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INTERAÇÕES FITOPLÂNCTON-BACTÉRIA: ASSOCIAÇÕES ESPECÍFICAS E POSSÍVEIS IMPLICAÇÕES ECOLÓGICAS

Inessa Lacativa Bagatini

Tese apresentada ao Programa de Pós-Graduação em Ecologia e Recursos Naturais da Universidade Federal de São Carlos, como parte dos requisitos para obtenção do título de DOUTOR EM CIÊNCIAS, área de concentração: ECOLOGIA E RECURSOS NATURAIS

Orientador: Prof. Dr. Armando Augusto Henriques Vieira

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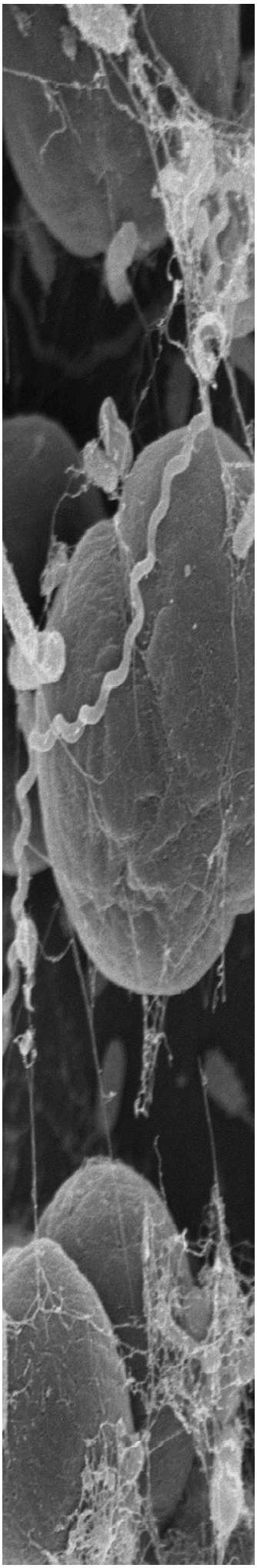

Adorava admirar a beleza das coisas, descortinar no imperceptível,
através do que é diminuto, a alma poética do universo
(Fernando Pessoa)

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RESUMO

INTERAÇÕES FITOPLÂNCTON-BACTÉRIA: ASSOCIAÇÕES ESPECÍFICAS E POSSÍVEIS IMPLICAÇÕES ECOLÓGICAS

A ocorrência de florações fitoplanctônicas é um problema mundial e que afeta a qualidade da água e a biota aquática. A comunidade bacteriana pode influenciar e interferir na formação, manutenção e término de tais florações por meio de diversas interações bióticas. Neste trabalho, por meio do sequenciamento massivo (pirossequenciamento) das regiões V3-V5 do gene RNAr 16S, foram caracterizadas as comunidades bacterianas associadas a 3 espécies fitoplanctônicas formadoras de *blooms:* as cianobactérias *Microcystis aeruginosa* e *Cylindrospermopsis raciborskii* e a diatomácea *Aulacoseira granulata.* Os objetivos do trabalho foram verificar se diferentes comunidades bacterianas desenvolvem-se com diferentes espécies hospedeiras ou em diferentes frações (aderidas e livres) e fases de crescimento (estados fisiológicos) de cada espécie fitoplanctônica. Ainda utilizando os dados de pirossequenciamento, a consistência dessas associações foi testada para *M. aeruginosa* e *A. granulata* por meio do cultivo de cada espécie fitoplanctônica com diferentes inóculos bacterianos (e meio de cultura para a cianobactéria). Além disso, os efeitos de bactérias aderidas isoladas de *A. granulata* e *M. aeruginosa* foram testados sobre o crescimento de ambas as espécies fitoplanctônicas. Comunidades bacterianas significativamente diferentes se desenvolveram: i) em resposta ao estado fisiológico e entre diferentes frações da mesma espécie fitoplanctônica, e ii) em resposta às diferentes espécies fitoplanctônicas hospedeiras, com maiores diferenças na proporção das UTOs (unidades taxonômicas operacionais) do que na presença/ausência de UTOs específicas. Diferenças entre experimentos (e inóculos bacterianos) alteraram significativamente a composição da comunidade bacteriana associada a uma mesma espécie fitoplanctônica. Algumas UTOs foram recorrentes nos diferentes experimentos, sugerindo uma associação mais consistente com a espécie hospedeira. A maioria das linhagens bacterianas isoladas de *M. aeruginosa* ou de *A. granulata* afetou negativamente o crescimento da diatomácea. No entanto, nenhuma linhagem ou mistura de linhagens apresentou efeito sobre a cianobactéria durante seu crescimento exponencial, sugerindo a importância da comunidade bacteriana para a dominância de *M. aeruginosa* no reservatório de Barra Bonita, ao menos em relação à competição com *A. granulata.*

ABSTRACT

INTERACTION PHYTOPLANKTON-BACTERIA: SPECIFIC ASSOCIATIONS AND POSSIBLE ECOLOGICAL IMPLICATIONS

The occurrence of nuisance phytoplankton blooms is a worldwide problem that affects water quality and other aquatic biota. Bacterial (non-cyanobacterial) communities can influence and interfere with the formation, maintenance and termination of such blooms via many biotic interactions. The bacterial communities associated with 3 bloom forming phytoplankton species, the *Cyanobacteria Microcystis aeruginosa* and *Cylindrospermopsis raciborskii* and the diatom *Aulacoseira granulata,* were assessed by the 454-Pyrosequencing of 16S rRNA gene amplicons (V3-V5 regions). The aims of this work were to verify if different bacterial communities would develop with different host species, or in different fractions (attached and free-living bacteria) and different growth phases (physiological state) of each phytoplankton species. Still using pyrosequencing data, the strength of phytoplankton-bacteria association was analyzed for *M. aeruginosa* and *A. granulata* by culturing the phytoplankton species with different bacterial inocula (and growth medium for the cyanobacteria). Furthermore, the effects of bacterial strains isolated from attached community of *A. granulata* and *M. aeruginosa* cultures were tested on the growth of the phytoplankton species. Significantly different bacterial communities developed: i) in response to physiological state and between fractions of the same phytoplankton culture, and ii) in response to the different host phytoplankton species, with major differences in the proportion of the OTUs between phytoplankton cultures rather than in the absence or presence of specific bacterial *taxa*. Differences between experiments significantly changed the BCC that developed associated with the same host species. However, some bacterial OTUs were found associated with the same phytoplankton species in different experiments, suggesting a tight association with the host species. Most of the strains either isolated from *M. aeruginosa* or from *A. granulata* affected negatively the growth of the diatom. However, the isolated strains had no effect on the cyanobacterial growth, suggesting the importance of bacterial community to the dominance of *M. aeruginosa* on Barra Bonita Reservoir, at least concerning to the competition with *A. granulata.*

