1989). The photosynthethic uptake of dissolved inorganic carbon by symbionts can increase pH at the surface boundary layer, thus buffering the effect of lowering seawater pH. However, Glas et al (2012) and Uthicke and Fabricius (2012) concluded that productivity increase would not be enough to compensate for the negative effects of ocean acidification on calcification.

The most striking finding from the present study is the evidence of an interactive effect of copper contamination and ocean acidification scenarios on the biomarkers analyzed. When acting alone, these stressors generally did not cause marked changes in the biochemical biomarkers analyzed, but evidence of inhibition increased substantially when the stressors were combined.

The Ca^{2+}-ATPase activity is involved in the biomineralization process in foraminifers by concentrating calcium and alkalinizing the calcification space (Erez, 2003), as reported for corals (Al-Horani et al., 2003). In water enriched with CO_2, the organism would have to spend more energy to alkalinize its microenvironment, which could lead to an increase in Ca^{2+}-ATPase activity. After 10 days of exposure, we observed a slight increase in Ca^{2+}-activity in foraminifers exposed to pH 7.5 without addition of copper into the seawater and in the presence of 3.2 µg/L Cu. A similar result was observed in foraminifers exposed to pH 7.2 in the presence of 1.6 µg/L Cu. However, organisms exposed to 2.3 µg/L Cu showed inhibition of Ca^{2+} activity at the lower pH values. These findings suggest that the interaction between copper contamination and seawater acidification is impairing Ca^{2+}-ATPase activity, and hampering the compensatory response that this enzyme could display to maintain the alkalinity of the calcification microenvironment. At this point, it is interesting to note that a complete restoration of Ca^{2+}-ATPase activity was observed after 25 days of exposure, although some inhibition was seen in foraminifers exposed to the two lower pH conditions with 1.6 µg/L Cu. Also, it is important to note that Ca^{2+}-ATPase activity was inhibited in *Amphistegina* spp. exposed to 1.6 µg/L Cu at pH 7.2 and in those exposed to 2.3 µg/L Cu at pH 7.5. This inhibitory effect can be explained considering copper speciation in seawater. Lower pH levels increase the bioavailability of copper ions, thus increasing its toxicity (Richards et al., 2011). Like other metals, copper is shown to inhibit Ca^{2+}-activity in mussels (Vijayavel et al., 2007). Also, Prazeres et al. (2012b) reported a negative correlation between Ca^{2+}-ATPase activity and dissolved copper concentration in Brazilian reef waters.

Mg^{2+}-ATPase activity in *Amphistegina* spp. was also affected by a combination of copper contamination and ocean acidification exposure. However, unlike Ca^{2+}-

ATPase, its response followed the same pattern in all experimental treatments tested. In foraminifers kept at control pH (8.1), copper exposure did not affect Mg^{2+}-ATPase activity. However, copper exposure inhibited these enzyme activity when foraminifers were kept in seawater at the lower pH levels tested (7.2 and 7.5). As noted above, many studies have shown that lowering pH increases metal bioavailability and toxicity (Richards et al., 2011; Nikinma, 2013). This could again explain the interactive and negative effect observed in the present study when foraminifers were exposed to low copper concentrations in more acidic conditions.

Exposure to divalent metals usually causes lipid peroxidation in invertebrates like mussels (Viarengo et al., 1996), corals (Vijayavel et al., 2012) and foraminifers (Prazeres et al., 2011; 2012a). Lipid peroxidation can modify membrane structure, thus affecting the functioning of membrane-bound enzymes. Also, it can inactivate the sulphydril groups of ATPases (Viarengo et al., 1993, 1996). These facts would explain the enzyme inhibitory effects observed in foraminifers exposed to copper in the present study. Independent of copper exposure, Mg^{2+}-ATPase activity was higher in organisms kept at pH 7.8, i.e., 0.3 units of pH lower than the control one. On the other hand, Mg^{2+}-ATPase activity was strongly inhibited in foraminifers kept in seawater at 7.2, the most acidic condition tested. Apparently, a compensatory response occurred under a mild acidification condition (pH 7.8), but was not enough to prevent enzyme inhibition in the most acidic condition (pH 7.2).

Mg^{2+}-ATPase plays an important role in the biomineralization process in foraminifers. Bentov and Erez (2006) have shown that Mg concentration must be lowered to promote calcification. Yanko et al. (1998) postulated that morphological abnormalities in foraminiferal shells may be related to higher rates of Mg incorporation. Thus, a lower Mg^{2+}-ATPase activity, as reported in the present study with *Amphistegina*

spp. exposed to copper contamination and/or ocean acidification, may lead to higher concentrations of Mg in foraminifers. This would consequently lead to the formation of weaker shells. This idea is consistent with Russel et al (2004) finding of higher Mg content in planktonic foraminifera grown in seawater of pH higher than 8.2. It is not well established how much Mg^{2+}-ATPase activity is required for Mg regulation in foraminifers, but its inhibition certainly can promote ionoregulatory disturbances.

