UNIVERSIDADE FEDERAL DO RIO DE JANEIRO
FACULDADE DE CIÊNCIAS BIOLÓGICAS
INSTITUTO DE BIOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIODIVERSIDADE DE BIOLOGIA EVOLUTIVA

MICROPARASITAS DO GÊNERO *AMOEBOPHRYA* SPP. INFECTANDO DINOFLAGELADOS MARINHOS: DIVERSIDADE GENÉTICA E POTENCIAL DE CONTROLE SOBRE POPULAÇÕES DE HOSPEDEIROS.

TATIANA VILLALBA VIANA

RIO DE JANEIRO

Julho de 2016

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TATIANA VILLALBA VIANA

Dissertação apresentada ao Programa de Pós-Graduação em Biodiversidade e Biologia Evolutiva do Instituto de Biologia da UFRJ, como parte dos requisitos necessários à obtenção do grau de Mestre.

Orientador: Professor Dr. Paulo Sérgio Salomon.
Co-orientador: Professora Dra. Cristiane Carneiro Thompson

Rio de Janeiro

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Aprovado em __ / __ / __

BANCA EXAMINADORA

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Prof.

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Prof.

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Prof.

Dedico este trabalho aos meus pais Luís e Ramália que estão comigo desde sempre. À Rose e ao Alexandre, meus pais por opção.

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Se vai tentar, vá até o fim.
Caso contrário, nem comece.
Se vai tentar, vá até o fim.
Pode perder namoradas, esposas, parentes, empregos e talvez até a cabeça.
Vá até o fim.
Pode ficar sem comer por três ou quatro dias.
Pode congelar no banco do parque.
Pode ser preso.
Pode receber escárnio, gozações, isolamento.
Isolamento é um presente, todo o resto é um teste da sua resistência, de quão forte é a sua vontade.
E você fará a despeito da rejeição e dos piores azares e será melhor do que qualquer coisa que possa imaginar.
Se vai tentar, vá até o fim.
Não há outra emoção como essa.
Você estará sozinho com os deuses e as noites queimarão como fogo.
Faça, faça, faça. faça,
até o fim, até o fim.
Você cavalgará a vida diretamente para o riso perfeito.
Essa é a única boa luta que existe.

Charles Bukowski

RESUMO

Membros do gênero *Amoebophrya* (Alveolata, Syndiniales) infectam dinoflagelados marinhos em zonas costeiras ao redor do mundo. Infecção por *Amoebophrya* spp. pode controlar o desenvolvimento de populações de dinoflagelados, incluindo espécies potencialmente nocivas. O grau de controle depende de características dos organismos bem como de fatores ambientais. Este estudo descreve aspectos (i) da diversidade e filogenia de *Amoebophrya* spp. e (ii) da prevalência de infecção em dinoflagelados planctônicos na Baía de Guanabara. Inicialmente foi feito um estudo *in silico* com uma sonda de rRNA (dap-1) específica para *Amoebophrya* spp. Sequências de 18S rDNA indubitavelmente originadas de *Amoebophrya* spp. e sequências relacionadas a estes organismos - originadas de bibliotecas de clones ambientais - foram comparadas com a sequência da sonda. O resultado da análise suportou a hipótese de que *Amoebophrya* spp. infectando dinoflagelados marinhos é restrita a um grupo monofilético conhecido como MALV II (Marine Alveolates Group II). Portanto, muitas sequências recuperadas em bibliotecas de clones ambientais são potencialmente de parasitas do tipo *Amoebophrya* spp. Ainda, a prevalência de infecção por *Amoebophrya* spp. em dinoflagelados da Baía de Guanabara foi investigada em amostras do plâncton coletadas mensalmente em dois locais durante 45 meses. Apenas 8 amostras apresentaram infecção por *Amoebophrya* spp. em dinoflagelados, com prevalência (porcentagem de células infectadas) tipicamente abaixo de 1%, com um máximo de 5% em uma amostra esporádica obtida fora do monitoramento de rotina. Diversas espécies de dinoflagelados encontrados em altas concentrações na Baía de Guanabara (e.g. *Levanderina fissa* e *Prorocentrum triestinum*) não apresentaram infecção detectável. A baixa frequência e prevalência de infecção por *Amoebophrya* spp. pode ser uma das causas do sucesso de populações de dinoflagelados na baía. Levanta-se a hipótese de que o avançado estado de eutrofização da baía desestabilize, a relação parasita-hospedeiro, de acordo com a teoria ecológica conhecida como paradoxo do enriquecimento. Os resultados desta dissertação contribuem para o melhor entendimento da diversidade e ecologia de microparasitas infectando microalgas planctônicas marinhas.

Palavras-chave: parasitismo, dinoflagellados, *Amoebophrya*, Baía de Guanabara, eutrofização.

ABSTRACT

Parasitoids of the genus *Amoebophrya* (Syndiniales) infect planktonic dinoflagellates in marine coastal areas. *Amoebophrya* infections can control host population development, including harmful species. Such control is variable, depending on characteristics of the organisms (host and parasite) as well as the environment. This study focused on (i) diversity and phylogeny of *Amoebophrya* spp. and (ii) infection prevalence of dinoflagellates by the parasite in an hipereutrophic estuary, Guanabara Bay (Southeast Brazil). Firstly, an *in silico* study of an rRNA probe specific for *Amoebophrya* spp. was conducted. Known *Amoebophrya* spp. 18S rDNA sequences and other sequences closely related to it - originated from environmental clone libraries - were compared with the probe sequence. This analysis lent support to the hypothesis that *Amoebophrya* spp. infecting marine dinoflagellates are restricted to a monophyletic cluster, MALV II (Marine Alveolates Group II, Syndiniales). This result suggests that many of the environmental 18S rDNA sequences from clone libraries clustering within MALV II are potentially *Amoebophrya* parasites. Infection prevalence of dinoflagellates by *Amoebophrya* spp. in Guanabara Bay was study on samples taken monthly at two fixed stations during 45 months. Only eight samples had detectable infections of dinoflagellates by *Amoebophrya* spp. with prevalence (percentage of infected hosts) typically below 1%, with maximum 5% in a sporadic sampling off the routine monitoring. Various potential host species found at high numbers during the survey (e.g. *Levanderina fissa* e *Prorocentrum triestinum*) had no detectable infections. These low infection frequency and prevalence by *Amoebophrya* in Guanabara Bay might be one of the reasons for the success of dinoflagellates in this environment, where blooms of these phytoplankters are recurrent. The hypothesis that the high organic load and advanced eutrophication levels of the bay destabilize the host-parasite interaction, as predicted by the ecological theory known as paradox of Enrichment, is here raised. The results of this dissertation contributed to a better understanding of the diversity and ecology of microparasites infecting marine planktonic microalgae.

Keywords: parasitism, dinoflagellates, *Amoebophrya* spp., Guanabara Bay, eutrophication.

SUMÁRIO

CAPITULO 1

1.1 INTRODUÇÃO

A dinâmica populacional de microalgas do plâncton depende do balanço entre fatores físicos e bióticos que determinam o aumento ou perda de células ao longo do tempo (SMAYDA 1997). Entre os fatores de perda, um que tem sido bastante estudado é a influência de patógenos, entre os quais figuram diversos parasitas e.g. bactérias, fungos e protistas (SALOMON e IMAI 2006; DOUCETTE *et al* 2006). Dinoflagellados marinhos estão entre os componentes do plâncton que servem de hospedeiros para diversos parasitas (CACHON e CACHON 1964, PARK e COATS 2002, NORÉN *et al* 1999). Esta dissertação trata de aspectos da diversidade e influência sobre populações naturais de hospedeiros de um tipo de parasita pertencente ao gênero *Amoebophrya* (Alveolata, Syndiniales) que infecta intracelularmente dinoflagelados marinhos.

1.1.2 DINOFLAGELADOS

Dinoflagelados compõem o superfilo Alveotala, juntamente com ciliados e apicomplexa (BACKVAROFF *et al* 2014). São protistas biflagelados e possuem como características específicas do grupo a presença de dinocarion, com cromossomos permanentemente condensados (exceto a subdivisão Syndinea) e o posicionamento dos dois flagelos, sendo um deles transversal à célula, formando uma espécie de cinto. e outro livre na base sulcal (FENSOME 1999). Dinoflagelados apresentam grande versatilidade nutricional com ampla variedade formas de obtenção de carbono e energia. Existem muitas formas exclusivamente heterotróficas, como membros dos gêneros *Oxyphysis* e *Protoperidinium*, enquanto outras são primariamente autotróficos fotossintetizantes, e muitos apresentam ambos os tipos de nutrição, sendo chamados mixotróficos (i.e. fixam carbono pela via fotossintética mas também possuem habilidade de absorver carbono dissolvido e mesmo particulado) (TAYLOR 1991). A maioria das espécies de dinoflagelados encontra-se no plâncton marinho, porém existem outras formas de vida, como epífitismo e simbioses com animais, como é o caso de membros do gênero *Symbiodinium* que colonizam tecidos de cnidários. e outros invertebrados marinhos (LESSARD e SWIFT, 1986).

1.1.2 DINOFLAGELADOS COMO PARASITAS: O GÊNERO *Amoebophrya* E OUTROS SYNDINIALES

Muitos dinoflagelados possuem hábitos parasíticos (CACHON 1964, COATS *et al* 1999), com destaque para a ordem Syndiniales, que contém uma série de pequenos parasitas infectando diversos organismos marinhos, incluindo outros dinoflagelados, copépodes, ciliados, entre outros (MORI *et al* 2007, SKOVGAARD *et al* 2009, KIM *et al* 2004). Reconstruções filogenéticas posicionam *Amoebophrya* na base do grupo monofilético que reúne os dinoflagelados (GUNDERSON *et al.* 1999, GUILLOU *et al.* 2008). Infecções por determinados membros da ordem Syndiniales em crustáceos e peixes podem ser extremamente prejudicais para a pesca e aquicultura (PARK *et al* 2013). Por outro lado, outros parasitas da ordem dos Syndiniales são vistos controlando varias espécies de dinoflagelados, podendo servir de mecanismos de controle biológico em caso de infecções em dinoflagelados formadores de florações. Dentro da ordem Syndiniales, estão inseridos, juntamente com *Amoebophrya*, outros gêneros com modo de vida parasitário, como *Syndinium* Chatton 1910, *Hematodinium* Chatton and Poisson 1930, *Ichthyodinium* Hollande and J. Cachon 1952, *Duboscquella* Chatton 1920 (FENSOME *et al* 1993), respectivamente parasitas de dinoflagelados, copépodos (SKOVGAARD *et al* 2005, 2012), lagostins e caranguejos (SMALL *et al* 2012, 2007), larvas de peixes pelágicos (MORI *et al* 2007) e ciliados tintínideos (HARADA *et al* 2007).

O gênero *Amoebophrya* é endoparasita de dinoflagelados e diversos metazoários (CACHON 1964; CACHON e CACHÓN 1987, COATS & BACHVAROFF 2014). Um grupo neste gênero, originalmente descrito como a espécie *Amoebophrya ceratii* (CACHON 1964) - por ter sido encontrado infectando dinoflagelados do gênero *Ceratium* -, mas atualmente reconhecido como um complexo de espécies, infecta mais de cinquenta espécies de dinoflagelados marinhos, muitas delas capazes de formar florações ou produzir toxinas, (CACHÓN 1964; COATS 1999; PARK, YIH, e COATS 2004, SALOMON *et al* 2003b, 2009).

Amoebophrya apresenta um ciclo de vida relativamente simples, com uma fase intracelular (trofonte) e outra de vida livre (CACHON e CACHON 1987). O desenvolvimento de *Amoebophrya* spp. dentro de células dos hospedeiros dinoflagellados inicia no citoplasma

ou no interior do núcleo (SALOMON *et al* 2003b, KIM *et al.* 2008, CHAMBOUVET *et al* 2011). Independentemente do local da infecção (nuclear ou citoplasmática), o hospedeiro infectado torna-se incapaz de se reproduzir e, com o desenvolvimento do parasita, acaba sendo destruído (CACHON & CACHON 1987). Portanto, *Amoebophrya* spp. apresenta características e dinâmica de infecção de um parasitoide (SALOMON e STOLTE 2010). Ao final do desenvolvimento da fase intracelular o parasita rompe a célula do hospedeiro na forma de um verme (Figura 1). Essa forma é multinucleada e multiflagelada, permanecendo assim por minutos e posteriormente se fragmentando, dando origem a células biflageladas conhecidas como dinósporos, a forma de vida livre de *Amoebophrya* spp. que tem entre 1,6 e 10 um (GROISILLIER *et al*. 2006, MONTAGNES *et al* 2008). Dinósporos de linhagens de *Amoebophrya* spp. isoladas de Cheasapeake Bay e cultivadas em laboratório juntamente com seus hospedeiros dinoflagelados não sobrevivem mais que 10 dias fora do hospedeiro (COATS e PARK 2002). Outros estudos apontam que os dinósporos são incapazes de permanecer vivos após duas semanas na água sem encontrar um hospedeiro (PARK *et al* 2013).

Amoebophrya spp. é comumente encontrado infectando dinoflagelados em águas costeiras. As taxas de prevalência de infecção em dinoflagelados (i.e. porcentagem de células infectadas em uma população do hospedeiro) têm sido registrada em vários locais do mundo e mostras grande variabilidade (COATS e PARK 2002, COATS *et al*, 1996, SALOMON *et al* 2003b, 2009; GUNDERSON *et al* 2002). Em alguns ambientes, como em águas abertas no Mar Báltico, a prevalência de infecção em *Dinophysis norvegica* raramente ultrapassa 2% durante um ciclo sazonal (SALOMON *et al.* 2003), enquanto outros ambientes, como Chesapeake Bay, MD, até 80% das células em populações do dinoflagelado *Akashiwo sanguinea* são encontradas infectadas pelo parasita (COATS *et al.* 1996). Entre os fatores associados a essa variabilidade estão características ambientais, e.g. temperatura e salinidade, disponibilidade de nutrientes, características do hospedeiro (e.g. produção de toxinas), florações e a predação dos dinósporos por ciliados (COATS e BOCKSTAHLER 1994, JOHANSSON e COATS 2002, MARANDA 2001).