LISTA DE SIGLAS E ABREVIATURAS

ARISA	Automated rRNA Intergenic Spacer Analysis
BCC	Bacterial Community Composition
CPS	Meio de cultura para bactérias com caseína, peptona e amido (Casein, Peptone and Starch)
CTAB	Brometo de cetil trimetil amônio (Cetyl Trimethylammonium Bromide)
DAPI	4',6-Diamidino-2-fenil-indol
DCMU	3-(3,4-dicloro-fenil)-1,1-dimetil-urea
DGGE	Denaturing Gradient Gel Electrophoresis (Eletroforese em gel com gradiente desnaturante)
DNA	Ácido desoxirribonucleico (desoxyribonucleic acid)
dNTPs	Desoxinucleotídeos Trifosfatados *(*Deoxynucleotide Triphosphates)
FW	Freshwater bacterial sequence database
MLAulac	Mistura de linhagens bacterianas associadas à *A. granulata*
MLMicro	Mistura de linhagens bacterianas associadas à *M. aeruginosa*
NCBI	National Center for Biotechnology Information
NGS	Next-Generation Sequencig
NMDS	Non-Metric Muldimensional Scaling
OTU	Operational Taxonomic Unit
PCR	Reação em cadeia da polimerase (Polimerase Chain Reaction)
RDP	Ribosomal Database Project
RFU	Unidades relativas de fluorescência (Relative Fluorescence Unit)
RNA	Ácido Ribonucleico (Ribonucleic Acid)
RNAr	Ácido Ribonucleico Ribossomal
rRNA	Ribosomal Ribonucleic Acid
T-RFLP	Terminal Restriction Fragment Length Polymorphism
UTO	Unidade Taxonômica Operacional
WDCM	World Data Center for Microorganisms

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APRESENTAÇÃO DA TESE

A tese será apresentada da seguinte forma: (1) Introdução geral; (2) Hipóteses e Objetivos; (3) Capítulos (com Resultados e Discussão); (4) Discussão geral; e (5) Conclusões.

Cada capítulo será apresentado no formato de artigo científico: com resumo, introdução, material e métodos, resultados, discussão, referências bibliográficas e material suplementar. Esse formato foi escolhido para facilitar a publicação dos resultados obtidos. Os dois primeiros capítulos estão em inglês e o terceiro em português. Uma vez que alguns capítulos estão em inglês, uma introdução geral, uma discussão geral e as conclusões em português foram necessárias. A divisão neste formato torna a publicação dos dados mais rápida, no entanto, as repetições nos capítulos são inevitáveis.

O primeiro capítulo encontra-se formatado para a publicação na revista à qual foi submetido (PLoS ONE), desta maneira, parte dos dados encontra-se como material suplementar. Neste capítulo, apresentamos os resultados de uma das hipóteses sobre a seleção de comunidades bacterianas por espécies fitoplanctônicas específicas, e as diferenças entre as comunidades bacterianas aderidas ao fitoplâncton e livres na cultura em diferentes fases de crescimento das cianobactérias (*Microcystis aeruginosa* e *Cylindrospermopsis raciborskii*) e da diatomácea (*Aulacoseira granulata*) estudadas.

No capítulo 2, testamos a consistência da especificidade das comunidades bacterianas associadas a duas espécies fitoplanctônicas, *M. aeruginosa* e *A. granulata*, quando cultivadas com inóculos bacterianos diferentes provenientes do mesmo ambiente, e meios de cultura diferentes para *M. aeruginosa.*

No terceiro capítulo abordamos o papel de algumas linhagens bacterianas isoladas das mesmas espécies fitoplanctônicas utilizadas no capítulo anterior. Os efeitos das linhagens bacterianas isoladas foram testados sobre culturas da espécie de onde as linhagens foram originalmente isoladas e sobre culturas da outra espécie.

Uma breve discussão geral e as conclusões foram elaboradas com base nos três capítulos apresentados.

Os dados de pirossequenciamento (apresentados nos dois primeiros capítulos) e de microscopia eletrônica de varredura (apresentados na introdução e no primeiro e no terceiro capítulos) foram obtidos durante o estágio de doutorado na Universidade de Uppsala (Suécia) e na Universidade de Oslo (Noruega), respectivamente, que totalizaram 4,5 meses. O doutorado sanduíche realizado na Universidade de Uppsala foi orientado pelos professores Dr. Stefan Bertilsson e Dr. Alexander Eiler, e proporcionou experiências com a preparação de amostras para pirossequenciamento e com utilização de softwares e métodos de análise de dados de pirossequenciamento (como o Qiime, Mothur e o R). O estágio na Universidade de Oslo, orientado pelo prof. Dr. Dag Klaveness, permitiu o contato com métodos para preparação de amostra e técnicas de microscopia eletrônica de varredura e de transmissão.

SUMÁRIO

INTRODUÇÃO GERAL

Interação fitoplâncton-bactéria

O represamento e a eutrofização antrópica de rios, associados ao grande potencial de crescimento de algumas espécies fitoplanctônicas, fazem com que esses organismos, especialmente as cianobactérias, produzam quantidades enormes de biomassa em curtos períodos, ou continuamente no caso de reservatórios tropicais e subtropicais (e.g. Dellamano-Oliveira et al. 2008, Sinha et al. 2012, Vieira et al. 2013). Esses *blooms* fitoplanctônicos podem ter grande impacto no fluxo de energia e nos ciclos biogeoquímicos dos ecossistemas aquáticos (Azam & Malfatti 2007). Além disso, ocasionam problemas como grande quantidade de biomassa flutuante, mau cheiro, depleção de oxigênio e, no caso das cianobactérias, por exemplo, produção de toxinas (Carmichael 2001, Paerl et al. 2001).

Variações na composição dos florescimentos fitoplanctônicos podem ser influenciadas por fatores abióticos como a competição por luz e disponibilidade de nutrientes (e.g. Huisman et al. 1999, Crossetti & Bicudo 2005), ou bióticos, como infecção por vírus (Padan & Shilo 1973, Manage et al. 1999) e interação com fungos (Canter 1951, Safferman & Morris 1963) e bactérias (não-cianobactérias) (Paerl 1992, Sher et al. 2011). Estas últimas, as interações bactérias-fitoplâncton, embora geralmente ocorram em escalas reduzidas (Blackburn et al. 1998, Barbara & Mitchell 2003, Eiler et al. 2006, Jiang et al. 2007, Seymour et al. 2010, Sher et al. 2011) ao redor da ficosfera (Bell et al. 1974), podem ter inúmeras funções ecologicamente relevantes, como remineralização de nutrientes e ciclagem de carbono (Bell et al. 1974, Cole 1982). Essas interações são importantes não somente para a biologia e fisiologia dos microrganismos e para o ambiente local, mas podem também influenciar os ciclos biogeoquímicos globais (Paerl 1996, Azam and Malfatti 2007). Embora o fitoplâncton e o bacterioplâncton sejam componentes chave do fluxo de energia e ciclagem de nutrientes nos ambientes aquáticos, nosso entendimento sobre a interação fitoplâncton-bactéria ainda é limitado (Grossart et al. 2005).