Divalent metal ions and H^+ can compete with Ca^{2+} and Mg^{2+} for the binding sites in the organism, modifying the functioning of ion transporters (Bianchini and Wood, 2003; Grosell et al., 2007). An increased availability of copper ions under acidic conditions would enhance this competition. Therefore, the interactive effect of metal contamination and ocean acidification, as observed in the present study, can potentially induce ionoregulatory disturbances.

In a broad view, Ca^{2+}-ATPase was found to be mainly affected by the interactive effect of both stressors. In fact, a significant effect of ocean acidification on Ca^{2+}-ATPase activity was only observed after 25 days of exposure. On the other hand, Mg^{2+}-ATPase was susceptible to acidification for a shorter time, showing a significantly reduced activity after 10 days of exposure. Moreover, Mg^{2+}-ATPase activity was even more affected in the presence of copper contamination. In turn, bleaching showed a late response to seawater acidification, and was also affected by the interactive effect of copper exposure. This combined effect indicates an increased sensitivity of *Amphistegina* spp. to ocean acidification in copper contaminated sites, as well as to copper contamination in acidic waters.

Impairment in ion transporters such as Ca^{2+}-ATPase and Mg^{2+}-ATPase may lead to ionoregulatory disturbances which can affect many physiological processes in foraminifers, including calcification. *Amphistegina* has tests composed of low-Mg-

calcite, which is the less soluble form of calcium carbonate found in marine calcifying organisms. For example, corals, sea urchins, soritid foraminifers (including chlorophyte- or dinoflagellate-bearing species), among other reef calcifying organisms, precipitate calcium carbonate in more soluble forms (aragonite and high-Mg-calcite). A scenario in which copper contamination is associated with ocean acidification may lead to severe impacts to the whole reef ecosystems, which are dependent on the biomineralization processes. Also, exposure to multiple stressors would increase the symbiont-bearing species susceptibility to mass bleaching, and consequently a decrease in the resilience of these organisms to diseases, infestations and many others adverse situations.

In the present study, more bleaching was observed in *Amphistegina* spp. individuals exposed to combined copper contamination and seawater acidification for 25 days. This finding is likely associated with a higher copper toxicity at lower pH levels. In fact, recent studies suggest that low pH/high pCO_2 induces bleaching in corals (Anthony et al., 2008) and foraminifers (Glas et al., 2012). Also, some authors reported coral bleaching related to copper exposure (Bielmyer et al., 2011; Prazeres et al.,2012a). The occurrence of a marked interactive effect of copper contamination and ocean acidification on foraminifer bleaching is of concern. Indeed, many authors have highlighted the role of interactive effects between global climate changes and chemical contamination in the observed damage to the photosystem apparatus in symbiont-bearing organisms (Negri et al., 2011; van Dam et al., 2012).

Although an adaptive response of the ionoregulatory enzymes from the holobiont was observed after 25 days of exposure, symbionts are likely not able to deal with the experimental conditions for a longer period of exposure. Despite the physiological cost of the adaptive response shown by the ATPase system is not known,

it is likely related to the photobiology system impairment. Bleaching is strongly correlated with oxidative stress in corals and foraminifers (Downs et al., 2002; Prazeres et al., 2011; 2012). Indeed, metal contamination alone can induce oxidative stress in invertebrates (Vijayavel et al., 2012; Giacomin et al., 2013), and the foraminifer *A. lessonii* (Prazeres et al., 2011; 2012). Furthermore, environmental hypercapnia was recently suggested to induce oxidative stress in oysters and mussels (Tomanek et al., 2011). These facts would help to explain the enhanced bleaching in *Amphistegina* spp. exposed to combined stress induced by copper contamination and ocean acidification. Also, oxidative stress conditions can induce membrane damage, leading to impairment of membrane-bound ATPase activity (Viarengo et al., 1993, 1996), as observed in the present study. The negative relationship between Mg^{2+}-ATPase activity after 10 days and bleaching percentages after 25 days of exposure may be an evidence of a possible oxidative stress condition under the experimental conditions. Also, it supports the idea that the biochemical biomarker (Mg^{2+}-ATPase activity) anticipates the physiological response (bleaching), as expected for a typical biomarker.

In this context, it is worth noting that changes in physiological (e.g., bleaching and other visual alterations) and biochemical (e.g., enzymes related to calcification) biomarkers can be earlier warning signs of damage than the occurrence of structural alterations (e.g. shell dissolution) and reduced biological function (reduced calcification rates). Also, biochemical biomarkers seem to show earlier responses than bleaching, a common endpoint used for monitoring purposes. In fact, Downs et al. (2005) proposed that this early warning potential is the main advantage of the use of cellular and biochemical biomarkers in environmental programs.