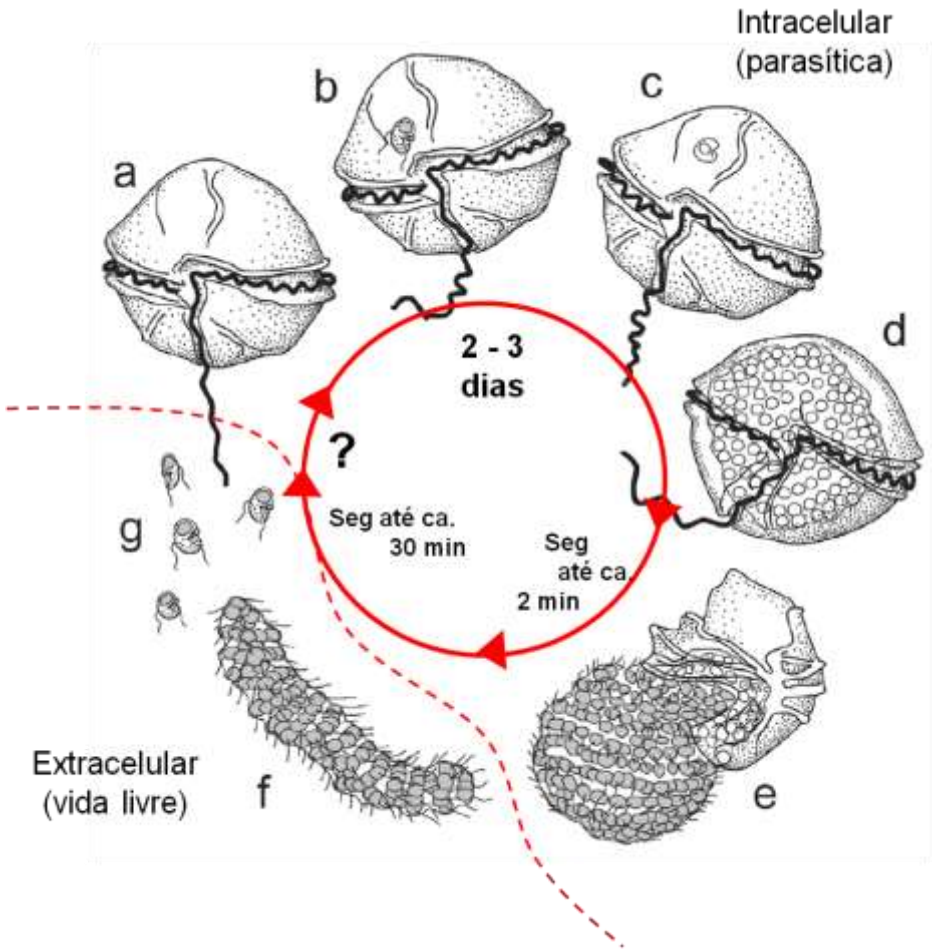

Figura 1. Ciclo de vida de *Amoebophrya* spp. infectando dinoflagelados. A fase infectiva (intracelular) inicia com o parasita penetrando uma célula do hospedeiro (a), estabelecendo-se no núcleo ou no citoplasma (b) onde começa a se desenvolver (c) formando um sincício (célula multinucleada) (d) até romper a célula do hospedeiro ao final do desenvolvimento (e) e tomar a forma de verme (f), que perdura por alguns minutos, completando a diferenciação celular (citocinese) liberando as pequenas formas de vida livre, denominados dinósporos (g) que irão infectar novas células dos hospedeiros, proliferando a infecção. O hospedeiro é invariavelmente destruído durante o desenvolvimento do parasita, que, por isso, assemelha-se a um parasitoide. Adaptado de Montagnes *et al.* (2008).

A característica letal da infecção de dinoflagelados por *Amoebophrya* spp. associada a altas prevalências observadas em populações naturais de hospedeiros durante epidemias, indica que este tipo de parasita tem potencial de controlar o desenvolvimento de florações de dinoflagelados, atuando como um importante fator de perda, por vezes comparável com a

predação por copépodos (MONTAGNES *et al.* 2008, HUDSON *et al.* 2006, TAYLOR 1968, MAZZILLO 2011). A especificidade de *Amoebophrya* em relação ao hospedeiro também tem sido estudada, tanto em experimentos de infecção cruzada em laboratório, quanto através da caracterização molecular do parasita em populações naturais e indicam que *Amoebophrya* spp, com pouquíssimas exceções, é altamente específica (CHAMBOUVET *et al.* 2008, COATS e PARK 2002, KIM 2006, COATS *et al* 1996; GUNDERSON *et al* 2002; JANSON *et al.* 2000; SALOMON *et al*, 2003a; SENGCO *et al* 2003). Essas observações ressaltam ainda mais a hipótese levantada por COATS *et al* (1996), de que *Amoebophrya ceratii* originalmente descrita como infectando diversas espécies de dinoflagelados é, na verdade, um complexo de espécies com alto grau de especificidade em relação aos hospedeiros.

1.1.3 DIVERSIDADE EM *Amoebophrya* E SYNDINIALES

O sequenciamento gênico da sub-unidade 18S do RNA ribossômico (18S rDNA) em linhagens de *Amoebophrya* spp. infectando dinoflagelados cultivadas em laboratório, somadas à sequências obtidas de células infectadas coletadas diretamente do ambiente, indicam alta diversidade dentro do grupo e levaram à conclusão que *Amoebophrya ceratii* (Koeppen) Cachon, na verdade corresponde a um complexo de espécies (COATS *et al* 1996; COATS e PARK 2002; GUNDERSON *et al* 2002; JANSON *et al.* 2000; SALOMON *et al*, 2003a; SENGCO *et al* 2003). Apesar de *Amoebophrya* spp. infectando dinoflagelados apresentarem similaridades no modo de infecção, são muito diversos em relação ao tempo de desenvolvimento dentro do hospedeiro, prevalência de infecção, número de dinósporos gerados ao final da infecção, local de infecção dentro da célula do hospedeiro, entre outras (COATS e PARK 2002, KIM *et al.* 2008), corroborando com os dados de diversidade genética.

Nos últimos 15 anos, o desenvolvimento de métodos de sequenciamento gênico mais eficientes e mais acessíveis permitiu a descoberta de uma diversidade de microrganismos marinhos até então subestimada por métodos tradicionais (LÓPEZ-GARCIA *et al* 2001, MOON-VAN DER STAAY *et al* 2001). O sequenciamento do marcador 18S rDNA em amostras do plâncton marinho de vários locais no mundo, revelaram uma ampla biodiversidade em pequenos eucariotos (< 10 µm), desde regiões costeiras à áreas de mar

aberto e oceano profundo (GUILLOU *et al* 2008, LÓPEZ-GARCIA *et al* 2001, MOON-VAN DER STAAY *et al* 2001, MASSANA *et al* 2004). Muitas das sequências destas amostras ambientais correspondem a alveolados marinhos não formalmente descritos e não cultivados, denominados MALV (Marine Alveolates), com diversos sub-grupos monofiléticos reconhecidos em reconstruções filogenéticas (LÓPEZ-GARCIA *et al*.,2001; MOON-VAN DER STAAY *et al*, 2001). Os grupos MALV-I e MALV-II são os mais abundantes e diversos (BACHVAROFF *et al* 2014, GROISILLIER *et al*. 2006, GUILLOU *et al*. 2008), mas pouco se sabe sobre a biologia e ecologia dos oganismos que os compõe. Alguns membros de MALV agrupam-se com outros Syndiniales já bem documentados, como o gênero *Ichthyodinium*, um parasita de ovos de peixes pelágicos (MORI *et al* 2007) e também linhagens de *Amoebophrya* spp infectando dinoflagelados (LÓPEZ-GARCIA *et al*.,2001; MOON-VAN DER STAAY *et al*, 2001). Estes estudos apontam para uma alta diversidade genética, grande abundância e distribuição ubíqua de *Amoebophrya* spp. e outros microparasitas de protistas e metazoários nos oceanos (GUILLOU *et al.* 2008).

1.1.4 REGISTROS DE *Amoebophrya* spp. NO ATLÂNTICO SUL

Em águas costeiras brasileiras, a presença de *Amoebophrya* spp. foi documentada pela primeira vez em populações de dinoflagelados coletados na região costeira próxima a Cabo Frio (SALOMON *et al.* 2006a). Um estudo subsequente relatou o processo de infecção durante o declínio de uma população do dinoflagelado *Ceratium falcatiforme* nesta mesma região, e concluiu que o ataque de parasites pode alterar significativamente fluxos de carbono na teia trófica microbiana, de especial importância durante períodos de subsidência no local (SALOMON *et al.* 2009). Estas são, até o momento, as únicas informações publicadas sobre o tema em águas costeiras do Atlântico sul. Na realidade, poucos são os relatos da infecção de dinoflagelados por este tipo de paraista em regiões costeiras tropicais, pois a maioria das publicações descreve infecção por *Amoebophrya* spp. em regiões temperadas.

1.2 OBJETIVOS

O objetivo geral deste estudo foi o de avaliar aspectos da diversidade e filogenia, e do impacto de parasitas do gênero *Amoeophrya* sobre populações naturais de dinoflagelados marinhos.

Os objetivos específicos foram:

- Avaliar a distribuição do gênero *Amoebophrya* dentro do grupo de pequenos alveolados marinhos de vida livre, MALV II.
- Avaliar a ocorrência e prevalência de infecção por parasitas do gênero *Amoebophrya* spp. em populações de dinoflagelados planctônicos da Baía de Guanabara.
- Estimar a contribuição da infecção por *Amoebophrya* spp. como fator de perda para populações de dinoflagelados da Baía de Guanabara.

1.3 ESTRUTURA DA DISSERTAÇÃO

Os resultados das pesquisas desenvolvidas nesta dissertação estão organizados na forma de dois artigos científicos, apresentados nos capítulos 2 e 3, a seguir.

No primeiro artigo, foi feito um estudo comparando-se a sequência de uma sonda de DNA especificamente desenhada para hibridizar à sub-unidade 18S do RNA ribossômico de parasitas do tipo *Amoebophrya* infectando dinoflagelados, com sequências de 18S rDNA de *Amoebophrya* spp. e membros do grupo MALV-II disponíveis em bancos de dados públicos. O artigo contém, também, uma compilação de imagens em microscopia (previamente disponível) que foi utilizada para comprovar a eficácia da sonda acima mencionada na detecção do parasita em dinoflagelados coletados em diversos locais ao longo do oceano Atlântico.

O segundo artigo baseia-se em um estudo sistemático de monitoramento da presença e prevalência de infecção por *Amoebophrya* spp. em dinoflagelados planctônicos na Baía de

Guanabara. Neste estudo foi utilizada a mesma sonda de rRNA, acima mencionada, para detectar o parasita, em conjunto com outras técnicas: coloração com DAPI, observação da auto-fluorescência natural do parasita em amostras vivas ao microscópio e com um sistema automatizado de imageamento (FlowCam). Os resultados da frequência de incidência e prevalência de infecção por *Amoebophrya* spp. são discutidos considerando-se características físico-químicas e bióticas da Baía de Guanabara, especialmente os altos níveis de nutrientes e microorganismos presentes na água, característicos de um ambiente com alto grau de eutrofização. Este estudo utilizou dados de qualidade de água (temperatura, salinidade, oxigênio dissolvido, concentração de nutrientes e clorofila) e abundância de microorganismos (bacterias totais e víbrios) obtidos em um programa de monitoramento da Baía de Guanabara (PELD-Guanabara) em cooperação com diversos grupos de pesquisa do Departamento de Biologia do IB-UFRJ.

Ao final, são apresentadas uma breve discussão e as conclusões do estudo.

CAPÍTULO 2

Are small marine Alveolates of the MALV II group parasites? Insights from *in situ* hybridization and *in silico* evaluation of an 18S rRNA probe specific to *Amoebophrya* parasites

Tatiana V. Viana[1], Giovana de Oliveira Fistarol[2], Cristiane C. Thompson[2], Fabiano L. Thompson[2], Paulo Sergio Salomon[1]

[1]Marine Phytoplankton Laboratory, [2]Marine Microbiology Laboratory, Biology Institute, Federal University of Rio de Janeiro, Rua Prof. Rodolpho P. Rocco, 211, CCS , Bloco A, Sala A1-81, CEP: 21941-902, Rio de Janeiro, RJ, Brazil.

Abstract

Recent research have shown that small eukaryotes are highly diverse and ubiquitous in the worlds ocean plankton. A group of these small eukryotes (Marine Alveolates group II or MALV-II) cluster together with parasitic of the genus *Amoebophrya* (Syndiniales), known for almost one century to infect marine dinoflagellates in marine waters. These observation lent credence to the hypothesis that most, if not all members of MALV II, have a parasitic life-style. In order to test this belief, we performed an in silico study comparing 18SrDNA sequences unmistakably originated from *Amoebophrya*, and sequences from other Syndiniales, with the sequence of a FISH probe (dap) specifically designed for *Amoebophrya*. The probe dap was also tested in 31 field samples where it successfully unveiled *Amoebophrya*-like infections in dinoflagellates collected in various marine areas. The *in silico* probe check showed that 22 known *Amoebophrya* parasites sequences match the probe with maximum 1 mismatch. The results of the *in situ* probe hybridization assays and *in silico* probe match analysis support the hypothesis that *Amoebophrya* parasites are restricted to MALV II. We also observed that several uncultured MALV II members match the probe sequence, and are likely to have parasitic life-styles infecting marine dinoflagellates. However, 20% of MALV-II sequences had very poor match with the probe (>4 mismatches) indicating that these sequences do not belong to typical *Amoebophrya* spp., and thus may or may not act as parasites. With the available data, we conclude that one should be careful when attributing gene sequences from environmental clone libraries as representing parasites of dinoflagellates, or of any other marine organism. More sequences of unmistakably parasitic *Amoebophrya* and other parasitic protists need to be produced to resolve this issue, enabling us to a better assessment of the importance of small marine eukaryotes in the sea. Whole-cell FISH probes assays, like the one used here, are excellent tools to help achieving this goal.

Keywords: marine Alveolates, MALV, parasitism, *Amoebophrya*, FISH probes

Introduction

A vast genetic diversity and abundance of small (in the nanoplankton size-classe) unicellular marine eukaryotes has been unveiled in the last decade by the use of molecular biology methods, especially cultivation-independent approaches, that retrieve the bulk genetic diversity (normally of ribosomal RNA genes, rRNA) of a whole community of microorganisms from the water in a given place of the ocean (López-Garcia *et al* 2001, Massana and Pedrós-Alió 2008, Keeling *et al* 2014). These findings have cast new light on the distribution and importance of small eukaryotes in the dynamics and carbon flow in marine microbial food webs (Salomon and Imai 2006, Kagami *et al* 2007, Jephcott *et al* 2015).

Many 18S rRNA gene sequences retrieved from environmental samples belong to uncultured Alveolates, a group of eukaryotes that encompasses common marine plankters e.g. dinoflagellates and ciliates (Romari and Vaulot 2004). Phylogenetic reconstructions based on 18S rRNA gene sequences divided these small, uncultured marine Alveolates in five groups, namely marine Alveolates group I (MALV I) to V (MALV V). Whereaas MALV-V is composed of known fish eggs and crab parasites sequences, like *Syndinium turbo* and *Hematodinium* spp (Guillou *et al* 2008), the other groups have very little correspondence to known organisms. Interestingly, since the first clone libraries began to be analysed, sequences of MALVII were observed to cluster together with parasites of the genus *Amoebophrya*, a small flagellated eukaryote that infects planktonic dinoflagellates in the sea (Cachon 1964, Cachon and Cachon 1987, López-Garcia *et al* 2001, Massana *et al* 2004).

Hitherto, *Amoebophrya* spp. infecting dinoflagellates are virtually the only known organisms belonging to these newly discovered uncultured Alveolates clustering together with MALV II in phylogenetic reconstructions. This observation has led to the hypothesis that many, if not the great majority, of the members of MALV II act as parasites of dinoflagellates, and perhaps of other protists and metazoans in the sea (Guillou *et al* 2010). However, the assumption that the diverse MALV-II is composed by parasitic microeukaryotes is based exclusively on sequence similarity and phylogenetic affiliation with known parasitic organisms, which are basically *Amoebophrya* parasites infecting marine Dinoflagellates. Because these parasites act in reorganizing carbon particles into smaller packages than their

hosts, and are then eaten by other protists e.g. tintinnid ciliates (Maranda 2001, Johanson and Coats 2002, Salomon *et al* 2009), their importance in determining carbon flows within microbial food webs would be extremely relevant if they are widespread and abundant in seawater (Salomon and Imai 2006, Guillou *et al* 2008, Jephcott *et al* 2015). Thus, determining the actual contribution of parasites within MALV II is relevant to increase our understanding of microbial web functioning.