A matéria orgânica liberada pelo fitoplâncton durante seu crescimento ou após a morte do *bloom* é uma fonte de carbono eficiente para o bacterioplâncton (Giroldo et al. 2003, Colombo et al. 2004) e as bactérias heterotróficas são as

principais responsáveis por transformar a matéria orgânica dissolvida liberada pelo fitoplâncton em matéria orgânica particulada, disponibilizando-a para níveis tróficos superiores (Azam et al. 1983, Azam 1998).

A liberação de matéria orgânica, principalmente polissacarídeos (Myklestad 1995), pelo fitoplâncton saudável, estimula o crescimento das bactérias. Com isso, uma espécie fitoplanctônica pode obter pequenas vantagens como remineralização e solubilização de nutrientes inorgânicos (Paerl 1992, Azam 1998, Jiang et al. 2007), produção de vitaminas e outros fatores promotores de crescimento (Croft et al. 2005, Liu et al. 2008, Sher et al. 2011), aumento na concentração de CO_2 e diminuição da concentração de O_2 e potencial redox na ficosfera (Paerl 1977, Sigee 2005). Essas pequenas vantagens, dependendo do ambiente, podem se traduzir em dominância temporária da espécie fitoplanctônica (Doucette 1995, Liu et al. 2008).

No entanto, as bactérias também podem afetar negativamente o crescimento do fitoplâncton, seja por competição por nutrientes (Sigee 2005, Danger et al. 2007) ou por produção de antibióticos e enzimas (Lovejoy et al. 1998, Manage et al. 2000, Mayali & Azam 2004, Sigee 2005, Shi et al. 2009a). Segundo Fukami et al. (1997), as bactérias com efeitos inibitórios têm um papel importante nas mudanças das espécies algais dominantes em ambientes aquáticos. Um esquema simplificado das interações fitoplâncton-bactéria pode ser visto na Figura I.

Desta maneira, em alguns casos, a interação entre fitoplâncton e bactérias, com gasto energético pelo fitoplâncton para manter populações bacterianas que podem apresentar feitos negativos em seu crescimento, pode ser considerada paradoxal (Bratbak & Thingstad 1985). Contudo, essa interação não parece ocorrer aleatoriamente, uma vez que as bactérias que interagem com determinadas espécies fitoplanctônicas também são selecionadas pela qualidade da matéria orgânica excretada (e.g. Teeling et al. 2012).

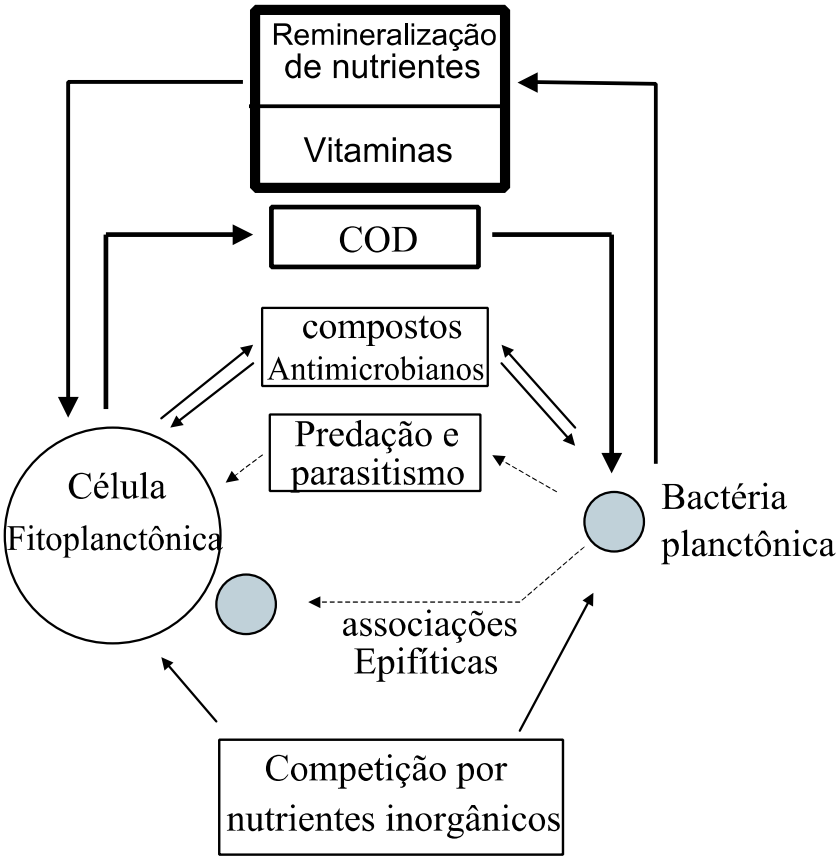

Figura I – Interações gerais entre fitoplâncton e bactéria (modificado de Sigee (2005).

Diversos trabalhos (e.g Kolmonen et al. 2004, Kent et al. 2007, Teeling et al. 2012, Paver et al. 2013) relatam a ocorrência de variações temporais e espaciais entre comunidades bacterianas fortemente associadas a variações nas comunidades fitoplanctônicas em ambiente natural. Da mesma maneira que o fitoplâncton influencia a dinâmica da comunidade bacteriana, esta também pode afetar a dinâmica das espécies fitoplanctônicas (Fukami et al. 1997, Mayali and Azam 2004).

A covariação entre mudanças fitoplanctônicas e comunidade bacteriana no ambiente é suportada por experimentos com culturas de algas e cianobactérias isoladas. Diferentes espécies fitoplanctônicas em culturas podem ser colonizadas por comunidades bacterianas distintas (Schäfer et al. 2002, Grossart et al. 2005, Shi et al. 2009b, Dziallas and Grossart 2011) e, em alguns casos, pode ocorrer quimiotaxia de grupos bacterianos a espécies fitoplanctônicas específicas (Lupton and Marshall 1981, Casamatta and Wickstrom 2000). Além disso, podem ocorrer

variações temporais nas comunidades bacterianas em diferentes fases de crescimento de uma espécie fitoplanctônica (Grossart et al. 2005), com diferenças entre as comunidades de bactérias que se aderem às células fitoplanctônicas e as bactérias livres na cultura (Grossart et al. 2005, Sapp et al. 2007).