As expected, the most pessimist scenario considered in the present study, i.e. a decrease of 0.9 units of pH in seawater, showed the most concerning results, although

this is the least probable scenario. The more likely future scenario (reduction of 0.3 to 0.6 units of pH) showed less alarming responses, except when combined with copper contamination. These findings highlight how local seawater contamination with metals can increase the vulnerability of reef-dwelling organisms to ocean acidification, especially in coastal zones.

It is also important to stress that the present study was performed in a mesocosm system, thus incorporating ecological complexity and providing more realistic data. In fact, seawater pCO_2 in shallow reefs changes daily due to tidal cycles, coastal runoff, and community respiration and photosynthesis (Moulin et al., 2014). The senoidal system used in the Coral Vivo Project's marine mesocosm allows the automatic and continuous addition of the experimental treatments to the seawater collected from the coral reef area. This system incorporates daily variations naturally found in the local reefs, and consequently provides an increased degree of environmental realism when compared to the traditional laboratory experiments. Furthermore, mesocosm facilities have been widely used in risk assessment of pesticides, metals and other xenobiotics (Shaw and Kennedy, 1996; Jokiel et al., 2008) because laboratory toxicity testing usually does not generate ecologically relevant information (Giesy and Hoke, 1989). Also, recent studies have highlighted the importance of conducting experiments under mesocosm condition as a key tool for the development of research on the ecological impact of climate changes (Stewart et al., 2013; Marangoni et al., 2014; Moulin et al., 2014; Santos et al., 2014).

Finally, is important to highlight the importance of experiments considering the potential combined effects of local and global impacts. As observed in the present study, organisms facing stress caused by chemical contamination may have their capacity to deal with climate changes hampered (Negri et al., 2011). On the other hand,

increasing temperature and acidification of surface waters can modify the toxicity of many contaminants (Nikinmaa, 2013). The interactive effects of current local impacts (chemical contamination sources) and the potential global impacts (rising temperature and ocean acidification) must be considered in research and management of coral reefs. In this context, findings reported in the present study indicate that physiological and biochemical biomarkers can be reliable and valuable tools to detect and monitor the ecological consequences of the combined changes in pH and metal contamination of seawater. Therefore, future studies should consider testing a larger suite of biomarkers and considering their responses over a longer period of exposure. This would enhance our knowledge about the effective link between the biomarker responses and their role in key biological functions in foraminifers, such as calcification, susceptibility to diseases, bleaching and mortality.

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Table 1.Summary of the analysis of variance (ANOVA) performed for Ca^{2+}-ATPase activity in foraminifers (*Amphistegina* spp.) exposed to different combinations of copper (Cu) concentrations and sea water pH levels for 10 and 25 days. ***: p<0.0001; **: p<0.001.

Effect	10 days of exposure					25 days of exposure			
	df	SS	MS	F	*p*	SS	MS	F	*p*
Cu	3	0.005	0.001	0.701	0.557979	0.012	0.004	2.027	0.129793
pH	3	0.007	0.002	0.927	0.438545	0.041	0.013	6.747	0.001179**
Cu+pH	9	0.127	0.014	5.313	0.000189***	0.064	0.007	3.496	0.004125**
Error	32	0.085	0.002			0.065	0.002		

Table 2. Summary of the Student-Newman-Keuls (SNK) *post hoc* test for Ca^{2+}-ATPase activity in the foraminifer *Amphistegina* spp. exposed to different combinations of copper (Cu) concentrations (µg/L) and sea water pH levels for 10 and 25 days. Different letters indicate significant different mean values among Cu concentrations within each sea water pH level ($p<0.05$).

Time of exposure	Sea water pH	Cu concentration (µg/L Cu)			
		1.0	1.6	2.3	3.2
10 days	7.2	a	a	b	a
	7.6	a	b	ab	a
	7.8	ab	a	a	a
	8.1	ab	a	b	a
25 days	7.2	a	a	a	a
	7.6	a	a	a	a
	7.8	a	ab	b	a
	8.1	a	a	a	a

Table 3.Summary of the analysis of variance (ANOVA) performed for Mg^{2+}-ATPase activity in foraminifers (*Amphistegina* spp.) exposed to different combinations of copper (Cu) concentrations and sea water pH levels for 10 and 25 days. ***: p<0.0001; #: p<0.05.