In this work we address the hypothesis of the wide-spread occurrence of *Amoebophrya* spp. parasitizing dinoflagellates within the group of small marine alveolates (MALV II). We did it by comparing the sequence of a fluorescently-labeled rRNA probe, used to detect *Amoebophrya* inside dinoflagellates in a marine waters of various parts of the globe, with carefully selected 18S rRNA gene sequences of *Amoebophrya* spp. as well as of uncultured members of MALV from clone libraries availabe in the database, and of marine dinoflagellates species known to serve as hosts to *Amoebophrya* spp. We also describe new sequences of *Amoebophrya* parasites infecting dinoflagellates from the North Sea and the Cabo Frio upwelling area in the South Atlantic ocean.

Material and Methods

Probe hybridization

A total of 31 samples of marine plankton were collected with a 20 um mesh-size plankton net in the photic zone in several locations in the South and North Atlantic, ranging from the coast of Santa Catarina state (Enseada Beach) in Southern Brazil, up to the cost of Iceland (Figure1 and Table 1). Cell suspensions were fixed with 4% paraformaldehyde in PBS (ph 7.0) for 1h at 4°C, rinsed with PBS and stored in either high grade methanol or 70% ethanol. These samples were used in whole-cell *in situ* hybridization assays (FISH) with a fluorescein-tagged probe, *dap-1* (5´-TTTTGGATGAYTCATAATAA-3´) (Salomon *et al* 2009), to unveil infections by *Amoebophrya* spp. in dinoflagellates. FISH assays were done as described elsewhere (Salomon *et al* 2003, 2009). Parasite infections in field samples were detected by the probe signal in an epifluorescence microscope with blue light excitation. Cells were counter-stained with 5 µg mL^{-1} DAPI (4',6-diamidino-2-phenylindole) to allow

observing hosts and parasite nuclei under UV excitation. Whenever possible, in samples with high dinoflagellete concentration, between 100 and 1000 hosts were inspected for infection and scored as infected or not infected.

18SrDNA amplification and sequencing

Single dinoflagellate cells infected with a mature *Amoebophrya* as determined by the the probe signal after FISH assay in samples from the North Sea and the Cabo Frio uppwelling region in the South Atlantic ocean, were individually transferred to PCR tubes using a microcapilar. PCR amplification on single cells was done by a semi-nested approach with primers and conditions as in Janson *et al* (2000) and Salomon *et al* (2003). Sequencing was done by the Sanger method with dye terminator chemicals.

In silico study of the dap-1 probe

The sequence of the probe dap-1 was tested *in silico* against 18s rRNA sequences of *Amoebophrya* spp., other members of the Syndiniales including marine Alveolates belonging to MALV-I MALV-II, MALV-III and MALV-V groups. Sequences were aligned in Muscle (Edgar 2004). Mismatches with the probe were inspected manually in the alignment using Seaview v4 (Gouy *et al* 2010). A total of 361 sequences were compared with the probe region and scored according to the number of mismatches. Sequences with five or more mismatches were pooled together.

Phylogenetic reconstructions

18S rDNA sequences for phylogenetic reconstructions were selected from GenBank and checked in the SILVA database (http://www.arb-silva.de) for quality. Only sequences with >1600 bp and good quality (pintail >80%) were used in the analysis. Phylogenetic reconstructions were done in MEGA 6.2 (Tamura *et al* 2013) after alignment in MUSCLE (Edgar 2004). The initial tree for the heuristic search was build with a Neighbor-Joining algorithm, BioNJ (Gascuel 1997).The maximum likelyhood method with a general time reversible model (Nei and Kumar 2000) with 10000 bootstrap was used.

Results and Discussion

Hybridization assays with probe dap revealed *Amoebophrya* infecting dinoflagellates in all 31 samples collected in the field, Figure 2 shows examples of probe signal unveiling the parasite inside host cells from four distinct marine areas across the Atlantic. DAPI counter-staining revealed the multinucleated syncytium typical of the parasite trophont (Coatas *et al* 1996, Gisselsson *et al* 2002, Salomon *et al* 2003a, 2003b). Overall, infected hosts observed in the samples belonged to the genera *Akashiwo, Alexandrium, Ceratium, Dinophysis, Heterocapsa, Prorocentrum,* and *Protoperidinium*, plus several non-identified dinoflagellates, including various gymnodinoid specimens. All these genera have been previously listed as suitable hosts for *Amoebophrya* (Park *et al* 2004, Jephcott *et al* 2015). Parasite prevalence (% of infected hosts in the population) was estimated in samples where dinoflagellates were abundant. The genus *Dinophysis* showed the highest prevalence, 20% in *Dinophysis norvegica* in the North Sea, 15 % in *Dinophysis acuminata* at Enseada Beach, a mussel-farming area in southern Brazil, and 10% in *Dinophysis acuminata* in Clam Cove ME, USA. In most of the samples analysed, dinoflagellates were abundant and at times dominant in the microplancton community, mostly because these samples were obtained within the frame of projects aiming to study the ecology of dinoflagellates, like the material from the Baltic and the North Seas (Gisselsson *et al* 2002, Salomon *et al* 2003a, 2003b) and from the upwelling region in the south Atlantic (Salomon *et al* 2009, this study). Noticeably, infections were observed even in conditions of low dinoflagellate abundance, in opportunistic samples, as for the one taken off the coast of Greenland during a spring bloom dominated by diatoms (Figure 2). The presence of *Amoebophrya* in all samples we analyzed agrees with this parasites widespread distribution in the oceans and highlights its importance in rearranging carbon within microbial food webs (Maranda 2001, Salomon and Imai 2006, Guillou *et al* 2008).

A comparison of the dap probe sequence with 22 18SrDNA sequences of unquestionably *Amoebophrya* parasites gave full and perfect match with 18 of them (82%) with the remaining 4 sequences (18%) with only one single mismatch (Figure 3). It is important to observe that this set of sequences that we are here calling *Amoebophrya* contains only sequences that could unambiguously be linked to *Amoebophrya* parasites, i.e. they

originated from parasites that were physically observed (in the microscope) infecting their hosts, either from cultured host-parasites systems, or in field samples from where single infected host cells were isolated and used for PCR amplification of the 18SrDNA, as e.g. in Salomon *et al* (2003a) and the new sequences from the North Sea and the Cabo Frio that described here. It does not include the majority of sequences of MALV II originated from clone libraries of environmental marine plancton that are tagged as *Amoebophrya* only on the basis of sequence similarity with known parasite sequences in the database. A considerable fraction on environmental sequences attributed to *Amoebophrya* within MALV II (20%) had 5 or more mismatches with the dap probe, whereas only 4% (one sequence out of 22) of recognized *Amoebophrya* parasites had this level of mismatch.

When compared against sequences of uncultured marine Alveolates belonging to MALV I, the dap probe showed very poor matches, with no perfect match with any of the sequences. Of the 66 MALV-I sequences contrasted with the probe, 9 (12%) had 4 mismatches whereas 66 (85%) had 5 or more mismatches. This is in line with the fact that no phylogenetic reconstruction available hitherto places *Amoebophrya* outside MALV-II. The same result was observed in our reconstruction (Figure 4). Four new sequences obtained from infected *Ceratium* spp. from the North Sea and two sequences from infected *Protoperidinium* spp. cells from the South Atlantic clustered together other *Amoebophrya* parasite and MALV-II sequences.

No discrepancies were ever found between probe and DAPI signals i.e. all infections revealed by DAPI were also positive for the probe signal. These observations from hybridization of field samples together with the *in silico* studies confirm the probe dap efficiency in detecting *Amoebophrya* parasites in marine waters, as previously tested by Salomon *et al* (2009).

Conclusion

Taken together, our results of in situ probe hybridization assays and in silico probe match anaysis, support the hypothesis that *Amoebophrya* parasites are restricted to MALV II. We conclude that several uncultured MALV II members are likely to have parasitic life-styles infecting marine dinoflagellates i.e. belong to *Amoebophrya*. However, the high number of

MALV-II sequences with poor match against the probe indicate that such sequences are unlike to belong to typical *Amoebophrya* spp., and thus may or may not act as parasites. The idea that a parasitic life-style is a common feature among the highly diverse group of small marine eukaryotes, especially the MALV group, has become widespread after new discoveries of their genetic diversity and abundance (Guillou *et al* 2008, Guillou *et al* 2010, Jephcott *et al* 2015). While this is a completely plausible and, above all, testable hypothesis, with the current data one should be careful when attributing gene sequences from environmental clone libraries as representing parasites of dinoflagellates, or any other oganism. There is a need for more studies where the parasitic phase of the alleged parasites is unmistakably observed, coupled to molecular identification to solve this issue. FISH probes like the one employed here and in other studies (Chambouvet *et al* 2008, Salomon *et al* 2006, 2009, Alves-de-Souza *et al* 2012) are a much useful tool for that purpose.

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Table 1. Geographical origin of marine plankton samples from where intracellular *Amoebophrya* spp. infections were detected with the fluorescein-tagged probe dap-1 in whole-cell FISH assays. See map in Figure 1 for locations.

Sample #	Geographical origin	Location	Lat	Long
1	N Atlantic	190 Km SE off Greenland	68°15.8'N	20°47.5'W
2	NE Atlantic	Ballsfjord-Norway	69°22.0'N	19°07.0'E
3	NE Atlantic	Sletvik-Norway	63°36.0'N	09°03.0'E
4	NE Atlantic	Sletvik-Norway	63°35.0'N	09°03.0'E
5	NE Atlantic	Halsafjord-Norway	62°57.2'N	08°22.8'E
6	NE Atlantic	Sognefjord-Norway	61°04.9'N	05°18.5'E
7	North Sea	174 Km W off Denmark	55°46.1'N	05°24.2'E
8	North Sea	147 Km W off Denmark	56°09.0'N	05°44.9'E
9	North Sea	94 Km W off Denmark	56°12.0'N	06°37.8'E
10	Baltic Sea	39 Km E off Sweden	56°04.9'N	16°29.4'E
11	Baltic Sea	11 Km E off Sweden	56°36.5'N	16°53.5'E
12	Baltic Sea	11 Km E off Sweden	56°36.5'N	16°53.5'E
13	Baltic Sea	11 Km E off Sweden	56°36.5'N	16°53.5'E
14	Baltic Sea	11 Km E off Sweden	56°36.5'N	16°53.5'E
15	Baltic Sea	3.3 Km E off Sweden	56°37.0'N	16°45.0'E
16	Baltic Sea	Gotland Deep	57°36.3'N	19°02.1'E
17	Baltic Sea	Gotland Deep	57°18.3'N	20°04.6'E
18	NE Atlantic	Sagres oyster farm-Portugal	37°00.6'N	08°55.6'E
19	NE Atlantic	2.6 Km E off Portugal	37°00.4'N	08°53.4'E
20	Mediterranean	Barcelona Harbor-Spain	41°22.5'N	02°10.9'E
21	Mediterranean	Barcelona Harbor-Spain	41°22.5'N	02°10.9'E
22	Adriatic Sea	Pesaro-Italy	43°57.6'N	12°51.0'E
23	Adriatic Sea	Fosso Sejore-Italy	43°53.1'N	12°57.9'E
24	NW Atlantic	Clam Cove-ME, USA	44°08.0'N	69°06.0'W
25	NW Atlantic	Greenwich Cove-RI, USA	41°39.5'N	71°27.0'W
26	Caribbean Sea	3.3 Km W off Barbados	13°15.5'N	59°40.5'W
27	Caribbean Sea	2.8 Km W off Barbados	13°13.0'N	59°40.2'W
28	SW Atlantic	Cabo Frio-Brazil	22°59.7'S	42°01.3'W
29	SW Atlantic	Cabo Frio-Brazil	23°00.0'S	42°00.4'W
30	SW Atlantic	Cabo Frio-Brazil	23°02.7'S	41°59.7'W
31	SW Atlantic	Enseada Bay-Brazil	26°13.2'S	48°30.5'W

Figure legends

Figure 1. Map with the location where plancton samples were obtained. See Table 1 for details.

Figure 2. Micrographs of dinoflagellate cells infected with *Amoebophrya* as unveilde by the probe dap-1. Each panel (a to d) is a composition of micrographs of the same cell observed under bright-field (left), epifluorescence with blue light excitation showing the fluoresceing-tagged dap-1 probe signal (middle), and epifluorescence with UV light excitation showintg hosts and parasites nuclei unveiled by the DAPI signal (right). **a.** An unidentified dinoflagellate host from a diatom dominated spring-bloom plancton community collected off the coast of Greenland; **b.** *Protoperidinium* sp. from Barcelona Harbour, Spain; **c.** *Dinophysis acuminata* from Greenwich Cove, MD, USA; **d.** *Prorocentrum* sp. cell from the Cabo Frio upwelling area, south-eastern Brazil. The probe signal is seen by the green fluorescence of its fluorochrome fluorescein (arrows). Scale bar: 10µm.

Figure 3. Probe match of 361 18SrRNA sequences of *Amoebophrya*, other syndinian parasites and representatives of marine alveolates (MALV) groups I, II, III, and V selected from the database.

Figure 4. Maximul Likelyhood phylogenetic reconstruction of 45 18SrDNA gene sequences of *Amoebophrya* spp., marine planctonic dinoflagellates, and representatives of the marine alveolates (MALV) groups I, II, III, and V. The tree is build base on 325 informative sites. Branch length is proportional to the number of substitutions per site. Numbers in the nodes are percentages of 1000 bootstrap replicates.

Figure 1.