As variações na qualidade da matéria orgânica excretada por diferentes espécies fitoplanctônicas (Fogg 1983, Gouvêa et al. 2005, Sarmento & Gasol 2012) e entre os diferentes estados fisiológicos da mesma espécie (Grossart et al. 2005, Bruckner et al. 2011), além da produção de antibióticos pelo fitoplâncton (Ostensvik et al. 1998) podem explicar a variação na comunidade bacteriana associada. A dinâmica da comunidade bacteriana deve-se à capacidade dos táxons bacterianos em degradar moléculas mais ou menos complexas liberadas pelo fitoplâncton, à ocorrência de quimiotaxia por compostos específicos e/ou resistência à produção dos antibióticos (Casamatta & Wickstrom 2000, Teeling et al. 2012). Diferentes linhagens bacterianas também podem promover excreção de compostos distintos pelo fitoplâncton (Bruckner et al. 2011).

A interação fitoplâncton-bactéria pode ocorrer com as bactérias livres no meio, que utilizam a matéria orgânica dissolvida liberada, assim como com bactérias se aderem às células, aos filamentos ou às colônias das espécies fitoplanctônicas. Nos anexos 1, 2 e 3 encontram-se algumas fotomicrografias eletrônicas das espécies estudadas neste trabalho, nas quais é possível observar bactérias aderidas aos filamentos e colônias, assim como livres no meio. Essas duas comunidades, aderidas e livres, associadas à mesma espécie fitoplanctônica, podem diferir em composição (Grossart et al. 2005, Rooney-Varga et al. 2005) e influenciar o fitoplâncton de maneiras diferentes (Mayali and Azam 2004, Morris et al. 2008). Por exemplo, algumas linhagens bacterianas necessitam de maior proximidade celular para apresentarem efeitos deletérios sobre espécies fitoplanctônicas (Sher et al. 2011), enquanto outras, embebidas na mucilagem da cianobactéria *M. aeruginosa* fazem da colônia um microambiente rico em fósforo (Jiang et al. 2007).

No ambiente natural, onde o efeito das bactérias livres pode ser menor devido à diluição de compostos produzidos (pelos dois organismos), ou como apontado por Vaqué et al. (1989) limitados pela probabilidade de encontro, o conhecimento das espécies aderidas às espécies fitoplanctônicas é bastante importante. Além disso, as bactérias aderidas podem apresentar uma associação mais específica com as espécies fitoplanctônicas do que as livres (Grossart et al. 2005, Sigee 2005).

Apesar da importância das interações entre fitoplâncton e bactérias para as espécies fitoplanctônicas, assim como para a dinâmica de florações e as funções dos ecossistemas aquáticos (Grossart et al. 2005, Paver et al. 2013), ainda sabe-se pouco sobre a composição bacteriana associada a diferentes espécies, sobre a especificidade das associações e sobre seus possíveis efeitos em associação a blooms de determinadas espécies.

Uma análise detalhada da composição das bactérias que são estimuladas pela excreção de compostos específicos de cada espécie fitoplanctônica é um dos primeiros passos (Sarmento & Gasol 2012), que associado ao estudo da função dessas bactérias (Teeling et al. 2012) e da adaptação das populações bacterianas expostas a frequentes florações de determinadas espécies fitoplanctônicas (Casamatta and Wickstrom 2000), pode fornecer subsídios para se entender o aparente paradoxo da interação fitoplâncton-bactéria. Além disso, a composição das espécies bacterianas associada a cada espécie fitoplanctônica pode fornecer informações sobre a ecologia dos microrganismos (Paver et al. 2013) e informações para a elucidação sobre a especificidade das associações.

A utilização do pirossequenciamento em estudos sobre diversidade bacteriana

Apesar das diferenças nas comunidades bacterianas entre espécies fitoplanctônicas, entre diferentes fases de crescimento e entre as diferentes frações (livres e aderidas) de uma cultura ou *bloom* fitoplanctônico, como apontado anteriormente, ainda há controvérsias sobre a seletividade e a especificidade das associações bactéria-fitoplâncton (Sapp et al. 2007) e sobre sucessão de comunidades em diferentes fases de crescimento das culturas (Schäfer et al. 2002). Além disso, mesmo para uma mesma espécie fitoplanctônica, as comunidades bacterianas parecem variar de acordo com a localização geográfica (Dziallas & Grossart 2011). Vários são os motivos para as diferenças relatadas entre os estudos, por exemplo, diferentes graus de interação e especificidade das bactérias com diferentes espécies fitoplanctônicas (Eigemann et al. 2012), inóculo bacteriano ou métodos para acessar a diversidade bacteriana associada (Dziallas and Grossart 2011) e desenho experimental (Grossart et al. 2005).

Em relação à variação nos resultados sobre a especificidade de comunidades bacterianas associadas a diferentes algas em um mesmo estudo (e.g Grossart et al.

2005, Sapp et al. 2007), uma das razões pode ser a profundidade de sequenciamento utilizada, quando não permite detecção de espécies menos abundantes e/ou limita a análise de proporções dos táxons bacterianos. Entre as técnicas utilizadas nos trabalhos comparando comunidades bacterianas associadas a espécies fitoplanctônicas isoladas ou em *blooms* naturais estão o T-RFLP (Li et al. 2011), ARISA (Guannel et al. 2011), clonagem (Eiler & Bertilsson 2004, Kormas et al. 2010), isolamento de linhagens seguido por sequenciamento (Berg et al. 2009) e, principalmente, DGGE (Grossart et al. 2005, Shi et al. 2009b, Dziallas & Grossart 2011, Shi et al. 2011, Shi et al. 2012). Essas técnicas fornecem um número pequeno de sequências de bactérias associadas quando comparadas às técnicas de sequenciamento de nova geração (ou NGS, do inglês Next-Generation Sequencing). O trabalho com maior número de sequências depositadas no GenBank, de Eiler & Bertilsson (2004), utilizando clonagem, possui menos de 1500 sequências considerando-se as leituras relacionadas às cianobactérias e menos de 600 sequências considerando-se somente as bactérias.