Effect	10 days of exposure					25 days of exposure			
	df	SS	MS	F	*p*	SS	MS	F	*p*
Cu	3	0.056	0.018	2.651	0.06548#	0.053	0.017	2.833	0.05376#
pH	3	0.415	0.138	19.524	0.00022***	0.036	0.012	1.928	0.14481
Cu+pH	9	0.136	0.015	2.136	0.05536#	0.113	0.012	1.994	0.07314#
Error	32	0.226	0.007			0.201	0.006		

Table 4. Summary of the Student-Newman-Keuls (SNK) *post hoc* test for Mg^{2+}-ATPase activity in the foraminifer *Amphistegina* spp. exposed to different combinations of copper (Cu) concentrations (µg/L) and sea water pH levels for 10 and 25 days. Different letters indicate significant different mean values among Cu concentrations within each sea water pH level ($p<0.05$).

Time of exposure	Sea water pH	Cu concentration (µg/L Cu)			
		1.0	1.6	2.3	3.2
10 days	7.2	a	a	a	a
	7.6	a	b	b	b
	7.8	a	ab	ab	b
	8.1	a	a	a	a
25 days	7.2	a	a	a	a
	7.6	a	a	a	a
	7.8	a	a	a	a
	8.1	a	a	a	a

Table 5.Summary of the analysis of variance (ANOVA) performed for bleaching percentage in foraminifers (*Amphistegina* spp.) exposed to different combinations of copper (Cu) concentrations and sea water pH levels for 10 and 25 days. **: p<0.001.

Effect	10 days of exposure					25 days of exposure			
	df	SS	MS	F	*p*	SS	MS	F	*P*
Cu	3	130.74	43.581	1.099	0.3635	413.67	137.89	2.004	0.133151
pH	3	52.43	17.478	0.441	0.7252	1323.73	441.24	6.413	0.001586**
Cu+pH	9	380.00	42.223	1.065	0.4135	582.42	64.71	0.940	0.504745
Error	32	1268.02	39.626			2201.66	68.80		

Table 6. Summary of the Student-Newman-Keuls (SNK) *post hoc* test for bleaching percentage in the foraminifer *Amphistegina* spp. exposed to different combinations of copper (Cu) concentrations (µg/L) and sea water pH levels for 10 and 25 days. Different letters indicate significant different mean values among Cu concentrations within each sea water pH level ($p<0.05$).

Time of exposure	Sea water pH	Cu concentration (µg/L Cu)			
		1.0	1.6	2.3	3.2
10 days	7.2	a	a	a	a
	7.6	a	a	a	a
	7.8	a	a	a	a
	8.1	a	a	a	a
25 days	7.2	a	ab	ab	b
	7.6	a	a	a	a
	7.8	a	a	a	a
	8.1	a	a	a	a

Figure Legends

Figure 1. Different levels of sea water pH employed in treatments throughout the 25-days period of experiment with the foraminifer *Amphistegina* spp. in the marine mesocosm of the Coral Vivo Project (Arraial d'Ajuda, BA, northwestern Brazil). C: control (general average pH = 8.1); C-0.3: acidification of 0.3 units of pH respect of the control (general average pH = 7.8); C-0.6: acidification of 0.6 units of pH respect of the control (general average pH = 7.5); c-0.9: acidification of 0.9 units of pH respect of the control (general average pH = 7.2).

Figure 2. Ca^{2+}- and Mg^{2+}-ATPase in foraminifers (*Amphistegina* spp.) after collection in the field (field reference) and acclimation in the marine mesocosm of the Coral Vivo Project (Arraial d'Ajuda, BA, northwestern Brazil). No significant difference was observed between the two groups of foraminifers.

Figure 3. Ca^{2+}-ATPase in foraminifers (*Amphistegina* spp.) after 10 days (A) and 25 days (B) of exposure to combined copper concentrations and sea water pH conditions in the marine mesocosm of the Coral Vivo Project (Arraial d'Ajuda, BA, northwestern Brazil). Results of the analysis of variance (ANOVA) are shown in Table 1, while those for the Student-Newman-Keuls (SNK) *post hoc* test are shown in Tables 2 and 3.

Figure 4. Mg^{2+}-ATPase in foraminifers (*Amphistegina* spp.) after 10 days (A) and 25 days (B) of exposure to combined copper concentrations and sea water pH conditions in the marine mesocosm of the Coral Vivo Project (Arraial d'Ajuda, BA, northwestern

Brazil). Results of the analysis of variance (ANOVA) are shown in Table 4,while those for the Student-Newman-Keuls (SNK) *post hoc* test are shown in Tables 5 and 6.

Figure 5. Bleaching in foraminifers (*Amphistegina* spp.) after 10 days (A) and 25 days (B) of exposure to combined copper concentrations and sea water pH conditions in the marine mesocosm of the Coral Vivo Project (Arraial d'Ajuda, BA, northwestern Brazil). Results of the analysis of variance (ANOVA) are shown in Table 7, while those for the Student-Newman-Keuls (SNK) *post hoc* test are shown in Tables 7 and 8.