Figure 2

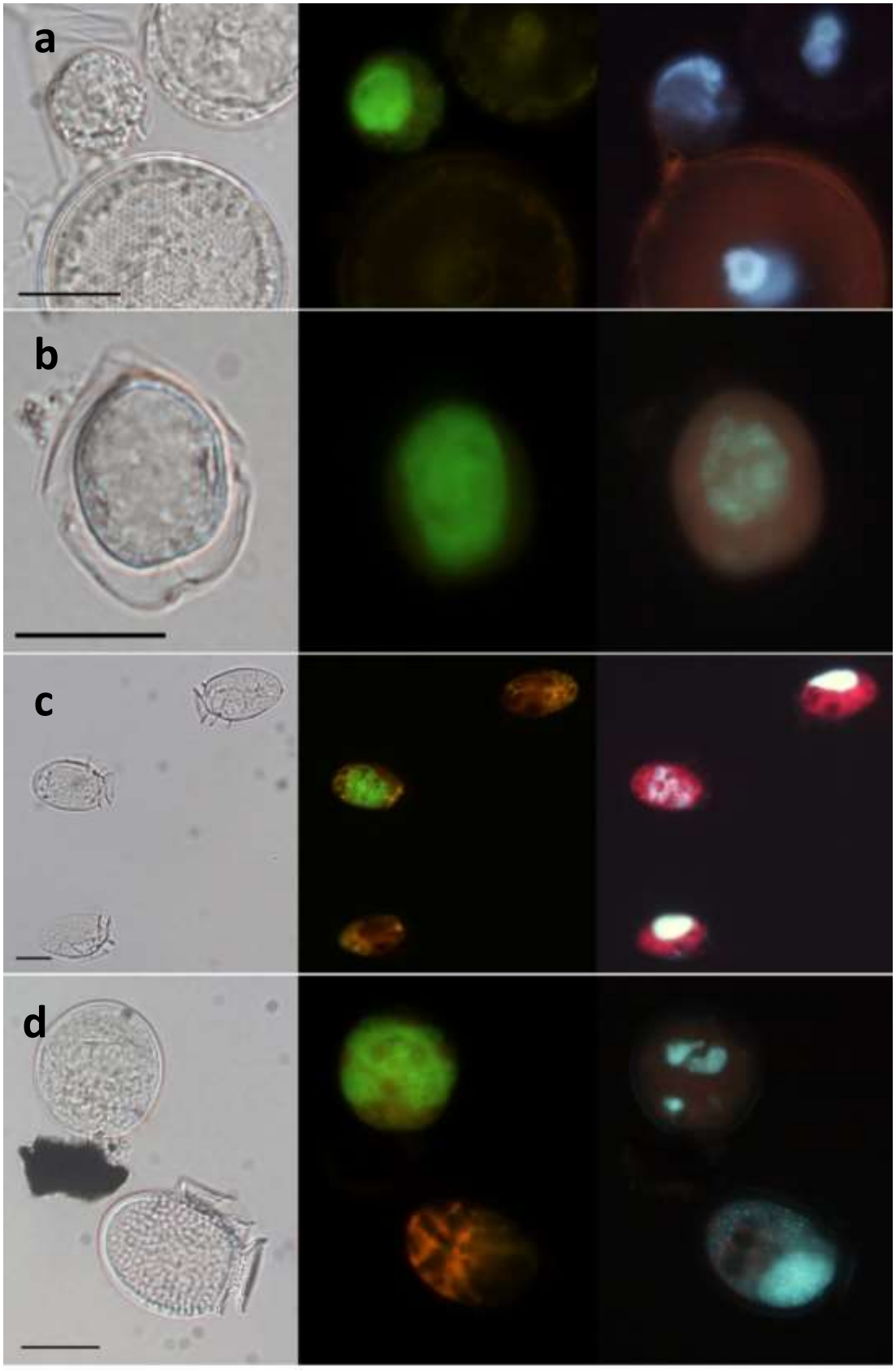


Figure 3

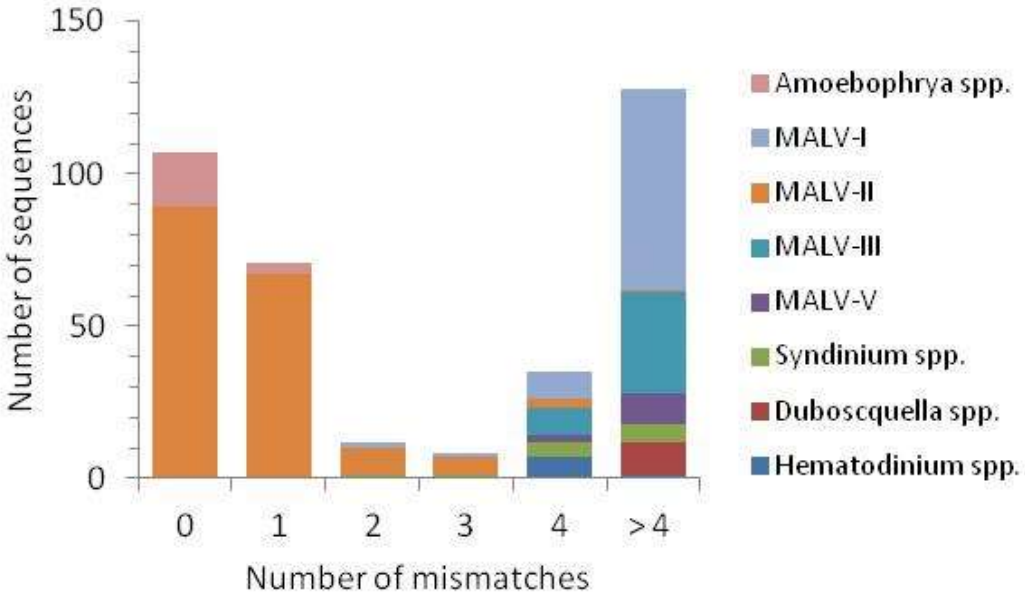
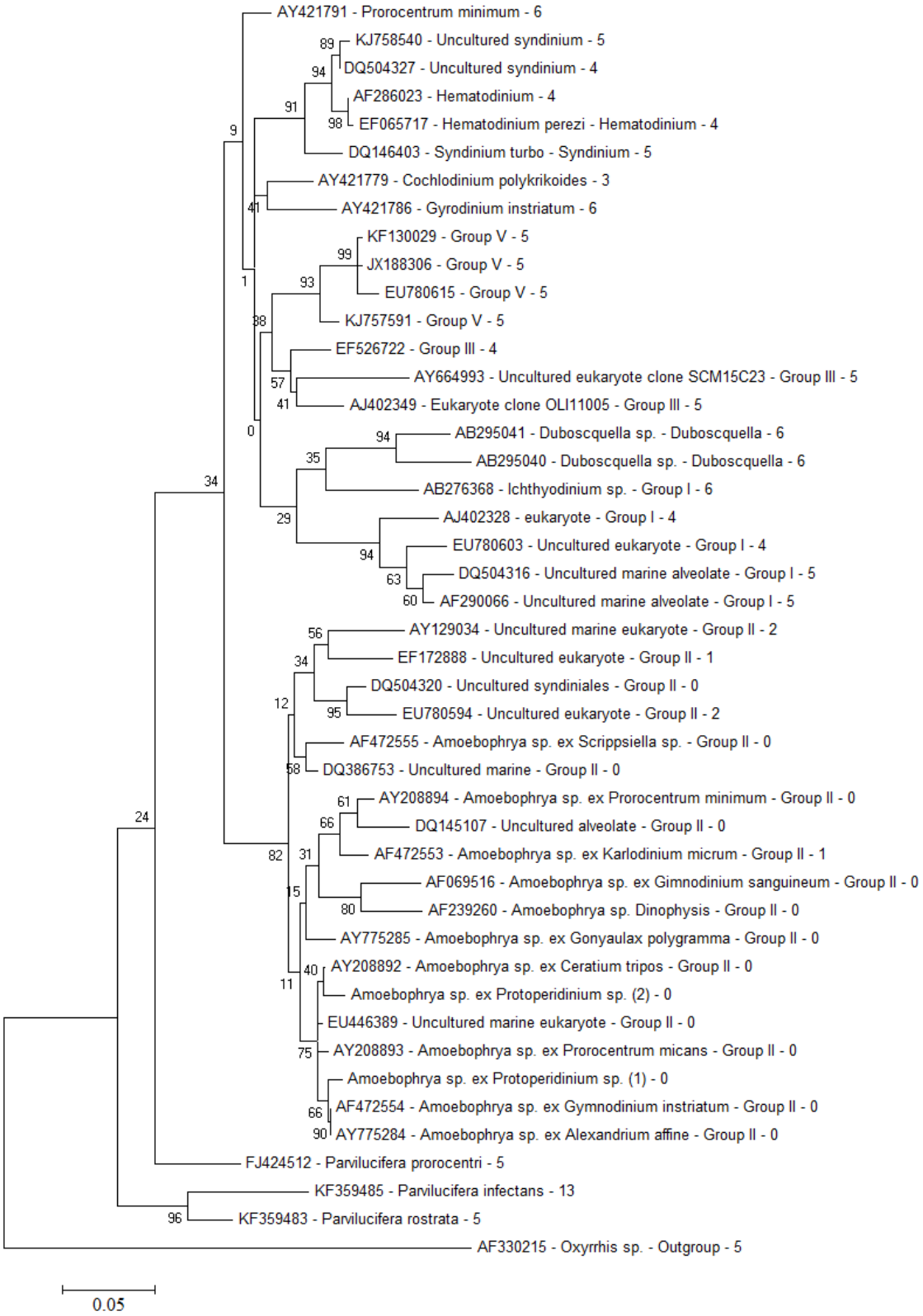

150
100
50
0
Number of sequences
0
1
2
3
4
>4
Number of mismatches
Amoebophrya spp.
MALV-I
MALV-II
MALV-III
MALV-V
Syndinium spp.
Duboscquella spp.
Hematodinium spp.

Figure 4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KJ758540 - Uncultured syndinium - 5
DQ504327 - Uncultured syndinium - 4
AF286023 - Hematodinium - 4
EF065717 - Hematodinium perezi - Hematodinium - 4
DQ146403 - Syndinium turbo - Syndinium - 5
AY421779 - Cochlodinium polykrikoides - 3
AY421786 - Gyrodinium instriatum - 6
KF130029 - Group V - 5
JX188306 - Group V - 5
EU780615 - Group V - 5
KJ757591 - Group V - 5
EF526722 - Group III - 4
AY664993 - Uncultured eukaryote clone SCM15C23 - Group III - 5
AJ402349 - Eukaryote clone OLI11005 - Group III - 5
AB295041 - Duboscquella sp. - Duboscquella - 6
AB295040 - Duboscquella sp. - Duboscquella - 6
AB276368 - Ichthyodinium sp. - Group I - 6
AJ402328 - eukaryote - Group I - 4
EU780603 - Uncultured eukaryote - Group I - 4
DQ504316 - Uncultured marine alveolate - Group I - 5
AF290066 - Uncultured marine alveolate - Group I - 5
AY129034 - Uncultured marine eukaryote - Group II - 2
EF172888 - Uncultured eukaryote - Group II - 1
DQ504320 - Uncultured syndiniales - Group II - 0
EU780594 - Uncultured eukaryote - Group II - 2
AF472555 - Amoebophrya sp. ex Scrippsiella sp. - Group II - 0
DQ386753 - Uncultured marine - Group II - 0
AY208894 - Amoebophrya sp. ex Prorocentrum minimum - Group II - 0
DQ145107 - Uncultured alveolate - Group II - 0
AF472553 - Amoebophrya sp. ex Karlodinium micrum - Group II - 1
AF069516 - Amoebophrya sp. ex Gimnodinium sanguineum - Group II - 0
AF239260 - Amoebophrya sp. Dinophysis - Group II - 0
AY775285 - Amoebophrya sp. ex Gonyaulax polygramma - Group II - 0
AY208892 - Amoebophrya sp. ex Ceratium tripos - Group II - 0
Amoebophrya sp. ex Protoperidinium sp. (2) - 0
EU446389 - Uncultured marine eukaryote - Group II - 0
AY208893 - Amoebophrya sp. ex Prorocentrum micans - Group II - 0
Amoebophrya sp. ex Protoperidinium sp. (1) - 0
AF472554 - Amoebophrya sp. ex Gymnodinium instriatum - Group II - 0
AY775284 - Amoebophrya sp. ex Alexandrium affine - Group II - 0
FJ424512 - Parvilucifera prorocentri - 5
KF359485 - Parvilucifera infectans - 13
KF359483 - Parvilucifera rostrata - 5
AF330215 - Oxyrrhis sp. - Outgroup - 5
0.05

CAPÍTULO 3

Low infection prevalence of planktonic dinoflagellates by *Amoebophrya* parasites in Guanabara Bay: a visit to the paradox of enrichment

Tatiana V. Viana[1], Piter D. F. Gomes[1], Felipe Coutinho[2], Denise R. Tenenbaum[1], Rodolfo Paranhos[3], Giovana O. Fistarol[2], Fabiano L.Thompson[2], Paulo S. Salomon[1]

[1]Marine Phytoplankton Laboratory, [2]Marine Microbiology Laboratory, [3]Hydrobiology Laboratory, Marine Biology Department, Institute of Biology, Federal University of Rio de Janeiro, Av. Carlos Chagas Filho, 373, Bloco A, CEP: 21941-902, Ilha do Fundão, Cidade Universitária, Rio de Janeiro-RJ, Brazil. [4]SAGE-COOPE-UFRJ

Abstract

Infections of planktonic dinoflagellates by eukaryotic parasitoids belonging to the genus *Amoebophrya* (Alveolata, Syndiniales) were studied in Guanabara Bay, a heavily eutrophicated marine coastal environment in south-eastern Brazil. Two locations in the bay were sampled monthly during nearly four years for dinoflagellate cell counts and parasite prevalence estimates (% of infected hosts cells). Dinoflagellates were abundant in the samples throughout the survey, including several bloom events such as *Prorocentrum triestinum* and *Levanderina fissa*, with cell densities in excess of 5.9 and 1.8 x 10^6 cells L^{-1}, respectively. Parasite infections were detected in 8 of the 45 sampling occasions at two fixed sites inside the bay, thought at extremely low prevalence levels - typically less than 0.5%. Maximum prevalence registered was 5% in an unidentified gymnodinoid host in one sporadic sampling obtained off the regular monitoring sites. Reasons for the low parasite frequency and infection prevalence, despite the high abundance of potential dinoflagellate hosts in Guanabara Bay, include (i) a direct effect of eutrophication resulting in increased carrying capacity of hosts due to high nutrient load, with consequent destabilization of the parasitic interaction, hindering parasite persistence in the environment and increasing its chances of local extinction (in line with the theory of the Paradox of Enrichment); (ii) indirect effects resulting from trophic interactions in the microbial food web that increase grazing pressure on free-living forms of the parasite, (iii) loss of fitness of the parasite due to the effect of pollution and pathogenic bacteria (e.g. vibrios) common in the organic matter- rich waters of Guanabara Bay; and (iv) the tidal regime and hydrodynamic characteristics of the bay that lead to oscillations in physical properties of the water to which parasites are more sensitive than hosts. These processes are not exclusive and may act together, with varying intensities in space and time, to suppress infection of planktonic dinoflagellates inside the bay. Our results point out to a low level of control of planktonic dinoflagellate by *Amoebophrya* parasite that is determined by biological and physical characteristics of the bay. Parasitism is not a significant loss factor for dinoflagellate populations in this environment. Together with the direct effect of eutrophication on host's carrying capacity, release from parasites might be an important factor that contributes for the recurrent and intense dinoflagellate blooms in Guanabara Bay.

Keywords: marine dinoflagellates, parasite, *Amoebophrya*, Guanabara Bay, eutrophication

INTRODUCTION

Heterotrophic marine eukaryotes of the genus *Amoebophrya* (Alveolata, Syndiniales) have a parasitic life-style infecting metazoan and protist hosts (Cachon 1964, Cachon and Cachon 1987). One such parasite, originally described as a single species, *Amoebophrya ceratii* Koepen, but now recognized as a highly genetic diverse species complex (Janson *et al* 2000, Gunderson *et al*. 2002, Salomon *et al*. 2003a, Kim *et al*. 2004, Kim *et al*. 2008), is known to infect dozens of marine dinoflagellate species in coastal waters worldwide, including several harmful (toxin-production and bloom-forming) host (Park *et al* 2004, Salomon and Imai 2006, Jephcott *et al* 2015). *Amoebophrya* spp. life-cycle has two distinct phases, a free-swimming phase represented by small (typically <5um), flagellated forms called dinospores; and a parasitic, trophic phase where the parasite develops inside the host cells from which it derives its food (Cachon & Cachon 1987). Abundance and diversity of the free-living stages in natural marine waters have only recently been reported with the help of molecular techniques (Chambouvet *et al* 2008, Diez *et al* 2001, Guillou *et al* 2008, Velo Suárez *et al* 2012). *Amoebophrya* spp. displays a typical parasitoid behaviour (i.e. the host is killed in the interaction with the parasite) (Cachon and Cachon 1987), with host-parasite population dynamics equivalent to a pray-predator interaction (Montagnes et a 2008, Salomon and Stolte, 2010). Infected hosts become metabolically and physiologically impaired and are ultimately killed at the end of the interaction (Park *et al* 2002).