No entanto, ainda não há estudos comparando comunidades bacterianas associadas a espécies fitoplanctônicas específicas utilizando tecnologias de sequenciamento de nova geração. Mesmo utilizando as outras técnicas citadas anteriormente, os dados sobre a diversidade da comunidade bacteriana associada a algumas espécies formadoras de florações em ambientes de água doce são escassos, como para a cianobactéria *Cylindrospermopsis raciborskii* (Pope and Patel 2008, Shi et al. 2009b), ou inexistentes, como para a diatomácea *Aulacoseira granulata.*

Devido à grande quantidade de âmplicons que pode ser sequenciada, associada à maior rapidez na obtenção dos resultados (Metzker 2009), o sequenciamento utilizando essas novas tecnologias permite uma comparação mais robusta das diferenças entre as comunidades bacterianas associadas a diferentes espécies fitoplanctônicas, assim como melhor detecção de espécies recorrentes associadas à mesma espécie fitoplanctônica em diferentes condições. Uma das técnicas de sequenciamento de nova geração que vêm sendo utilizada para a caracterização de comunidades bacterianas por meio do sequenciamento do gene RNAr 16S é o 454-pirossequenciamento (Cheng et al. 2011, Zhang et al. 2011, Peura et al. 2012). Esta técnica permite o sequenciamento simultâneo de comunidades bacterianas de diversas amostras por meio da utilização de iniciadores

(*primers*) associados a *barcodes* (Tamaki et al. 2011) e tem sido considerada revolucionária (como outras técnicas de NGS), uma vez que pode fornecer um número suficiente de sequências para se analisar com profundidade a diversidade microbiana em virtualmente qualquer tipo de amostra (Cheng et al. 2011).

No caso de análises das associações fitoplâncton-bactéria, especialmente das bactérias aderidas (ou epifíticas), a utilização de técnicas mais poderosas como o pirossequenciamento é importante para a melhor resolução da comunidade bacteriana, uma vez que uma grande quantidade de sequências relacionadas ao RNAr 16S das cianobactérias ou dos cloroplastos também é amplificada e interfere na quantidade de sequencias bacterianas obtidas.

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HIPÓTESES E OBJETIVOS

Hipóteses

A) A diversidade específica encontrada no fitoplâncton implica também na diversidade de compostos da matéria orgânica excretada por essas espécies, o que pressupõe diversidade nas espécies de bactérias heterótrofas que orbitam suas ficosferas e diferenças entre as comunidades associadas a espécies diferentes. Por outro lado, em uma mesma espécie de microalga as mudanças fisiológicas advindas com a idade celular também impõem alterações na composição dos excretados e, consequentemente, também determinam mudanças na composição específica das bactérias associadas. Além disso, uma vez que a proximidade entre as células pressupõe maior tolerância e/ou quimiotaxia e/ou, no caso de formação de uma matriz polissacarídica, capacidade de degradação de polímeros mais complexos, esperam-se diferenças entre as comunidades livres e aderidas.

B) Através de mecanismos evolutivos presume-se que tais associações seriam naturalmente direcionadas para alta especificidade com base na interação bactéria/substrato, com seleção de espécies benéficas para ambos os organismos nas interações. Desta maneira, mesmo com diferenças nas condições de cultivo ou inóculo bacteriano, deveria ocorrer certa similaridade entre as comunidades bacterianas que se associam à mesma espécie.

C) Entre tais relações o fornecimento pela espécie fitoplanctônica de substratos apropriados para o crescimento de bactérias que por sua vez promoveriam retorno de algum nutriente específico, proteção ou vantagem competitiva, é bem plausível. Assim como também é plausível que algumas das relações traduzam-se em eventos deletérios. Uma vez que é esperada maior especificidade entre bactéria aderida e fitoplâncton, é possível que as bactérias aderidas tragam mais benefícios diretos para a espécie fitoplanctônica do que para outra espécie competidora com a qual a linhagem bacteriana não apresenta interação tão próxima.

Objetivos Específicos

Utilizando uma técnica de sequenciamento (o 454-pirossequenciamento) para caracterizar e comparar comunidades bacterianas associadas a diferentes espécies fitoplanctônicas, pretendeu-se:

1) Verificar se culturas unialgais de 3 espécies formadoras de *blooms* (duas cianobactérias, *Microcystis aeruginosa* e *Cylindrospermopsis raciborskii* e uma diatomácea, *Aulacoseira granulata*), quando inoculadas com a mesma comunidade bacteriana inicial, selecionam comunidades bacterianas diferentes, com UTOs (unidades taxonômicas operacionais) bacterianas específicas para cada espécie. Além disso, determinar se ocorre sucessão bacteriana ao longo do ciclo biológico das espécies fitoplanctônicas e se há diferenciação entre comunidade bacteriana livre e aderida a cada espécie hospedeira.

2) Testar se a especificidade na associação fitoplâncton-bactéria varia para as espécies *M. aeruginosa* e *A. granulata* quando são utilizados inóculos bacterianos e/ou condições de cultivos diferentes nas culturas fitoplâncton-bactéria. Pretendeu-se também determinar as UTOs recorrentes em experimentos com inóculos diferentes a fim de encontrar UTOs que possivelmente apresentem uma interação mais forte com as espécies hospedeiras.

Utilizando linhagens bacterianas aderidas isoladas de diferentes espécies fitoplanctônicas pretendeu-se:

3) Verificar se as bactérias aderidas a uma espécie fitoplanctônica possuem efeitos distintos sobre a própria espécie e sobre uma possível espécie competidora.

CAPÍTULO 1:

HOST-SPECIFICITY AND DYNAMICS IN BACTERIAL COMMUNITIES ASSOCIATED WITH BLOOM-FORMING FRESHWATER PHYTOPLANKTON*

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Keywords: bacterial community composition, pyrosequencing, diatom, cyanobacteria, phytoplankton-bacteria interaction

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1.1. Abstract

Many freshwater phytoplankton species have the potential to form transient nuisance blooms that affects water quality and other aquatic biota. Heterotrophic bacteria can influence such blooms via nutrient regeneration but also via antagonism and other biotic interactions. We studied the composition of bacterial communities associated with three bloom-forming freshwater phytoplankton species, the diatom *Aulacoseira granulata* and the cyanobacteria *Microcystis aeruginosa* and *Cylindrospermopsis raciborskii*. Experimental cultures incubated with and without lake bacteria were sampled in three different growth phases and bacterial community composition was assessed by 454-Pyrosequencing of 16S rRNA gene amplicons. *Betaproteobacteria* were dominant in all cultures inoculated with lake bacteria, but decreased during the experiment. In contrast, *Alphaproteobacteria,* which made up the second most abundant class of bacteria, increased overall during the course of the experiment. Other bacterial classes responded in contrasting ways to the experimental incubations causing significantly different bacterial communities to develop in response to host phytoplankton species, growth phase and between attached and free-living fractions. Differences in bacterial community composition between cyanobacteria and diatom cultures were greater than between the two cyanobacteria. Despite the significance, major differences between phytoplankton cultures were in the proportion of the OTUs rather than in the absence or presence of specific taxa. Different phytoplankton species favoring dissimilar bacterial communities may have important consequences for the fate of organic matter in systems where these bloom forming species occur. The dynamics and development of transient blooms may also be affected as bacterial communities seem to influence phytoplankton species growth in contrasting ways.