Figure 1

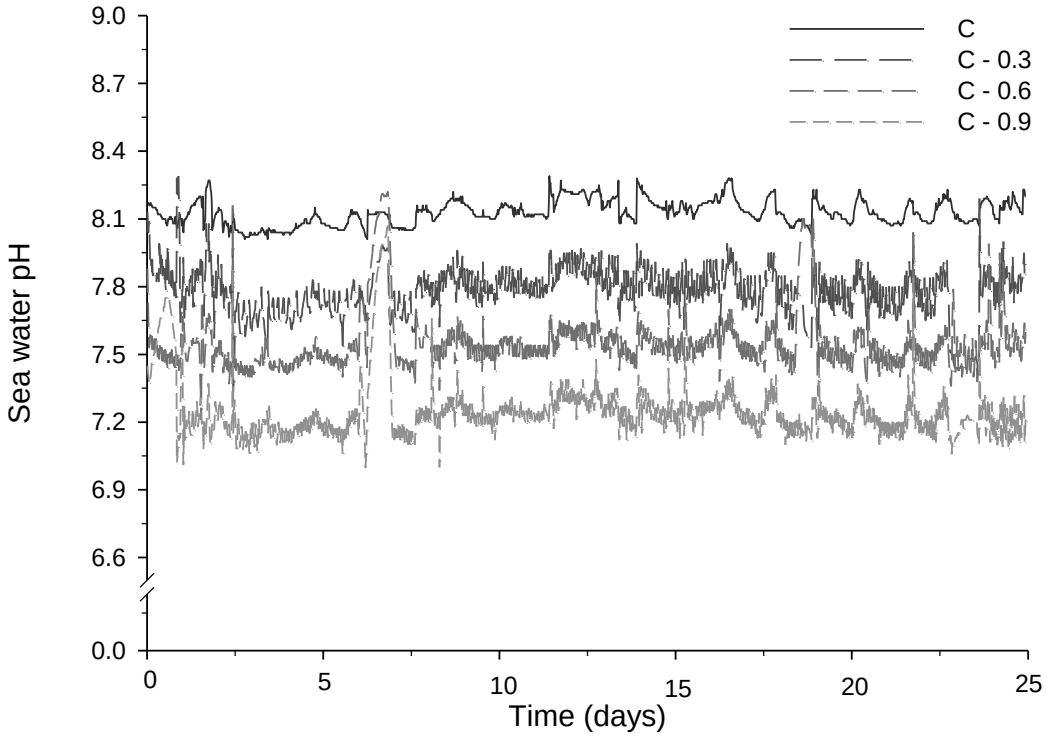

9.0
8.7
8.4
8.1
7.8
7.5
7.2
6.9
6.6
0.0
Sea water pH
0
5
10
15
20
25
Time (days)
C
C - 0.3
C - 0.6
C - 0.9

Figure 2

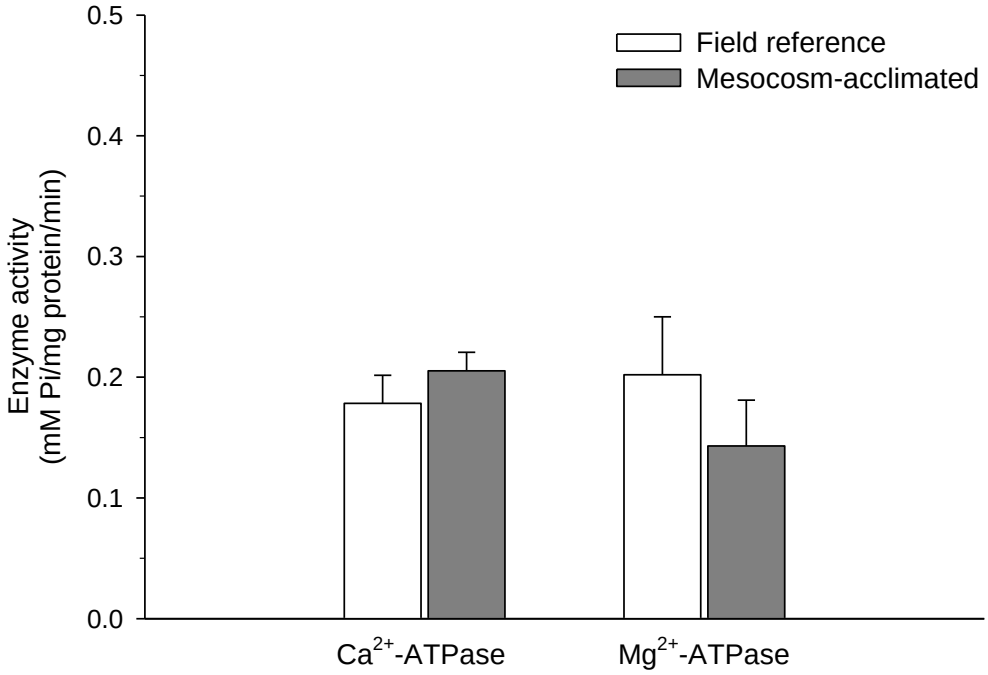

0.5
0.4
0.3
0.2
0.1
0.0
Enzyme activity
(mM Pi/mg protein/min)
Field reference
Mesocosm-acclimated
Ca2+-ATPase
Mg2+-ATPase