The highest impact on host populations due to infections by *Amoebophrya* occurs during periods of parasite epidemic outbreaks (Nishitani *et al*. 1985, Coats *et al*. 1996, Velo Suarez *et al* 2015). Quick spread of infection is favoured at elevated host cell densities, e.g. during blooms, when encounter between parasite and host cells are more likely to occur (Coats *et al* 1996). Parasite prevalence in nature differs among different *Amoebophrya*- host dinoflagellate systems. For example, infection of the thecate dinoflagellate *Dinophysis norvegica* in the Baltic Sea is frequent but normally below 2 %, whereas the same host in infected at higher prevalence (up to 20%) in the adjacent North Sea (Gisselson *et al*. 2002, Salomon *et al*. 2003b). *Akashiwo sanguinea* populations in Chesapeake Bay frequently show localized, high prevalence levels up to 80% (Coats *et al*. 1996).

Amoebophrya spp. infecting dinoflagellates vary in their host range, the majority showing relatively high host-specificity (Salomon *et al* 2003b 2009, Coats *et al* 1996, Coats and Park 2002; Chambouvet *et al* 2008). High virulence associated with narrow host range of

several *Amoebophrya* spp. observed both in laboratory and field studies support the idea that these parasites can contribute to host population decline, being potential candidates for mitigating agents against harmful dinoflagellate blooms (Taylor 1968, Coats and Park 2002). Modelling and field observations have shown that, in cases of frequent and highly prevalent infections, *Amoebophrya*-like parasites might be as or more important than grazing in controlling dinoflagellate blooms (Chambouvet *et al*. 2008, Montagnes *et al*. 2008). Moreover, the influence of highly host specific parasites in natural populations has the potential to shape phytoplankton community structure, drive seasonal species succession (Ibelings *et al*. 2004), as well as the very presence of certain phytoplankter hosts in coastal marine environments (Chambouvert *et al*. 2008).

Environmental conditions, both physical and biological, influence host and parasite population dynamics and thus have the potential to modulate infection prevalence. Planktonic dinoflagellate populations dynamics is influenced by many biotic and abiotic factors (Smayda 1997). The same factors affect the parasites, in addition to other particularities intrinsic to the interaction with its host. For example, growth rate of *Amoebophrya* is likely temperature dependent and its reproductive output and infectivity are known to be influenced by the host nutritional status (Yih and Coats 2000). Control of dinoflagellate blooms by *Amoebophrya*-like parasite is predicted by mathematical models to be directly influenced by eutrophication (nutriente load) and also strongly dependent on biotic interactions resulting from the particular structure of the plankton community as a whole (Montagnes *et al* 2008, Alves-de-Souza *et al* 2015).

Despite the acknowledged widespread occurrence of *Amoebophrya* spp. infecting dinfolagellates in marine waters, most records come from temperate regions in the Northern hemisphere (Coats and Bockstaher 1994, Maranda 2001, Salomon *et al* 2003a, 2003b, Chambouvet *et al* 2008, Mazzillo *et al* 2011, Park *et al* 2013, Siano *et al* 2010, Velo Suarez *et al* 2015), and very few from Southern oceans (Salomon *et al* 2009, Alves-de-Souza *et al* 2012). Tropical marine waters have also not received much attention in this regard (Salomon *et al* 2009). In the present study, by means of an extensive field monitoring program with monthly samplings during nearly 4 years, we investigated *Amoebophrya* infections in planktonic dinoflagellates in two fixed sites in Guanabara Bay, a 400 Km^2, heavily eutrophicated tropical estuary connected to the Atlantic ocean in south-eastern Brazil. Water quality parameters and microbial community abundance (total bacteria and vibrio counts)

were also determined to characterize the physical, chemical and biological environment with which hosts and parasites were interacting.

Material and Methods

Study area

The study area was located in the Guanabara Bay (22°50'S; 43°10'W), a hypereutrophic estuarine system in the Rio de Janeiro State, south-eastern Brazil, near one of the largest metropolitan areas of the world. The bay's margins and catchment area include the city of Rio de Janeiro and other 15 municipalities that, together, have a human population of ca. 6 million inhabitants that cause a variety of human-related impacts to the bay, especially increased nutrient loads (Fistarol *et al* 2015).

Sampling and sample processing

From February, 2012 to October, 2015, plancton samples were collected at approximately monthly intervals in Guanabara Bay. Plain water samples and net tows for quantification of dinoflagellates and estimates of parasite prevalence were obtained from a regular monitoring program (Long Term Ecological Monitoring Program, PELD-Guananabara Bay) in two sites, one in a shallow embayment (station BG-A, depth 18m) and another in the bay's central channel (station BG-D, depth ca. 10m). Samples for microbial analysis (total bacteria and vibrio counts), physical and chemical water parameters (nutrients, temperature, and salinity), and chlorophyll measurements were obtained in a parallel monitoring program (Guanabara Bay's Microbial Observatory) at three sites (Gregoracci *et al* 2012), one at the bay's mouth, close to its connection with the Atlantic ocean (station BG-01, depth 40m), one in the central channel (station BG-07, depth 25 m) coinciding with station BG-A, and one ca. 1.5 Km from station BG-D (station BG-34, depth 1.5 m) within a shallow water embayment (Figure 1). On both series, plain water samples were taken at the water surface (ca. 0.5 m) using a 5-L Niskin bottle sampler. For phytoplancton cell counts, volumes of 250 ml of each sample were fixed with 2%, sodium cacodilate buffered, formaldehyde

immediately after sampling. Net tows (20 µm mesh size) were taken at each sampling site. One aliquot of the net tow cells was fixed with formaldehyde as above, whereas other was diluted in filtered seawater and kept alive. Additionally, 5L of water were collected, filtered through a 200 um nylon net to remove large particles and potential grazers, kept cool and protected from direct sunlight within ice-cooling boxes, and brought to the laboratory alive for plating to count vibrios, counts of live dinoflagellates and other phytoplanters in an automated imaging system, and further concentration and fixation for downstream parasite detection by FISH assays.

Upon arrival to the laboratory, 1 to 2 litres of the live plankton sample as well as an aliquot of the live cell suspension from the 20 µm mesh-size net tows from each sampling site were further concentrated in a 20 µm mesh nylon net down to 7 mL. Cells were fixed with 4 % paraformaldehyde in phosphate buffered saline (PBS, pH 8.0) for 1 h, rinsed once with PBS, twice with 70 % (v/v) ethanol, transferred to 70 % ethanol and stored at -20 °C (Salomon *et al*. 2003a).

Quantification of dinoflagellates

Dinoflagellate cells were counted either in formaldehyde-fixed samples in an inverted microscope - for samples taken from February, 2012 to January, 2014 - or alive with an automated, in-flow image acquisition and analysis system (FlowCam, Fluidic Imaging) - for samples collected from February, 2014 to October, 2015.

For the counts in the microscope, the most abundant dinoflagellates present in the water were quantified in the formaldehyde-fixed samples by the settling technique (Utermöhl 1958). Briefly, volumes of ca. 10 to 20 ml of samples were transferred to settling chambers and the cells let to settle for 72h. Cells were then counted in an inverted microscope (Olympus model BX51) at 20 or 40X magnification, depending on the cell size. A total of 400 cells of each target cell type were counted in each sample.

For the FlowCam counts, the equipment was first calibrated for size measurements using size-certified, 10 and 20 µm silicon beads, according to the manufacturer instructions. The equipment was fit with a 90 µm field-of-view flow-cell and a 10X magnification objective. Live plankton samples were analysed within 3 h of sampling. Samples were kept cool in dim light between sampling and analysis. Samples were filtered through a 100 µm nylon mesh before being aspired into the capillary line of the FlowCam. Images were

acquired in auto-imaging mode, at a flow rate of 0.1 mL per minute, an acquisition rate of 20 frames per second, for 30 minutes. Threshold for image acquisition was set to 3 um diameter as ESD (equivalent spherical diameter).

Parasite detection

For the whole survey period, detection of *Amoebophrya* infections in dinoflagellates was done in three different ways: (i) in live material in the microscope by the characteristic green autofluorescence elicited by the parasite upon blue light excitation, (ii) in live cell images collected with the FlowCam, and (iii) by means of a (whole-cell) in situ hybridization (FISH) assay with a fluorescein-tagged, rRNA-based oligonucleotide probe.

Firstly, live cells concentrated in the 20 um mesh-size net tows, or in plain water when dinoflagellate blooms where present, were investigate for infections on the same day of the sampling, immediately upon arrival of the samples in the laboratory (i.e. within 3 h of sampling). Aliquots of cells suspensions were transferred to 2-mL settling chambers and analysed in an inverted microscope (Olympus model BXi51) equipped with epifluorescence (λ_{ex} 470-490 nm, λ_{em} > 550 nm). Intermediate to mature *Amoebophrya* infections are detected by the characteristic green autofluorescence of the parasite upon blue light excitation (Salomon *et al* 2003b).

Secondly, for samples taken at sites BG-A and BG-D between February, 2014 and October, 2015, images of dinoflagellate cells (> 15 μm) collected alive with the FlowCam were inspected for infections based on the typical beehive shape of medium to mature *Amoebophrya* trophonts, associated with lack of pigmentation of the host due to consumption of host cellular material by the parasite, and other prominent parasite-induced changes in host cell morphology such as deformations and enlargement (Cachon and Cachon 1987, Salomon *et al* 2006, Salomon and Imai 2006, Salomon *et al* 2003b, Kim and Park 2014). For that, up to 500 well focused cells (selected with the edge gradiente filter of the analysis software) from each host species of interest were inspected. When a host species was present at low abundance, all focused cell images were analysed.

Finally, we used a (whole-cell) in situ hybridization (FISH) assay with a fluorescein-tagged, rRNA-based oligonucleotide probe (5'-TTA TTA TGA (AG)TC ATC CAA AA -'3) specific for *Amoebophrya* parasites infecting dinoflagellates, designed for *Amoebophrya* and previously used to detect the parasite infecting dinoflagellates in the nearby region of Cabo

Frio, ca. 150 Km north of Guanabara Bay (Salomon *et al* 2009). Cells from the vertical net tows fixed with paraformaldehyde and stored in 70 % ethanol or concentrated from plain water during blooms were used in the FISH assays as previously describe (Salomon *et al*. 2003a, 2009). After hybridization, cells were counter-stained for 15 min with 0.5 to 1 μg ml^{-1} DAPI, resuspended in an antifade solution (SlowFade Antifade, Molecular Probes) and mounted on microscope slides with cover slips sealed with nail polisher. Detection was done in an Olympus BX40 epifluorescence microscope fitted with filter cubes suitable for fluorescein (λ_{ex} 470-490 nm, λ_{em} > 550 nm) and DAPI (λ_{ex} 360-370 nm, λ_{em} > 420 nm) fluorescence detection. Up to 500 cells of each dinoflagellate species of interest present in the samples were analysed and scored as infected or non-infected.

Total bacteria and vibrio counts

For total bacteria abundance estimates, aliquots of 3 ml were fixed with 0.05% glutaraldehyde and 1% paraformaldehyde for 10 minutes at RT in the dark and snap-frozen in liquid nitrogen for analysis of bacteria for flow cytometry. Bacterial abundance was determined in two replicates of seawater by flow cytometry after staining with the nucleic acid dye SYBR green-I (LifeTechnologies, Carlsbad, CA) according to Andrade *et al*. (2003), with minor modifications. For vibrio counts, freshly collected seawater samples (within max 2h after sampling sampling) were serially diluted with sterile seawater by factors of 10 and plated in triplicate (100 μL) in Thiosulphate Citrate Bile Salts Sucrose (TCBS) agar plates. Colony counts were performed 24 h after incubation at 28°C.

Biological, physical and chemical water parameters

Physical and chemical environmental parameters were analyzed by standard oceanographic methods (Grasshoff *et al*. 1999). Chlorophyll a analyses were performed after vacuum filtration (<25 cm of Hg) of 2L of seawater. The filters (Glass fiber Millipore AP15) were extracted overnight in 90% acetone at 4 °C, and analyzed by spectrophotometry or fluorimetry. Temperature and salinity were evaluated by using CTD or salinity meters from YSI. Inorganic nutrients analyzed were : 1) ammonia by indophenol, 2) nitrite by diazotization, 3) nitrate by reduction in Cd-Cu column followed by diazotation, and 4) orthophosphate by reaction with ascorbic acid.

The environmental parameters and data on bacteria and vibrios were analyzed as boxplos, with the aid of R and RStudio program (version 0.98.1103). In them contains information on the estimates of Whisker, the first line of the box as the first quartile, the median (thicker line in the middle of the box) and the third quartile to the top of the box. In the graphs are also presented outliers (small spheres).

Results

Physical and chemical characteristics of the sampling sites

Average water temperature over the whole sampling period differed among sites, ranging from 22.7 °C (max 27.2°C) at the entrance of the bay (site BG-01), close to the ocean, to 23.8°C (max 27.9°C) in the central channel station (site BG-07), and 25.7°C (max 31.7°C) in the shallow station (site BG-34) (Figure 2). Salinity had an opposite trend compared to temperature, with higher values at the bay's entrance and central channel stations, and lower values in the shallow water site. Besides higher average values, the shallow water site showed much more variation in temperature and, notably, salinity, than the other two sites. Dissolved inorganic nitrogen (DIN: nitrite + nitrate + ammonium) averaged 13.3 ± 8.9 µM (max 36.2 µM) at the entrance of the bay, 23.5 ± 17.1 µM in the central channel and 247.9 ± 89.1 µM (max 436.5 µM) in the shallow site. Ammonium was normally the major form of inorganic nitrogen present in the water, especially in the shallow site where it accounted for 98 ± 2% of the DIN. Average inorganic phosphorus (orthophosphate) also increased from the bay's mouth (1.0 ± 0.5 µM) towards the central channel (1.5 ± 1.0 µM), to ca. 10 times more at the shallow site (11.6 ± 5.3 µM). Dissolved oxygen concentrations were ca. 4 mgL^{-1} both at the entrance of the bay and in the central channel, whereas the shallow station had levels averaging ca. 2.5 mgL^{-1} (Figure 2).

Microbial community and chlorophyll concentrations

Total bacteria counts by flow cytometry showed a seasonal pattern with higher concentrations in the austral summer months (December to March, data not shown). Bacteria concentrations at the bay's mouth and the central channel sites averaged 4.1 ± 3.7 X 10^6 cells L^{-1} and 9.0 ± 7.8 X 10^6 cells L^{-1}, respectively, whereas the shallow sampling site had on

average 23.0 ± 10.8 X 10^6 cells L^{-1} (Figure 2). Vibrio counts estimated by the plating technique showed highly distinct patterns between the two deep stations (mouth and central channel) compared to the shallow site. Whereas in the former average vibrio concentrations were similar at ca. 0.15 X 10^6 cells L^{-1}, in the latter several outbreaks that occurred typical, but not exclusively, during the summer season, were observed, with concentration averaging 2.5 X 10^6 ± 4.5 cells L^{-1} throughout the whole survey period, and maximum in excess of 21 X 10^6 cells L^{-1}in discrete samples. Chlorophyll *a* concentration was highly variable within and among sites with a minimum of 1 µg L^{-1} measured at the bay's mouth up to 623 µg L^{-1}, detected in the shallow water site. Average chlorophyll concentration in the shallow site (average 168 ± 148 µg L^{-1}) was 5 to 10 times higher than in the other two sites, with several peaks above 300 µg L^{-1} (Figure 2).