1.2. Introduction

The diatom *Aulacoseira granulata* and the cyanobacteria *Microcystis aeruginosa* and *Cylindrospermopsis raciborskii* are common phytoplankton species in eutrophic freshwater lakes and reservoirs around the world [1-3]. These and other phytoplankton can form transient blooms that greatly impact the energy and nutrient flux in aquatic environments because of the locally high amount of organic matter being produced. This may cause additional problems such as oxygen depletion and some species may also form surface scums and produce toxins [1]. The occurrence and dynamics of phytoplankton blooms can be explained by a combination of abiotic and/or biotic factors [4,5], where phytoplankton-bacterial interactions may also play a central yet largely overlooked role [6-8].

Cellular interactions between microorganisms typically occur at the nano- to micrometer scale [7-10] and are important for the dynamics of microbial populations while also influencing microbially mediated biogeochemical cycles [5,11]. Despite the suggested importance of cellular interactions between freshwater phytoplankton and heterotrophic bacteria, our understanding of such interactions is still very limited [12] and little is known about the taxon specificity of such associations.

Phytoplankton can interact with free-living bacteria but also maintain attached bacteria on their cellular surface. Such communities may differ significantly in composition from their free-living counterparts [12,13] and may influence the phytoplankton in contrasting ways [8]. For example, Sher et al. [14] found that bacterial strains could enhance the growth of *Prochlorococcus* and that, in some cases, this positive effect was caused by diffusible compounds; however, they also identified antagonistic effects requiring close cellular proximity.

Physical attachment of bacteria to the surface of microalgae may therefore suggest a tight functional association [12,15] which is likely to be more specific compared to functional interactions between the microalgae and free-living bacteria. Nevertheless, free-living bacteria can also be associated to specific microalgae, largely because different phytoplankton species release contrasting spectra of dissolved organic compounds to the surrounding medium [16]. These potential substrates may then select for certain subsets of the bacterial community capable of efficiently using these compounds to fuel growth, in culture or in natural environment [17-19]. Specific associations can additionally be due to production of antibiotics by the microalgae or cyanobacteria, where selection is governed by differential resistance of bacterial populations [20,21].

Bacterial communities may also vary with microalgae growth phase and physiological status, as shown for experimental cultures [12] or natural phytoplankton blooms [18] and this has been linked to changes in the organic matter released during the different stages of phytoplankton growth [22]. Such community shifts may in turn have feedback effects on phytoplankton growth [14,23] and stimulate changes in the quality and quantity of compounds that will be released by the algae [22,24]. Therefore, relationships between bacteria and phytoplankton can be defined according to spatial (proximity of cells) and temporal scales as well as the degree of specificity of the interaction [6].

One important gap in knowledge with regards to microalga-bacteria interactions concerns the taxonomic composition and diversity of bacterial communities associated with distinct algal populations and taxa [12]. Previous studies have revealed the identity of some dominant bacterial taxa that seem to be associated with *M. aeruginosa* [e.g. 25-27]. Information on bacterial taxa associated

with *Cylindrospermopsis sp*. [27,28] is on the other hand very limited. For freshwater diatoms a few studies have focused on the associated microflora [24] and there are no studies for *A. granulata*. These studies are however based on a small number of sequences to describe and compare bacterial communities from bloom-forming microalgae. In general, most previous work describing the diversity of bacterial communities associated with freshwater cyanobacterial and marine diatom blooms have been based on less than 150 sequences [e.g.12,29,30], with the notable exception of Eiler and Bertilsson [25], Berg et al [31], and Li et al [32]. Because NGS (Next Generation Sequencing) approaches typically yield a much larger number of sequences, it enables a more robust comparison between bacterial communities compositions associated with phytoplankton species. Furthermore, the attached bacterial communities can be better resolved despite a high background of phytoplankton derived sequences.

Using high throughput 454 pyrosequencing of 16S rRNA amplicons, we characterized the bacterial communities associated with three important freshwater phytoplankton species, and tested the hypotheses that (1) bacterial community composition (BCC) is characteristics for different phytoplankton host species, (2) bacterial communities will change gradually during the different phytoplankton growth phases and (3) that there are differences between attached and free-living bacteria in phytoplankton-heterotroph co-cultures.

1.3. Material and Methods

Phytoplankton species and bacterial inocula

Two cyanobacterial species, *Microcystis aeruginosa* Kützing (BB005) and *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya & Subba Raju (BB048), and one diatom (Bacillariophyceae) *Aulacoseira granulata* var. *granulata* (Ehrenberg) Simonsen (BB001), were isolated from the hypereutrophic Barra Bonita Reservoir in São Paulo State, Brazil, they form frequent blooms [33-35]. The isolates were made axenic and maintained in the culture collection of the Botany Department at the Federal University of São Carlos (World Data Center for Microorganisms No. 835). No specific permission was required for sampling the phytoplankton species and the bacteria studied.

C. raciborskii was originally axenic, but was contaminated prior to the experiment. Hence, the culture was washed 3 times by normal centrifugation and once by density gradient centrifugation with Percoll (see below) 2 days before the experiment to minimize bacterial contaminants. The algal inocula were checked for axenic conditions before the experiment in WC (Wright's cryptophyte) medium [36] supplemented with peptone and glucose (250 $mg.L^{-1}$ of each, WC p+g) and in CPS (casein, peptone and starch) broth and agar media.

A bacterial inoculum was obtained from Barra Bonita Reservoir, on 06^{th} of March-2012. The inoculum consisted of a mixture of equal proportion of the water collected at different depths (0.5, 1, 5, 10 and 15 m), using a sterilized sampling system with a bottle connected to a vacuum pump and an 18 m silicone hose. In the laboratory, the collected water was filtered through 1.2 µm glass fiber filter to remove larger phytoplankton, zooplankton and detritus. Since nanoflagellates can even pass 0.8 µm pores [37] the bacterial inoculum was incubated for 2 hours with

cycloheximide (100 mg.L^{-1}) and then 0.4 L was washed by first capturing the cells on 0.22 µm polycarbonate membrane filters and then rinsing them three times with the same volume of sterile WC medium. The cells were resuspended to the same volume in WC medium and used as bacterial inoculum in the experiment. A fraction (ca. 120 mL) of this inoculum was filtered again through 0.22 µm polycarbonate membrane filters and the filtrate was inoculated in the axenic phytoplankton cultures to serve as controls for the effects of viruses and potentially residual cycloheximide. No flagellates were found in the cultures during the experiment and the effects of any possible remaining cycloheximide was not significant in previous test for *M. aeruginosa* (ANCOVA, F=2.59 p= 0.12) and *A. granulata* (ANCOVA, F=0.012, p=0.91) in liquid culture. The bacteria captured on the polycarbonate membrane were resuspended in WC medium, centrifuged (16000 *xg*, 25 min) and stored in -20 °C for DNA extraction and sequencing of 16S rRNA genes from the bacterial inoculum.