Figure 3

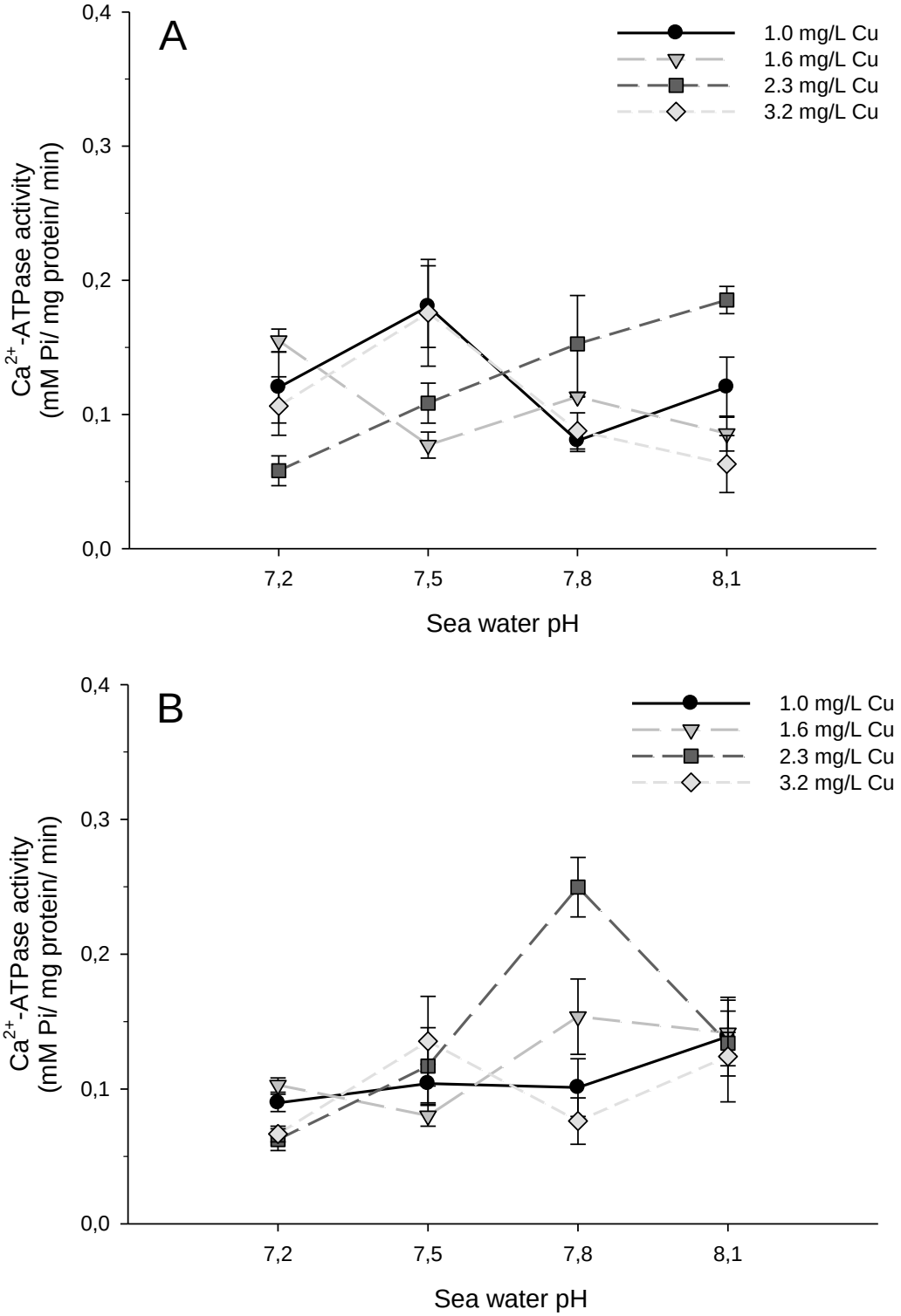

A
1.0 mg/L Cu
1.6 mg/L Cu
2.3 mg/L Cu
3.2 mg/L Cu
Ca2+-ATPase activity
(mM Pi/ mg protein/ min)
0,0
0,1
0,2
0,3
0,4
7,2
7,5
7,8
8,1
Sea water pH
B
1.0 mg/L Cu
1.6 mg/L Cu
2.3 mg/L Cu
3.2 mg/L Cu
Ca2+-ATPase activity
(mM Pi/ mg protein/ min)
0,0
0,1
0,2
0,3
0,4
7,2
7,5
7,8
8,1
Sea water pH