Diversity and abundance of dinoflagellates

Dinoflagellates were systematically detected in all samples analysed. The most frequent and abundant dinoflagellate taxa detected at stations BG-A and BG-D are listed in Table 1. This set of species was present on both sites, though at different concentrations in any given sampling time and with high variability over time (Figure 3 and 4). The most representative species included armoured forms like *Prorocentrum triestinum*, present in 93% of the samples on both sites, at densities up to 5.9 X 10^6 cells L^{-1}, and in less amounts its relatives *P. dentatum* and *P micans*; and also *Scrippsiella* spp. Among representatives of naked dinoflagellates the relatively large *Levanderina fissa* (ø ca. 50 µm), present in 60% of the samples from station BG-A and 65% from station BG-B, at densities up to 1.8 X 10^6 cells L^{-1}, and small, unidentified gymnodinoids of the nanoplankton size-class, were the most abundant taxa. Other frequently observed dinoflagellate taxa were *Dinophysis* spp., *Gyrodinium* spp., *Polikrykus* sp., *Oxyphysis* sp., and *Protoperidinium* spp.

Parasite infections

Infection of dinoflagellates by *Amoebophrya* parasites was observed in only 8 sampling occasions within the regular monitoring on sites BG-A and BG-D. Most observations (7) were made on site BG-A, and only once in site BG-D. Other three

observations were made in samples obtained off the regular sampling program in opportunistic samples by other researchers (Figure 3). Very few hosts were found infected, either by inspection of live cells in the epifluorescence microscope, DAPI-staining, or with the FISH-probe assay (Table 2). Infection was only observed in small dinoflagellates of the nanoplancton size-class. The highest infection prevalence recorded during the whole period was 5% in a dense population (25.5 x 10^3 cells L^{-1}) of an unidentified gymnodinoid of ca. 17 μm in an opportunistic sampling at site E, off the regular sampling program sites (see map in Figure 1). At this occasion, the emergence process of a mature *Amoebophrya* trophont from its host cells was observed, as in a sample from site BG-07 from Augusts, 2015. Early infections were also detected in live samples (Figure 5). None of the large, microplanctonic dinoflagellates common in the samples, markedly *Levanderina fissa*, were found infected.

Discussion

Field observations of infection of free-living marine dinoflagellate species by the parasite *Amoebophrya* spp. have accumulated in the literature since the early reports in the end of the 19th century (see reviews by Park *et al*. 2004 and Jephcott *et al* 2015). Most observations, however, still originate from temperate coastal marine areas of the northern hemisphere, whereas much less information exists for the southern oceans, especially for tropical regions. The Southern Atlantic ocean is one of the least studied areas in this aspect. The only reports on the occurrence of *Amoebophrya* spp. in Southern Atlantic coastal waters comes from the upwelling area around Cabo Frio, ca. 120 Km east of the Guanabara Bay, in the northernmost limit of the South Brazil Bight (Salomon *et al*. 2006, Salomon *et al* 2009). In the Cabo Frio area, populations of the thecate dinoflagellate *Neoceratium falcatiforme* (former *Ceratium falcatiforme*) were found infected by the parasite, that was detected using the same FISH probe as we used here (Salomon *et al* 2009). This study, therefore, expands the knowledge on the distribution of *Amoebophrya* spp. in marine waters and cast new light in the mechanisms that regulate parasite control of planktonic dinoflagellate populations in highly eutrophic coastal systems.

With no doubt, the most striking finding of this study was the low frequency (% of samples with detectable infection) and prevalence (% of infected cells in host populations) by *Amoebophrya* spp. in dinoflagellates throughout almost four years of monthly sampling in Guanabara Bay. *Amoebophrya* infection prevalence in host populations in natural marine environments is highly variable. Prevalence observed at any given time depends primarily on the intrinsic dynamics of each particular host-parasite system, with periods of low prevalence intercalated with peaks of parasite outbreaks that exert severe control on hosts (Coats and Bockstahler 1994, Chambouvet *et al* 2008, Salomon and Stolte 2010). High *Amoebophrya* spp. prevalence in natural dinoflagellate populations is usually observed when host cell densities are in the order of 10^6 L^{-1} (Nishitani *et al* 1985, Coats *et al*. 1996, Velo Suárez *et al* 2015,). Biotic interactions within the microbial food web, notably grazing on parasite's free-living forms by ciliates that remove parasites from the water (Maranda *et al* 2001, Johansson and Coats2002, Montagnes *et al* 2008, Kagami *et al* 2007), have the capacity to hider infection outbreaks. High abundance of phytoplankton cells other than dinoflagellate hosts might have a significant dilution effect on the control of dinoflagellate blooms by parasites, either by resource competition with dinoflagellates, that reduces the number of potential hosts, or by affecting numerical-functional responses of grazers that increase consumption of free-living parasite stages (Alves-de-Souza *et al* 2015). Parasite-induced changes in the physiology and behaviour of host cells, such as photosynthetic performance and diel vertical migration patterns that segregates infected from non-infected populations (Coats and Bockstahler 1994), also influences the outcome of prevalence. Moreover, physical properties of the water (e.g. temperature and nutrient concentrations) influence infection dynamics by determining important features of the infective cycle, such as the parasite development time inside the host and parasite reproductive output i.e. number of parasites produced per infected hosts (Nishitani *et al* 1985, Salomon and Stolte 2010, Alves-de-Souza *et al*. 2015). Low infection prevalence by *Amoebophrya* on dinoflagellates in certain environments, such as the Baltic Sea were mid to mature infections are consistently below 2% in *Dinophysis* spp., has been associated to the presence of an inefficient parasite type and unfavourable environmental setting (Gisselson *et al* 2002, Salomon *et al* 2003a and 2003b). Nevertheless, even at low levels, infections are frequently observed in natural marine systems in studies with considerably less sampling effort than we made in Guanabara Bay (e.g. Salomon *et al* 2003a, Salomon *et al* 2009). We searched for *Amoebophrya* infections in more than 150 plankton samples over a time span of almost 4 years, throughout a wide range of dinoflagellate

concentrations, including several bloom events, and using different techniques. The fact that the FISH probe did not unveil *Amoebophrya* infections could be due to the presence of genetically distinct parasites with too many mismatches in the probe site. Though this possibility cannot be completely ruled out, it is very unlike. First, the probe targets a conserved region of the 18SrRNA, and has been successfully used to detect *Amoebophrya* infecting several dinoflagellate taxa in the Atlantic coast nearby Guanabara Bay (Salomon *et al* 2009) and various other marine areas (unpublished data). Moreover, prior to and during the course of this work, we checked the probe sequence against the database. As of July, 2016, the dap-1 probe matches all known *Amoebophrya* sequences in the database with maximum 1 mismatch. Secondly, staining of cells with nucleic acid dyes (such as DAPI and SYTOX) is an efficient technique to visualize medium to late trophonts inside host cells, though early infective stages might be missed (Gisselsson *et al* 2002, Salomon *et al* 2003a, b). Likewise, mature trophonts are readily spotted by their beehive shape when infecting hosts. Had an *Amoebophrya* outbreak occurred, we would have easily detected at least the late infection stages, as we observed in few samples at low prevalence.

In addition to low or undetectable infections by *Amoebophrya* spp., no other parasites known to infect dinoflagellates were observed in Guanabara Bay, as for example Perkinsids of the genus *Parvilucifera* that are easily recognized by a characteristic cocoon-like structure (sporangium) in mature infections (Norén *et al* 1999) and are also widespread in marine waters though much less host-specific than *Amoebophrya* (Norén *et al* 2000, Jephcott *et al* 2015).

Because no significant infection prevalence by *Amoebophrya* was observed in planktonic dinoflagellates of the Guanabara Bay during the almost 4-year survey with monthly sampling in two locations, the question of whether this parasite is present in its free-living form in the bay's waters arouse. *Amoebophrya*-related sequences are commonly found in DNA clone libraries of microbial marine communities in size fractions smaller than 10 or 5 um (Massana *et al* 2015). This evidence of the presence of the parasites in the water indicates a scenario for potential encounter between parasite and dinoflagellate hosts. Though we did not estimate abundance of the free-living parasites in the water, quantification of these forms are possible with the use of FISH probes similar to the ones that we used (Chambouvet *et al*. 2008).

Amoebophrya-host interactions can be modelled as a typical Lotka-Volterra prey-predator association (Montagnes *et al* 2008, Salomon and Stolte 2010, Alves-de-Souza *et al*

2015). In this type of inter-specific interaction, trophic dynamic model simulations predict the destabilization of the system at increased carrying capacity of the pray/host, producing increasingly higher and more time sparse oscillations in population densities of both players, with high probability of extinction of the organisms involved. This model-predicted phenomenon is known as the Paradox of Enrichment (Rosenzweig 1971). In case of parasitism, due to the unidirectional dependence of the parasite for its host, as is the case for *Amoebophrya* (Coats and Park 2002), the risk of local extinction relies all on the parasite side, whereas the hosts is free to consume resources and grow (Salomon and Stolte 2009).

For marine dinoflagellate populations, carrying capacity is increased when availability of resources for growth is high, condition met in highly eutroficated, nutrient rich waters (Smayda *et al* 1997). Using a modelling approach of the marine microbial food web, Alves-de-Souza *et al* (2015) have shown that eutrophication, besides having direct influence on both dinoflagellate hosts and parasites growth, has also the effect of hindering parasite infections by affecting numerical-functional responses such as of grazers that consume free-living parasite stages (Johansson and Coats 2002). Tight control of dinoflagellates by *Amoebophrya* has been observed in small, enclosed aquatic systems where encounter between host and parasites is facilitated, like reported for *Alexandrium fundyense* populations in a small (82,200 m^2), shallow saline pond (Salt Pond, Nauset Marsh System, Eastham, MA, USA) (Velo Suárez *et al* 2013). In larger and more hydrodynamic systems such as the Guanabara Bay, encounter between hosts and parasite is likely to be disturbed by e.g. freshwater flushes from rivers entering the bay, and tidal mixing. The shallow embayment we sampled in Guanabara Bay (stations BG-D and BG-34) is much more eutrophicated and shows more variable hydrology (e.g. wider oscillations of temperature and salinity) than the central channel site. *Amoebophrya* was detected only in one occasion throughout the survey, despite the fact that dinoflagellates are, on average, twice as abundant there than in the central channel site. This fact corroborates the hypothesis that heavy eutrophication hinders parasite infections, lending support to the Paradox of Enrichment Theory (Rozensweig 1971).

Guanabara Bay is an estuarine system highly impacted by human activities (Fistarol *et al* 2015). High loads of nutrients promote the growth of primary producers and shape the abundance and diversity of the components of the microbial food web (Gregoracci *et al* 2012). Although continuous, long term time series of Guanabara Bay's plancton communities are not available, there are evidences that point out to an increase in the relative contribution of flagellated forms in detriment to diatoms in this system (Villac and Tenenbaum, 2010;

Fistarol *et al* 2015), a typical consequence of eutrophication in marine waters. The inner, shallow parts of the bay like our stations BG-D and BG-34, which receives higher nutrient inputs than the more hydrodynamic channel areas, favor higher microbial growth, including many vibrios (Gregoracci *et al* 2012). Several vibrios are known pathogens of a variety of marine animals (Austin *et al* 2005, Thompson *et al* 2004). Whether and by which mechanisms this high prokaryotic cell load influences *Amoebophrya* infections needs further investigation.

Besides high eutrophication, Guanabara Bay has several maritime ports that deals ships from all over the world. These are vectors for introduction of indigenous dinoflagellate species via ballast water. Success of invasive species is due to, in part, escape of enemies (predators, parasites) in the new environment, according to the Enemy Release Hypothesis. Escape from *Amoebophrya* parasites has been claimed as one of the main reason for the success of *Alexandrium* spp. in Thau lagoon, France (Chambouvet *et al* 2011) and it might be also the case for some of the common parasites in Guanabara Bay.

Conclusions

During our survey we observed several dinoflagellate blooms in the Guanabara Bay's waters. Epidemics of *Amoebophrya* infections in these common (bloom-forming) dinoflagellate species are rare, and possibly inexistent. The destabilization of *Amoebophrya*-host systems due to eutrophication, as predicted by the theory of the Paradox of Enrichment by Rozensweig (1971) is here evoked as a mechanism that hampers *Amoebophrya* proliferation in Guanabara Bay, favouring the development of dinoflagellate populations. Human-induced changes in marine systems through eutrophication might have much more profound consequences for the functioning of microbial food webs than the immediate, direct increases in primary and secondary productivity. By disrupting interactins such as between *Amoebophrya* spp. and its dinoflagellate hosts, nutrient enrichment of marine coastal waters can lead to changes in carbon flow in sea, resulting in an unbalanced status where dinoflagellate blooms will become even more frequent.

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Tables

Table 1. Summary of cell concentration (cells mL^{-1} X 10^3) statistics of selected dinoflagellate taxa that where more frequent and abundant in the samples of stations BG-A and BG-D during the sampling period. Data for total dinoflagellate concentration is also shown. Max: maximum; SD: standard deviation. The last four columns represent the frequency (percentage) out of all samples analysed in each site where each particular taxon was present above detection limit (>0), and above 100, 1000, and 5000 cells mL^{-1} X 10^3, respectively.

Station BG-A Taxon	Max	Mean	SD	% >0	% >100 x10^3	% >1000 x10^3	% >5000 x10^3
Levanderina fissa	1833	134	403	60	14	14	0
Dinophysis spp.	43	2	7	33	0	0	0
Prorocentrum triestinum	2076	158	337	93	37	37	0
Prorocentrum micans	36	4	7	58	0	0	0
Prorocentrum dentatum	267	14	48	44	5	5	0
Gyrodinium spp.	140	12	24	81	2	2	0
Scrippsiella spp.	396	24	63	77	5	5	0
Protoperidinium spp.	5	0	1	5	0	0	0
Gymnodinoids <20 µm	417	65	97	76	17	17	0
Total dinoflagellates	***3868***	***516***	***750***	***100***	***79***	***79***	***0***

Station BG-D Taxon	Max	Mean	SD	% >0	% >100 x10^3	% >1000 x10^3	% >5000 x10^3
Levanderina fissa	1226	72	210	65	12	12	0
Dinophysis spp.	36	4	8	53	0	0	0
Prorocentrum triestinum	5898	706	1190	93	63	63	2
Prorocentrum micans	992	35	151	63	7	7	0
Prorocentrum dentatum	283	10	44	30	2	2	0
Gyrodinium spp.	568	40	87	84	2	2	0
Scrippsiella spp.	2046	116	315	93	23	23	0
Protoperidinium spp.	19	1	4	11	0	0	0
Gymnodinoids <20 µm	1553	161	267	81	40	40	0
Total dinoflagellates	***7692***	***1548***	***1765***	***100***	***88***	***88***	***7***

Table 2. *Amoebophrya* infections observed in Guanabara Bay plankton samples collected from February, 2012, to October, 2015.

Sampling date	Sampling site	Parasite prevalence	Host
01/02/2012	BG-A	n.c.	n.i.
28/03/2012	BG-A	n.c.	n.i.
01/02/2013	BG-A	n.c.	n.i.
27/03/2013	BG-A	0,5	small gymnodinoid
15/05/2013	E	5,0	small gymnodinoid*
18/11/2013	BG-A	n.c.	n.i.
19/03/2014	BG-A	n.c.	small gymnodinoid*
14/04/2014	BG-A	n.c.	small gymnodinoid
11/08/2014	BG-D	n.c.	large gymnodinoid
21/01/2015	Near BG-01	0,25	small gymnodinoid
21/01/2015	Near BG-01	0,25	*Prorocentrum cordatum**
21/01/2015	Near BG-01	0,5	*Protoperidinium* sp.
21/01/2015	Near BG-01	0,5	*Scripsiella* sp.
07/08/2015	BG-07	n.c.	*Prorocentrum cordatum*

*infected cells from Figure 6.