Experimental design

Each exponentially growing phytoplankton species was inoculated in 4 L WC medium. For the diatom, the medium was supplemented with silica (twice the original concentration – final $Na_2SiO_3.9\ H_2O$ concentration 56.84 mg L^{-1}). The WC medium [36] contains, ~20.7 higher $P\text{-}PO_4$ and 6.4 times higher $N\text{-}NO_3$ concentrations than usually found in Barra Bonita Reservoir [34]. Higher nutrient concentration was used to extend the phytoplankton growth curve and obtain sufficient biomass production. All the procedures were done under aseptic conditions. Each culture was immediately split in 2 flasks, and either inoculated with 0.1 L (5% v/v) bacterial inoculum (non-axenic) or amended with a similar volume of the filter-sterilized control medium. Subsequently, each culture was homogenized and 150 mL aliquots were aseptically transferred to 250 mL culture flasks that were incubated at 23 ± 2 °C

under illumination of 90±10 µmol photons m^{-2} s^{-1} in 12h:12h light-dark cycle. For each sampling day, 3 independent replicate flasks each from the bacterial treatment and the controls were analyzed. Sampling dates varied between the phytoplankton species: *Aulacoseira granulata* (days 2, 9 and 16 – early exponential, exponential and stationary phases), *Microcystis aeruginosa* (days 2, 15 and 20 - early exponential, late exponential and stationary phases) and *Cylindrospermopsis raciborskii* (days 2, 15 and 17 - early exponential, stationary and early senescent phases). Controls were checked for axenic condition in WC p+g medium on the same days, except for *C. raciborskii* where only the inoculum was checked and found to be not axenic. These contaminant bacteria in the control cultures were identified. Analyses of chlorophyll *a* and absorbance were also carried out on the initial day, and absorbance was measured on at least 4 more occasions during the experiment.

Phytoplankton growth analyses

Phytoplankton pigments were extracted using hot 90% ethanol [38]. Chlorophyll-a was measured spectrophotometrically and quantified according to Lorenzen [39]. Phytoplankton growth was followed by *in vivo* chlorophyll absorbance calibrated to extracted chlorophyll-*a* levels.

In vivo chlorophyll absorbance for the diatom cultures was measured at 680 nm with correction for particles at 750 nm. For Cyanobacterial cultures, the absorbances were also measured at 680nm, but corrected for particles at 730 nm. These wavelengths were chosen to correlate better with absolute chlorophyll-*a* concentrations (measured as described above) for *A. granulata* (R^2=0.91) and *M. aeruginosa* (R^2=0.98) in previous study.

Nutrient concentrations

Nitrate and phosphate were analyzed using Dionex ICS-1100 (Ion Chromatography System, Thermo Scientific) in 0.22 µm filtered samples.

Bacterial cell counts

Slides for total bacterial counting were prepared using 4'6-diamidino-2-phenylindole (DAPI) (0.5 µg mL^{-1}) [40]. At least 400 cells were counted in each sample using images obtained by epifluorescence microscopy (Zeiss Axioplan 2 microscope, Zeiss AxioCam HRc camera) processed with AxioVision release 4.5 software (Zeiss). In order to obtain the density of *Actinobacteria*, total bacterial density was multiplied by the proportion of actinobacterial reads obtained by sequencing.

Scanning electron microscopy

Samples obtained during the second sampling were filtered and centrifuged with Percoll (see below). A drop of a suspension containing phytoplankton cells with adhered bacteria was then inoculated in fresh WC medium and monthly transferred to new tubes for 2 months. These cultures were used for scanning electron microscopy (SEM) preparation. Control cultures were also maintained for 2 months. A drop of the sample (10 or 20 µL) fixed in 2% glutaraldehyde was deposited onto coverslips, which were previously coated with poly-l-lysine solution [41], and left overnight in a moist chamber. The excess of water was then carefully dried and the coverslips washed twice in distilled water and dehydrated by 10 minutes treatment with increasing ethanol concentrations (1x 50, 70, 80, 90 and 96% and 4x 100% ethanol). Samples were then critical point dried by using liquid carbon dioxide (BalTec CPD 030). The coverslips were mounted onto SEM stubs with carbon sticky tabs and the samples coated with carbon using a Cressington 308UHR sputter

coater, before observation in a Hitachi S4800 scanning electron microscope (Hitachi, Tokyo, Japan) operated at 5.0 kV.

Fractions separation: free-living and attached bacteria

For the DNA-based analyses, 30 to 40 mL of each culture sample was first aseptically filtered (under a vacuum < 200 mmHg) through an 8 µm pore-size cellulose acetate membrane and washed once with 20 mL of sterile WC medium. The free-living bacteria were defined as the fraction < 8 µm. None of the phytoplankton species used in the experiment passed through this pore size, and the use of this larger pore size compared to the filter used for the original preparation of the inoculum allowed for a more robust separation of bacteria attached to the phytoplankton cells and their free-living counterparts. The filtrate (free-living bacteria) was centrifuged for 25 min at 16000 *x*g, and the pellet maintained frozen until DNA extraction. To recover the phytoplankton-attached bacteria, the fraction retained by filtration was washed twice with 30 mL of sterile WC medium and resuspended in 2 mL of the same medium using a sterile Pasteur pipette to recover most of the cells. The recovered cells were centrifuged in a density gradient with Percoll (GE-HealthCare) in 15 mL falcon tubes with 10 mL of final volume, for 1h at 15500 *xg*, 10°C in a fixed angle-rotor centrifuge (Centrifuge 5804R, Eppendorf). The Percoll concentration was 30% for the Cyanobacteria and 80% for the diatom in WC medium. The unique band for the diatom and the intermediate band for the cyanobacteria, in which there were more cells, were aspirated with a sterile pipette, centrifuged again for 30 min, 16000 *xg* in a 1.5 mL tube filled with WC medium, and the pellet stored at -20°C until the DNA extraction. For the second sampling of adhered bacteria from *C. raciborskii* (C_d02_ab) and free-living bacteria of *A. granulata* (A_d02_fb) there were only two replicates for bacterial community analysis.

The controls were extracted and analyzed without replicates and without any fractionation procedure: *M. aeruginosa* day 20, *A. granulata* day 16, and all sampling days (02, 15 and 17) for *C. raciborskii* since it was not axenic.