Figure 4

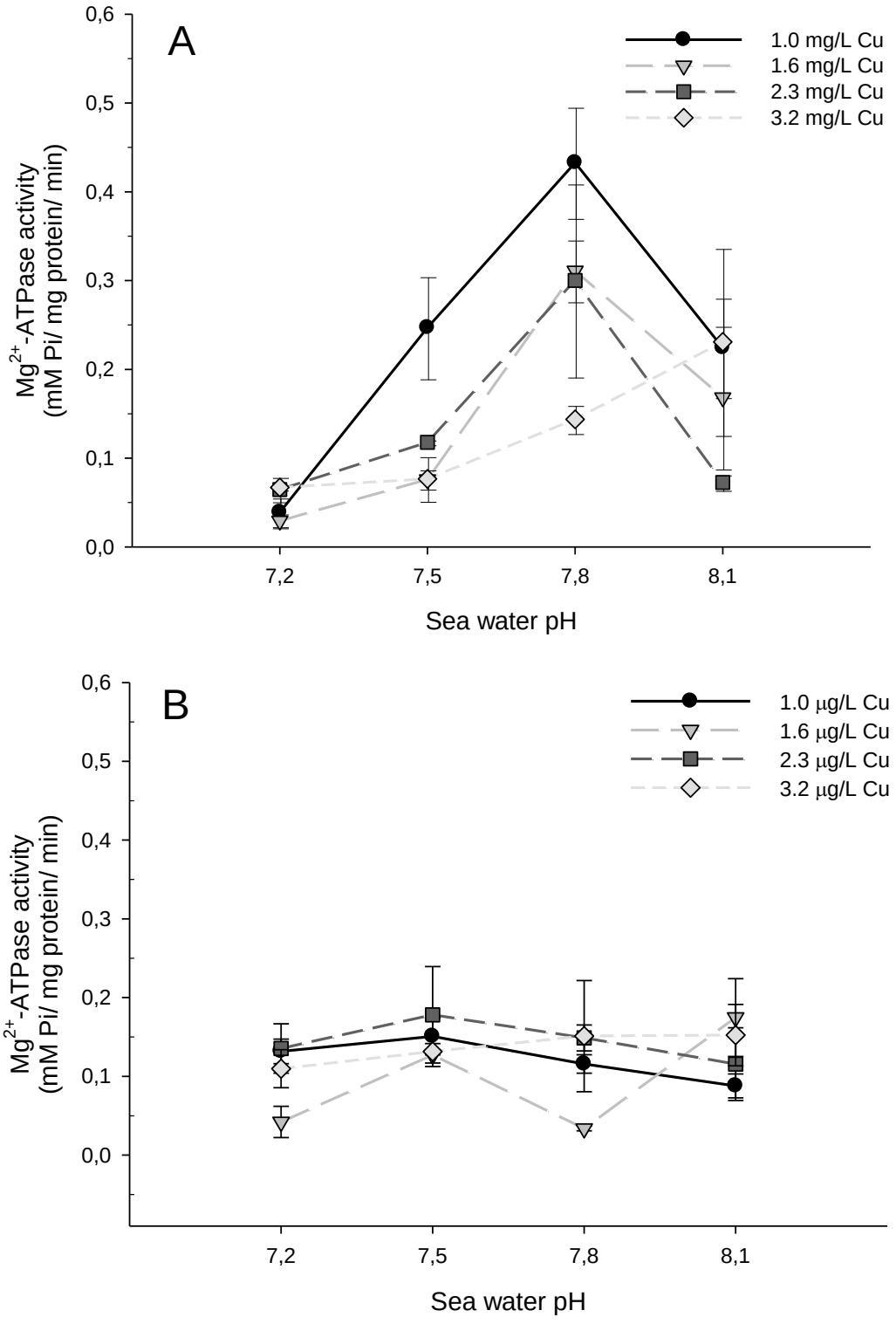
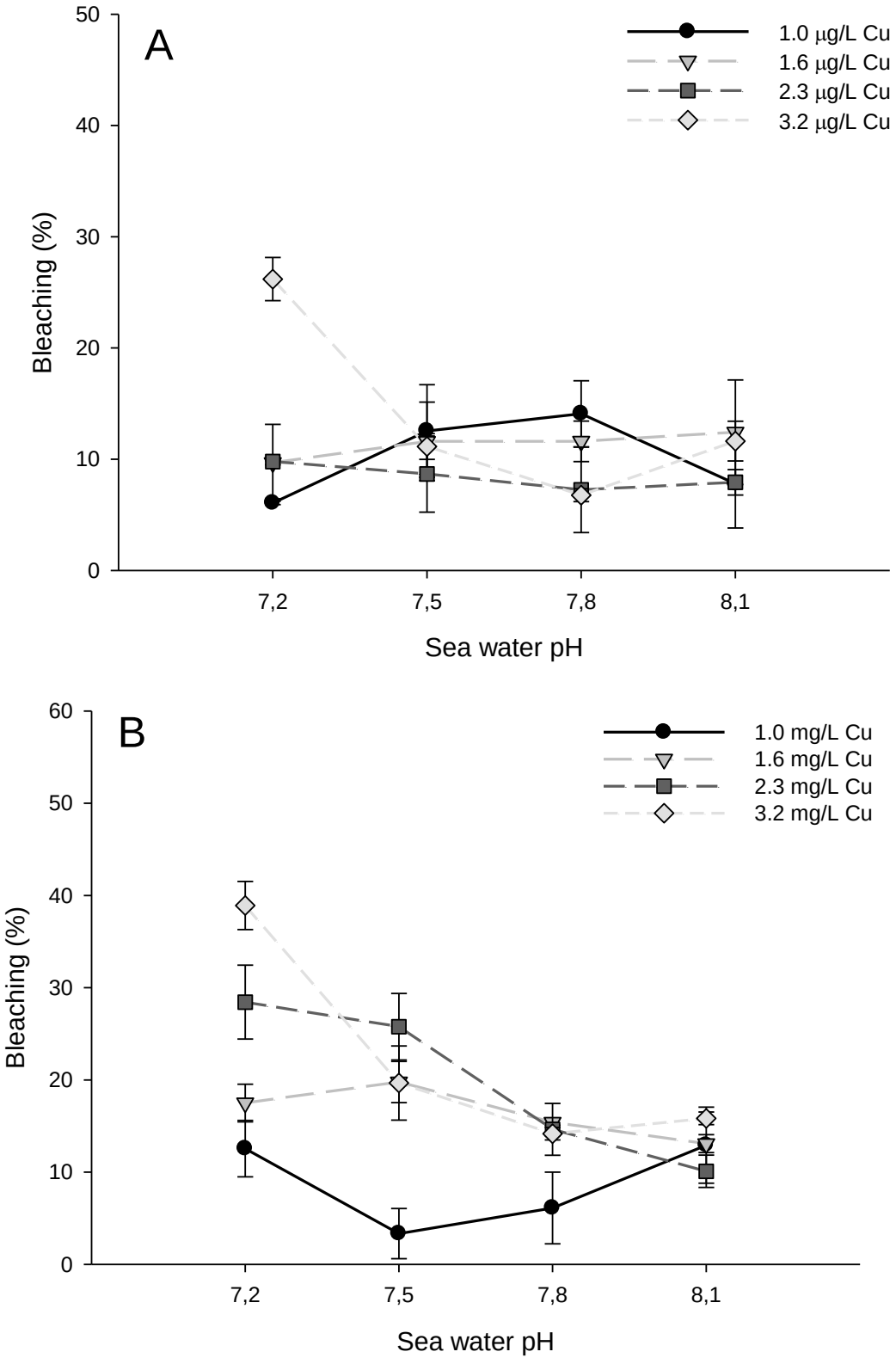

A
1.0 mg/L Cu
1.6 mg/L Cu
2.3 mg/L Cu
3.2 mg/L Cu
Mg2+-ATPase activity
(mM Pi/ mg protein/ min)
0,0
0,1
0,2
0,3
0,4
0,5
0,6
7,2
7,5
7,8
8,1
Sea water pH
B
1.0 µg/L Cu
1.6 µg/L Cu
2.3 µg/L Cu
3.2 µg/L Cu
Mg2+-ATPase activity
(mM Pi/ mg protein/ min)
0,0
0,1
0,2
0,3
0,4
0,5
0,6
7,2
7,5
7,8
8,1
Sea water pH

Figure 5


A
1.0 µg/L Cu
1.6 µg/L Cu
2.3 µg/L Cu
3.2 µg/L Cu
Bleaching (%)
0
10
20
30
40
50
7,2
7,5
7,8
8,1
Sea water pH
B
1.0 mg/L Cu
1.6 mg/L Cu
2.3 mg/L Cu
3.2 mg/L Cu
Bleaching (%)
0
10
20
30
40
50
60
7,2
7,5
7,8
8,1
Sea water pH