Figure Legends

Figure 1. Map of Guanabara Bay showing the stations sampled in this work. Continuous, monthly sampling was done at stations BG-01, BG-07, BG-34 within the Microbial Observatory monitoring program (Gregoracci *et al* 2012); and BG-A and BG-D. One sample was obtained at site E.

Figure 2. Water temperature and salinity, total inorganic nitrogen (TIN = nitrite + nitrate +ammonia), phosphate, dissolved oxygen, chlorophyll, total bacteria, and vibrios concentrations at three sites monitored in Guanabara bay from January 2012 to December, 2015. Chlorophyll *a* concentration, total bacteria and vibrio counts during the sampling period at two stations in Guanabara Bay.

Figure 3. Total dinoflagellates, small gymnodinoid forms, and *Prorocentrum triestinum* and *Levanderina fissa* cell concentrations during the sampling period of February, 2012, to October, 2015, at two sites in Guanabara Bay (BG-A and BG-D, see map in Figure 1 for details). Arrows and the numbers close to them indicate cell concentrations beyond the scale of the graph for particular sampling occasions. Diamonds in the upper panel represent sampling occasions where *Amoebophyra* spp. infections were detected in dinoflagellates according to Table 2 (green: station BG-A, orange: station BG-D, blue: sporadic samplings obtained elsewhere in the bay).

Figure 4. Cell concentration of the dinoflagellate taxa *Scrippsiella* spp., *Gyrodinium* spp., *Prorocentrum micans*, and *Dinophysis* spp. during the sampling period at two sites in Guanabara Bay. Sampling sites color code as in Figure 3. Arrows and the numbers close to them indicate cell concentrations beyond the scale of the graph for particular sampling occasions.

Figure 5. Micrograhps of dinoflagellate cells collected in Guanabara Bay infected with *Amoebophrya* spp. parasites. Hybridization Prorocentrum sp and Protoperidinium in Guanabara Bay. Cells were labeled probe hybridized and counterstained with DAPI. In addition to the common micrograph, UV light and blue light were used. **A to C**: *Prorocentrum* with mature infection *Amoebophrya* found in an occasional sample, in January

2014. **D to F**: infection in *Protoperidinium* sp. also that occasional sample, although with somewhat different aspects of previous infection. Scale: 10um. **G, H and I:** Sequence of micrographs of the emergence of *Amoebophrya* from a *Prorocentrum cordatum* cell collected at station BG-07 in August, 2015.**J and K:** Early infection of a gymnodinoid dinoflagellate by *Amoebophrya* unveiled by the green autofluorescence of the parasite (arrow) inside the host. Two non infected hosts are seen in the same frame. Bright field (d) and epifluorescence (e) microscopy of the same field of view. The red color comes from the chlorophyll fluorescence elicited upon blue light excitation of life cells. Cells from net tow collected at station BG-A (central channel) in March, 2014. f to g: Scale bar: 10µm.

Figure 1

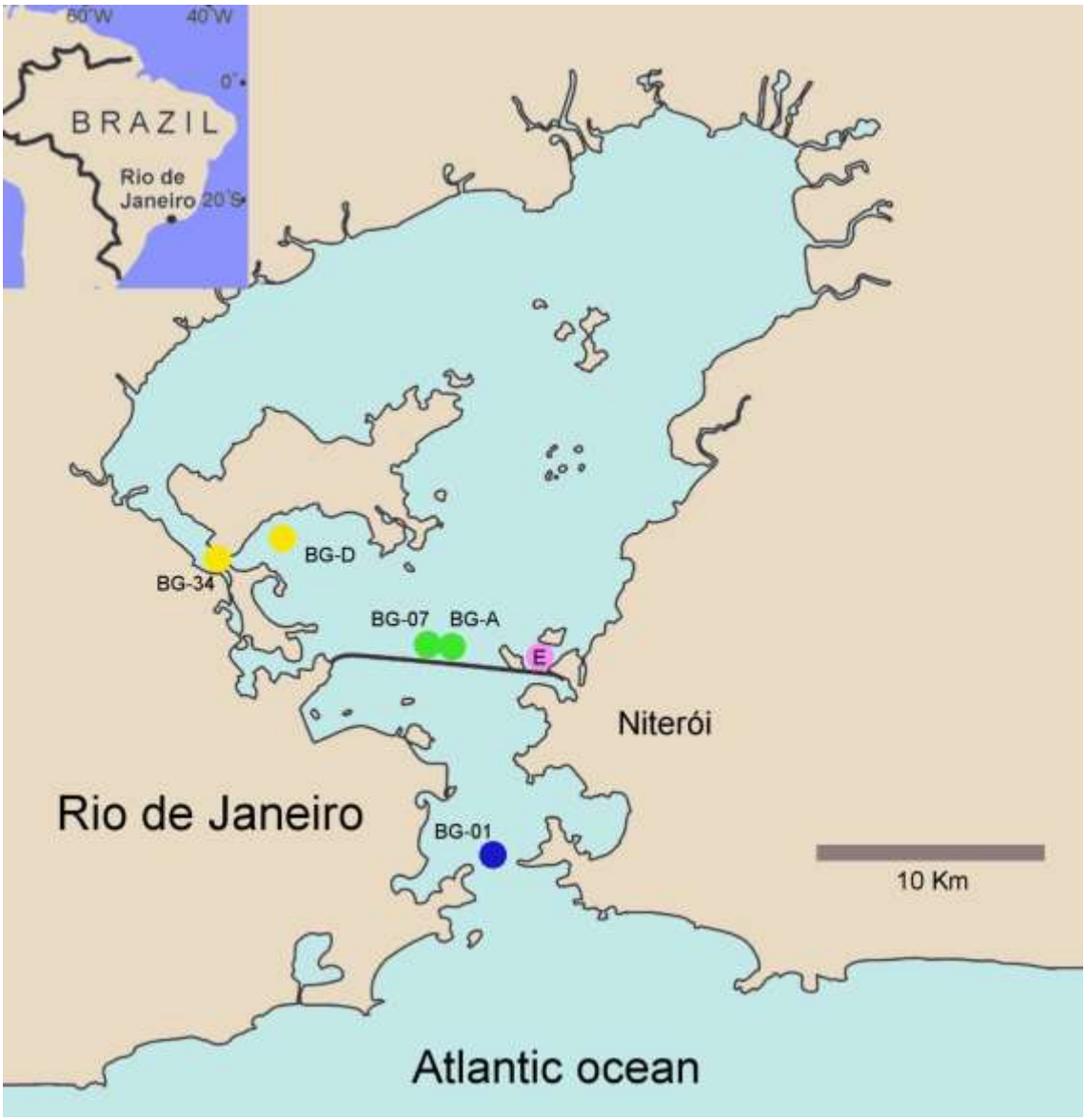

60°W
40°W
0°
BRAZIL
Rio de Janeiro 20°S
BG-D
BG-34
BG-07
BG-A
E
Niterói
Rio de Janeiro
BG-01
10 Km
Atlantic ocean

Figure 2

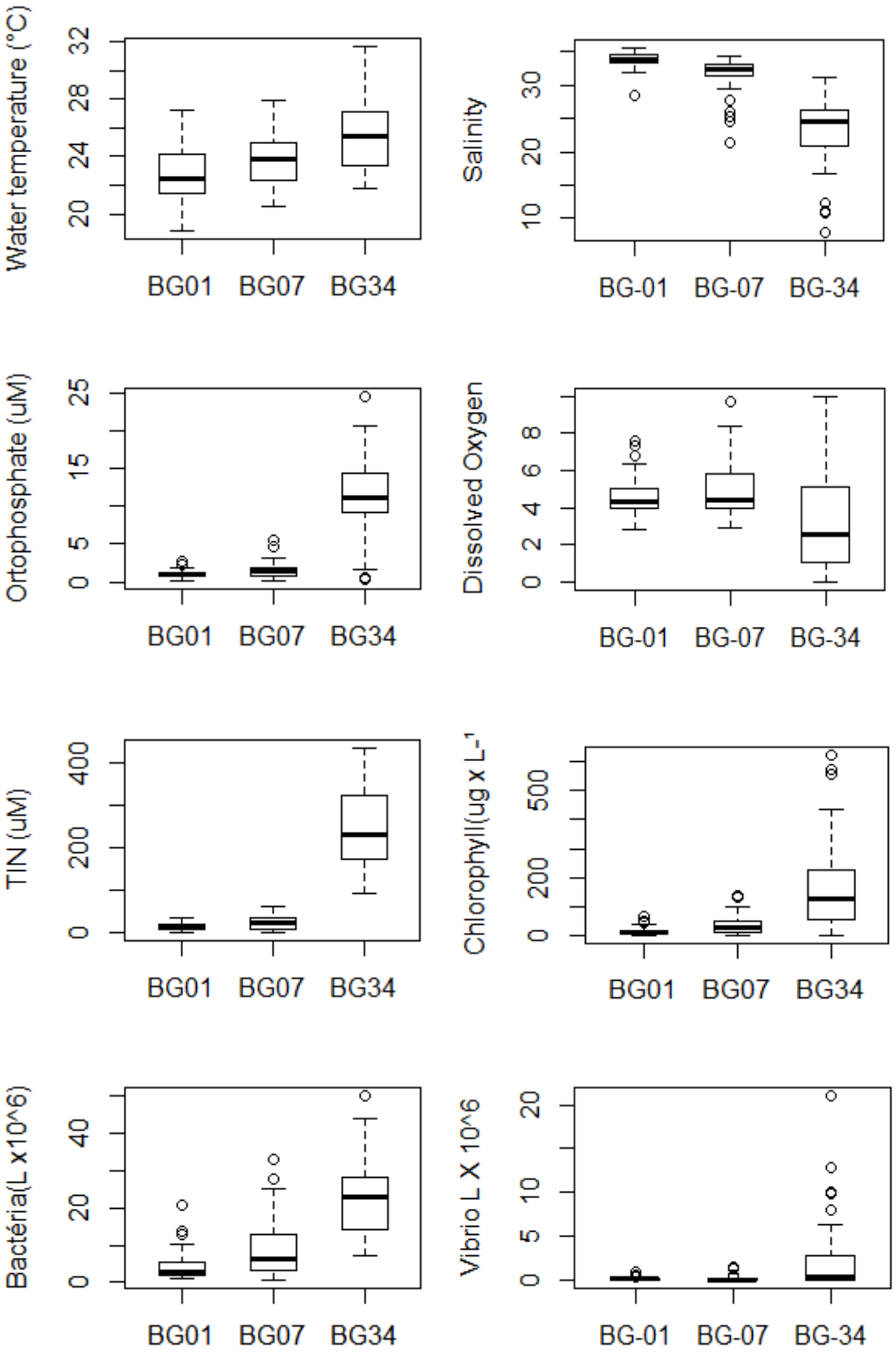

Water temperature (°C)
20
24
28
32
BG01
BG07
BG34
Salinity
10
20
30
BG-01
BG-07
BG-34
Ortophosphate (uM)
0
5
15
25
BG01
BG07
BG34
Dissolved Oxygen
0
2
4
6
8
BG-01
BG-07
BG-34
TIN (uM)
0
200
400
BG01
BG07
BG34
Chlorophyll(ug x L-1
0
200
500
BG01
BG07
BG34
Bactéria(L x10^6)
0
20
40
BG01
BG07
BG34
Vibrio L X 10^6
0
5
10
20
BG-01
BG-07
BG-34

Figure 3

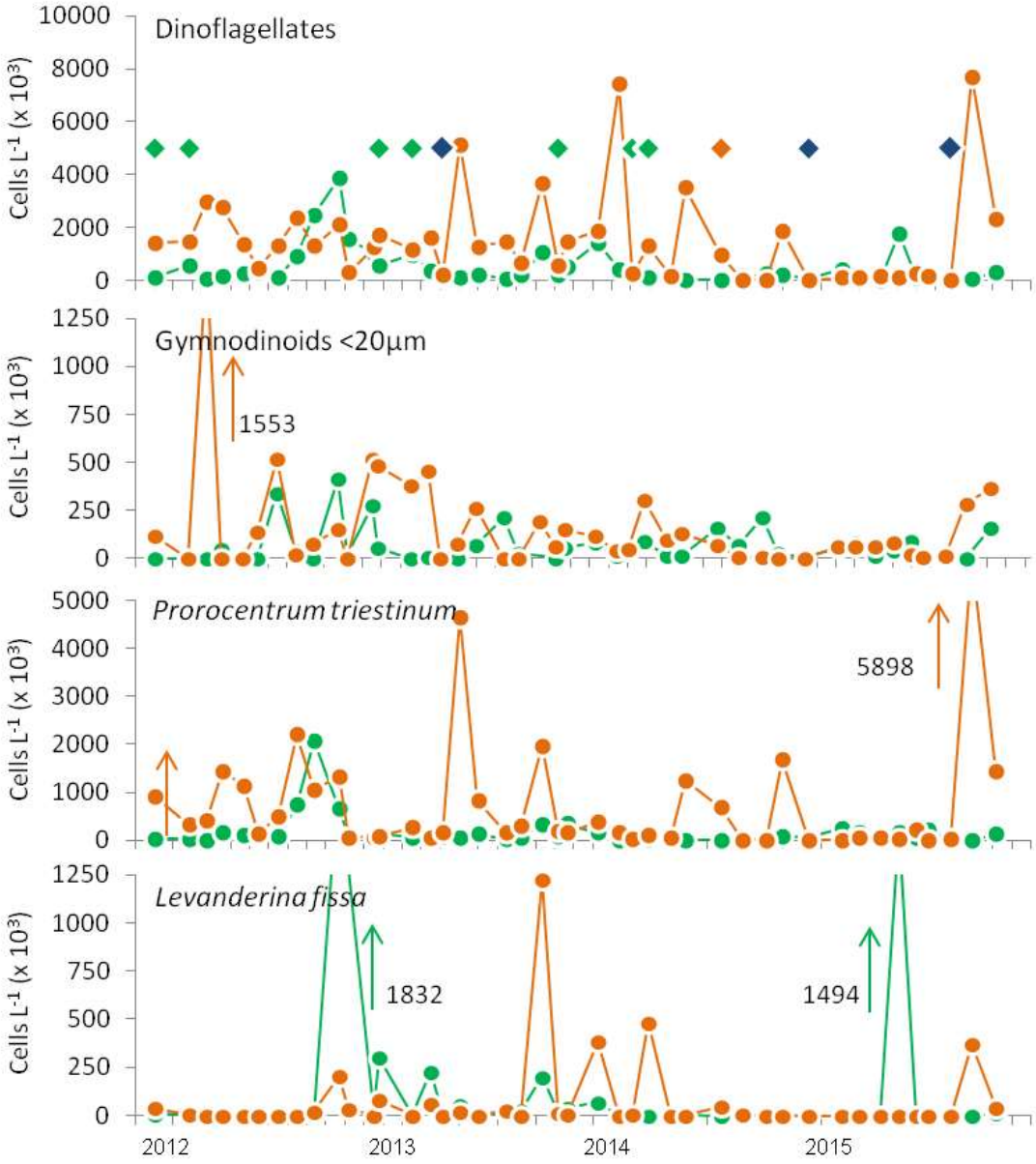

Dinoflagellates
Cells L-1 (x 10³)
10000
8000
6000
4000
2000
0
Gymnodinoids <20µm
1250
1000
750
500
250
1553
Prorocentrum triestinum
5000
4000
3000
2000
1000
5898
Levanderina fissa
1832
1494
2012
2013
2014
2015

Figura 4

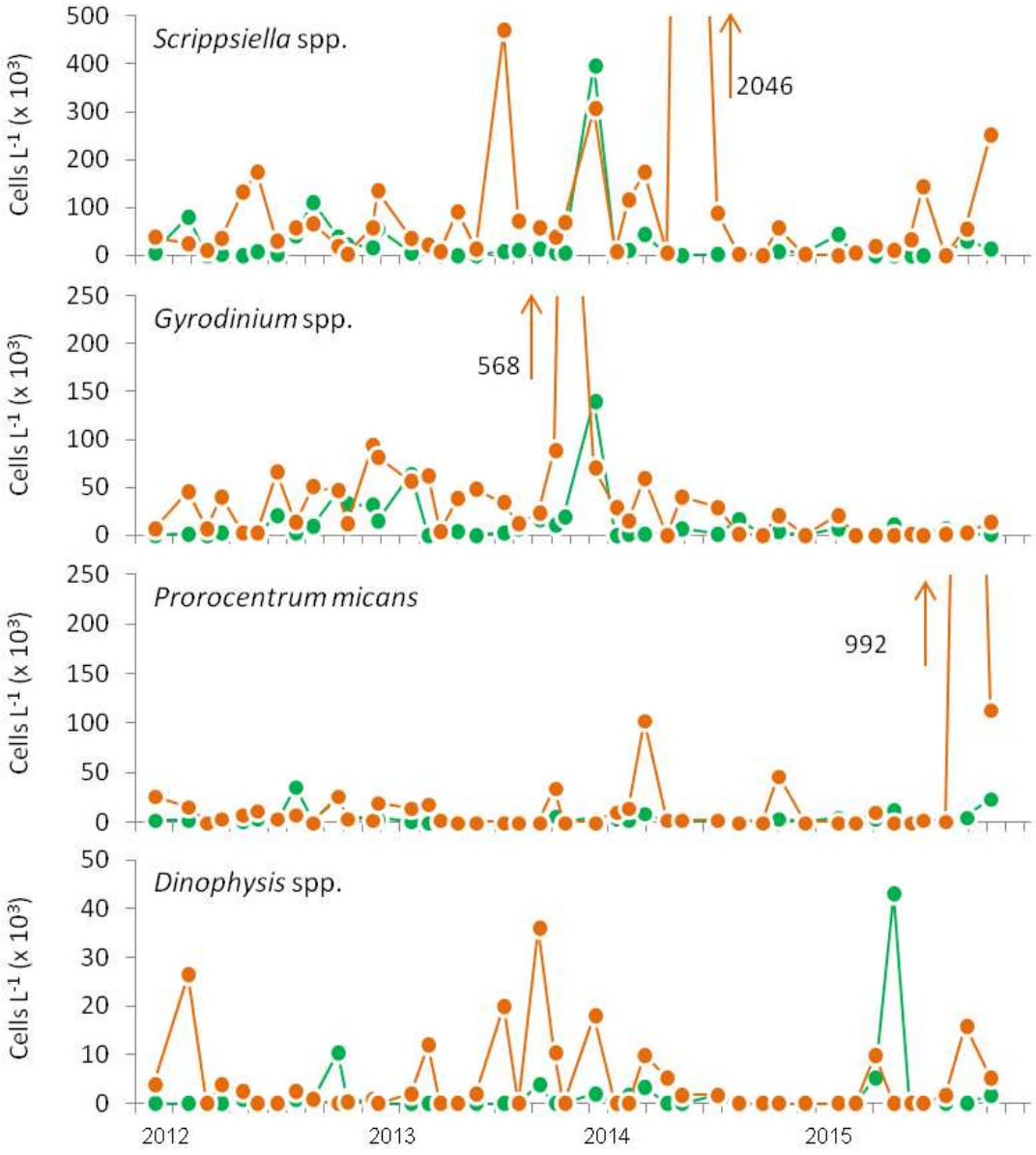
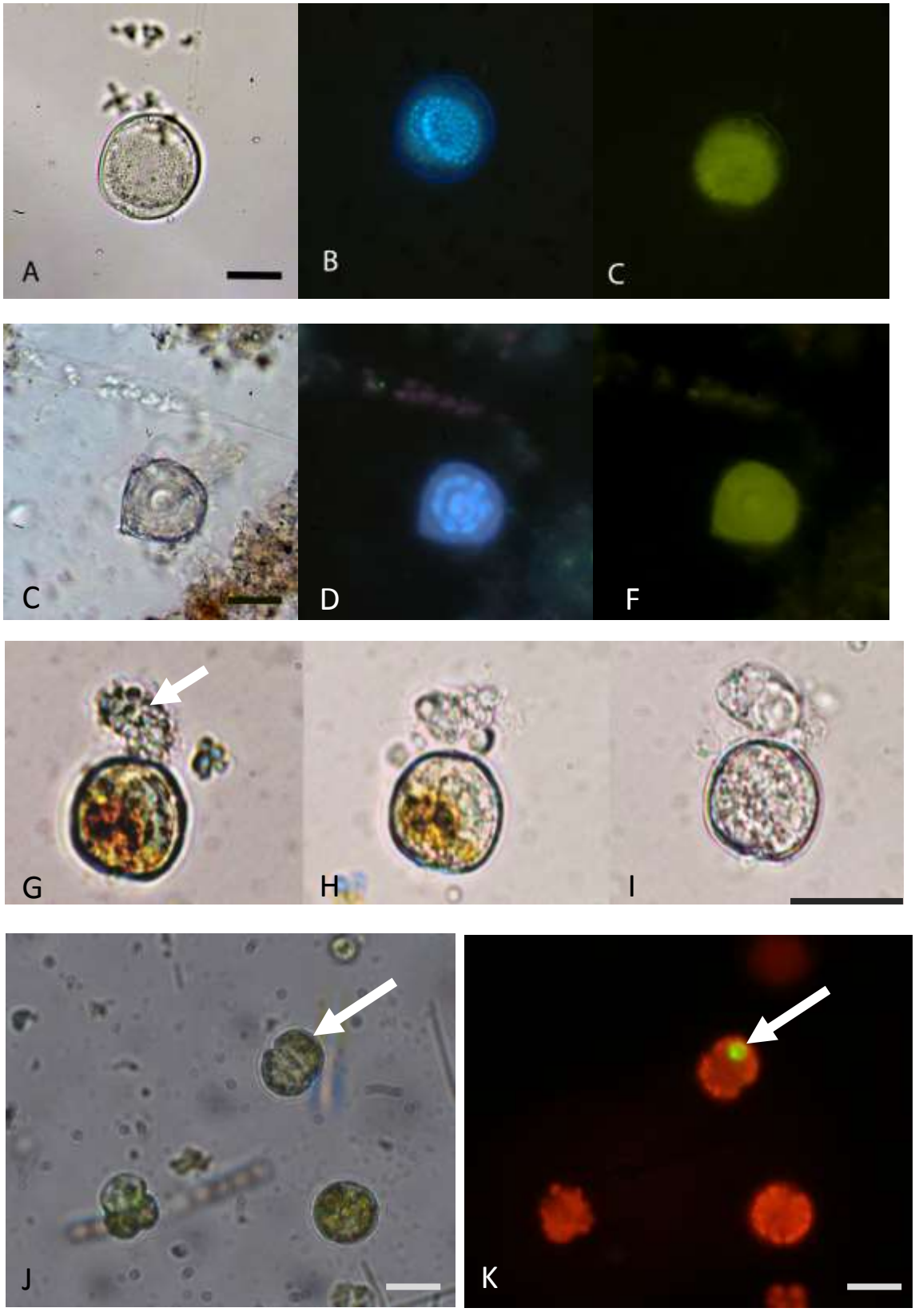

Scrippsiella spp.
2046
Gyrodinium spp.
568
Prorocentrum micans
992
Dinophysis spp.
Cells L-1 (x 10³)
2012
2013
2014
2015

Figure 5



CAPITULO 4

4.1 DISCUSSÃO

Nesta dissertação foi investigada pela primeira vez, de forma sistemática, a hipótese de que *Amoebophrya* spp. infectando dinoflagelados marinhos está restrita ao grupo de pequenos eucariotos marinhos conhecido como MALV-II. Apesar da recorrente ocorrência de sequências de 18S rDNA similares a *Amoebophrya* spp. em bibliotecas de clones de amostras marinhas (MASSANA et al 2004, MOON-VAN DER STAAY et al 2001, MOREIRA e LÓPEZ-GARCIA 2002), não existem evidências para afirmar que estas sequências sejam de organismos com hábitos realmente parasíticos. A combinação do uso da sonda dap-1, que especificamente detecta a diversidade de *Amoebophrya* spp. em dinoflagelados marinhos, com a análise *in silico* da cobertura da sonda em sequências de MALV de bibliotecas de clones (i.e. organismos nunca observados) e em sequências indubitavelmente oriundas de parasitas *Amoebophrya* spp. que infectam dinoflagelados (cuidadosamente seleciondas da literatura), indicou que este tipo de parasite está mesmo restrito ao grupo MALV-II. Também de forma inédita, foi feito um estudo de longa duração da prevalência de infecção por *Amoebophrya* spp. em dinoflagelados planctônicos da Baía de Guanabara, um dos poucos estudos deste tipo de fenômeno em ambientes tropicais. Os resultados mostraram que populações de dinoflagelados abundantes na Baía de Guanabara são pouco afetados por parasitismo, possivelmente devido ao avançado estado de eutrofização do ambiente que interfere de forma direta e indireta na interação parasita-hospedeiro.

A diversidade de pequenos eucariotos marinhos tem sido largamente documentada nos últimos 15 anos, principalmente por meios moleculares i.e. biblioteca de clones construídas a partir de DNA extraído de toda a comunidade microbiana de um local (MASSANA et al 2004, MOON-VAN DER STAAY et al 2001, MOREIRA e LÓPEZ-GARCIA 2002). Dentre os grupos documentados, alveolados marinhos (grupo MALV) tem recebido muita atenção por serem extremamente diversos e amplamente distribuídos no meio marinho (LOPÉZ-GARCIA *et al* 2001, LOVEJOY *et al* 2006, CHAMBOUVET *et al* 2011). Além disso, esse grupo engloba diversos microparasitas com papeis importantes no controle de seus hospedeiros (CACHON *et al* , CHAMBOUVET *et al* 2008), embora muitas das sequências

obtidas venham de análises ambientais onde os organismos não foram observados ou identificados (BACHY *et al* 2011, ALVES-DE-SOUZA *et al* 2012,). A análise filogenética feita com sequências ambientais pertencentes a MALV II e sequências previamente confirmadas como sendo de *Amoebohrya* spp, incluindo novas sequências deste parasita oriundas de dinoflagelados infectados coletados na região de Cabo Frio (Capítulo 2), ficaram restritas ao grupo MALV-II. Estes resultados não excluem a possibilidade de que membros de MALV-II não seja parasitas, mas reforçam a ideia de que muitos deles o sejam.

Níveis de prevalência de *Amoebohrya* spp. documentados para populações naturais de dinoflagelados marinhos são bastante variáveis, desde valores muito baixos e.g. em torno de 0,5%, até extremamente altos, onde ca. 80% das células de uma espécie de hospedeiro foram observadas infectadas (COATS *et al*. 1996; KIM *et al*. 2004, ALVES-DE-SOUZA *et al* 2012; CHAMBOUVET *et al* 2008, 2011; COATS *et al*., 1996, SALOMON *et al* 2003b). Picos de infecção, independentemente do nível de prevalência, perduram por alguns dias, pois o parasita leva em torno de 2 a 3 dias para completar um ciclo na fase intracelular e produzir novas formas infectivas (TAYLOR 1968, NISHITANI *e al* 1985, COATS e BOCKSTAHLER 1996, SALOMON *et al* 2003a, 2003b, 2009). Mesmo em ambientes com baixa prevalência, como em populações de *Dinophysis* spp. no mar Báltico (GISSELSON *et al* 2002, SALOMON *et al* 2003a), infecções pelo parasita são frequentemente observadas durante a persistência de hospedeiros no ambiente. Por isso, a baixa frequência de ocorrência e os baixos níveis de prevalência de *Amoebophrya* spp. em inoflagelados na Baía de Guanabara foram de certa forma surpreendentes. Diversas espécies de dinoflagelados encontradas na Baía de Guanabara, já foram observadas como sendo hospedeiros para *Amoebophrya* spp. em outros locais do mundo (PARK *et al* 2004, KIM *et al* 2006, 2008, MARANDA *et al* 2001). Fatores apontados para a baixa prevalência incluem características ambientais, e não só aspectos inerentes ao hospedeiro. Características abióticas (temperatura, nutrientes) relacionados à qualidade da água podem ser limitantes para a permanência do parasita, tanto por aumentar a capacidade suporte dos hospedeiros, de modo que estes consigam escapar do controle do parasita, segundo a hipótese do paradoxo do enriquecimento (ROSENZWEIG 1971), como por promover o desenvolvimento de populações de predadores (tipicamente ciliados) que se alimentam dos dinósporos de *Amoebophrya* spp, controlando sua proliferação (JOHANSSON e COATS 2002, SIANO *et al* 20011).

Conforme observado no por Villac e Tenenbaum (2010), dinoflagelados são um importante componente da Baía de Guanabara que vem tendo sua representatividade aumentada no microplâncton, possivelmente devido a mudanças no estado trófico do sistema. Um exemplo deste fenômeno foi observado durante este estudo, onde o dinoflagelado *Prorocentrum triestinum* esteve presente em mais de 90% das amostras analisadas, e na coleta do mês de Agosto de 2014, representou quase que a totalidade de dinoflagelados da Baía naquele momento. Apesar da alta frequência de ocorrência deste dinoflagelado, nenhuma infecção por *Amoebophrya* spp. ou outro microparasita foi observada, apesar desta ser uma espécie de dinoflagelado encontrada infectada em outros lugares do mundo (KIM *et al* 2008). Outras espécies de dinoflagelados abundantes na Baía de Guanabara, como o gimnodinoide *Levanderina fissa*, também não foram encontrados com infecção, apesar do intenso esforço amostral (milhares de células foram analisadas) e utilização de diferentes técnicas de detecção (FISH, DAPI, observações por microscopia e com a FlowCam em células vivas). Em comparação, uma espécie morfologicamente similar e taxonomicamente próxima a *L. fissa*, *Akashiwo sanguinea*, é frequentemente encontrada infectada por *Amoebophrya* spp. em Chesapeake Bay (MD), em níveis de até 80% (COATS e BOCKSTAHLER 1994). Portanto, resta pouca dúvida que as condições reinantes na Baía de Guanabara impedem frequentes e intensos episódios de infecção das populações de dinoflagelados pelo parasita.

4.2 CONCLUSÕES

- Parasitas do gênero *Amoebophrya* que infectam dinoflagelados são restritos ao grupo de alveolados marinhos MALV II.
- *Amoebophrya* spp. infecta dinoflagelados planctônicos na Baía de Guanabara, porém com frequência de ocorrência e prevalência de infecção extremamente baixas, mesmo em espécies de hospedeiros comumente encontrados infectados em outras regiões costeiras do mundo. O elevado estado de eutrofização da baía é a provável causa destes baixos níveis de infecção.
- Dada a infrequente e baixa prevalência, infecção por *Amoebophrya* spp. não se configura como um fator significante de perda para populações de dinoflagelados na

Baía de Guanabara. Esta baixa pressão por parasitismo pode ser um dos fatores que permitem o desenvolvimento de populações de dinoflagelados neste ambiente.

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