DNA extraction, PCR amplification

DNA extraction was performed as follows: 100 µL of proteinase K solution (50 ng μL^{-1} of proteinase K in TE at pH 8) and 0.2 g of glass beads (150-212 µm, Sigma-Aldrich) were added to each 1.5 mL tube. The samples were incubated in a water bath for 15 minutes at 45 °C and subsequently vortexed at 2500 rpm for 15 seconds. CTAB and NaCl were added to each tube at a final concentration of 2% CTAB [42] and 1.35 M NaCl. The samples were incubated for 20 minutes at 65°C and 15 minutes at 55°C followed by centrifugation at 12000 x *g* for 15 min. The supernatant was transferred to a new tube and extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform, followed by DNA precipitation with cold 100% ethanol with sodium acetate (final concentration 1M) followed by a washing step with cold 70% ethanol [43]. The DNA was resuspended to 25 µL in TE-4 buffer and the DNA concentration was determined by absorbance at 260 nm on a NanoDrop 2000C spectrophotometer. Partial bacterial 16S rRNA genes (*Escherichia coli* position 341-805, V3-V5 regions) was amplified using universal bacterial primers 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC) [44]. Primer 341F carried a 454FLX adaptor B at the 5' end and primer 805R carried a 7-bp molecular barcode specific for each sample followed by a 454FLX adaptor A at the 5' end [45]. PCR amplification was performed under the following conditions: initial denaturation at 98°C for 30 s, 25 to 29 cycles at 98°C for 10 s, 53°C for 30 s and 72°C for 30 s, and final extension at 72°C for 7 min. Each sample was amplified in duplicate 30 µL reactions using 10-45 ng of template DNA, 0.02 UμL^{-1} of Phusion

DNA Polymerase (Finnzymes, Espoo, Finland), 1x Phusion HF buffer, 0.2mM dNTPs and 0.25 µM of each primer. Each amplification was checked by electrophoresis on a 1% agarose gel [25]. The replicate PCR reactions were pooled and purified using Agencourt AMPure XP PCR purification kit according to manufacturer instructions (Beckman Coulter Inc., Brea, CA, USA). Purified amplicons were quantified using the Quant-iT PicoGreen dsDNA assay Kit (Invitrogen) and pooled in a known concentration to obtain at least 3,000 sequences for the free-living community and 9,000 sequences for attached community of each sample.

Pyrosequencing and sequences analyses

Amplicons from each sample were sequenced from adaptor A on a 454-FLX system using Titanium chemistry (454 Life Sciences, Brandford, CT) at the SNP/SEQ platform hosted by SciLife Lab, Uppsala, Sweden.

From a total of 1,295,767 reads for the total run, 549,029 reads passed quality control and were assigned to the different samples in the present study based on their barcodes (Table 1. S1). The sequences removed were reads from samples not included in this study and ambiguous sequences, i.e. reads with low quality as inferred from their flowcharts and those that did not carry the exact primer sequence. After implementation of these quality control criteria, the remaining reads were denoised using AmpliconNoise Version 1.24 [46]. AmpliconNoise implements algorithms that remove PCR and 454 pyrosequencing noise as well as the chimera removal tool Perseus. Less than 1.1% of the reads were removed by Perseus using settings (alpha=-15, beta=0.25) for false positive detection, resulting in an average number of 8765 sequences per sample with a range of 2,324 to 16,308 reads per sample (in total 543,428).

To assign reads into OTUs (operational taxonomic units), UCLUST [47] was applied with a 97% sequence similarity cutoff. A representative sequence from each of the resulting OTUs was classified using the naïve Bayesian classifier (Ribosomal Database Project – RDP classifier,) [48] implemented in MOTHUR [49] in combination with the greengenes database gg_OTU_97 and the taxonomy after Hugenholtz [50]. In addition, the reads were annotated against a local freshwater bacterial sequence database (FW) that included almost 12,000 sequence entries [51]. In the text we use the taxonomic placement assigned by naïve Bayesian classifier, followed by the number of the OTU and FW classification in parentheses.

After removal of contaminations as inferred from sequenced blanks as well as phytoplankton reads (cyanobacterial and diatom chloroplasts), 217,653 reads remained with a minimum sequence length of 350 bp length. The complete 454 run has been deposited in the NCBI Short Read Archive under accession number SRR873436.

Statistical analyses

All statistical analyses were conducted using R [52]. Phytoplankton growth curves (absorbance) of axenic and non-axenic cultures were compared by Covariance Analysis (ANCOVA) using time as covariate [12].

Treatment effects on bacterial OTUs were visualized in heatmaps [53] after resampling with the perl script daisychopper.pl [54] to 86 sequences, the lowest number of reads for one of the samples. The bacterial heatmap included the 51 most abundant OTUs in the combined dataset.

The Morisita-Horn distance was ordinated in two dimensions using non-metric multidimensional scaling (NMDS) to visualize the dynamics in community structure (β-diversity) using the function metaMDS in R and the none-resampled OTU matrix.

This metric was chosen based on its robustness with samples of differing sample size [55], since the number of reads of associated bacteria was low in some samples as a result of high number of reads from *Cyanobacteria* and/or chloroplast. Statistical significance of the differences in community composition between free-living and particle associated bacteria, sampling day and different phytoplankton species (pairwise comparison or between the 3 phytoplankton species) were investigated in an ANOVA experimental design on the basis of the Morisita-Horn distance measure, using permutation methods [56] using the function Adonis in R. The same analysis was performed using distinct cutoffs of abundance (total proportion of the reads of the OTUs in the dataset considering all bacterial reads of the 3 phytoplankton species) and frequency presence (presence of OTUs in the samples). The p-values were corrected by the method of false discovery rate using the function qvalue in R [57].

1.4. Results

Growth of phytoplankton species and sampling days

There were no significant differences in *M. aeruginosa* growth in response to the bacterial inocula (ANCOVA, p=0.11, F=2.62) (Figure 1.1). In contrast, a significant negative difference in the growth of *A. granulata* (ANCOVA, p<0.001, F=31.82) was observed (Figure 1.1). *C. raciborskii* cultures inoculated with lake bacteria exhibited significantly higher growth yield compared to the control (ANCOVA, p<0.001, F=16.34) (Figure 1.1). Differently from *M. aeruginosa*, that presented the highest chlorophyll *a* concentrations, both *A. granulata* and *C. raciborskii* did not seem to be nitrate and/or phosphate limited during growth (Table 1. S2).

All three phytoplankton species had bacteria directly attached to the cells. In the case of *M. aeruginosa,* colonies only formed in co-cultures with bacteria, as under this conditions a polysaccharide matrix was produced (Figure 1.2). *M. aeruginosa* single cells without this polysaccharide layer did not have attached bacteria. Bacteria were not detected by SEM in *M. aeruginosa* and *A. granulata* control cultures (Figure 1.2 a, c). Nevertheless a few reads of non-cyano/chloroplast origin were detected in the controls of *A. granulata* (23 non-chloroplast reads from 10884 reads in total) and *M. aeruginosa* (3 non-cyano reads from 6947 reads in total) (Table 1. S3) that most likely represent contamination during DNA extraction, PCR reactions, library preparation and/or sequencing, but we cannot rule out that they originate from the cultures themselves.

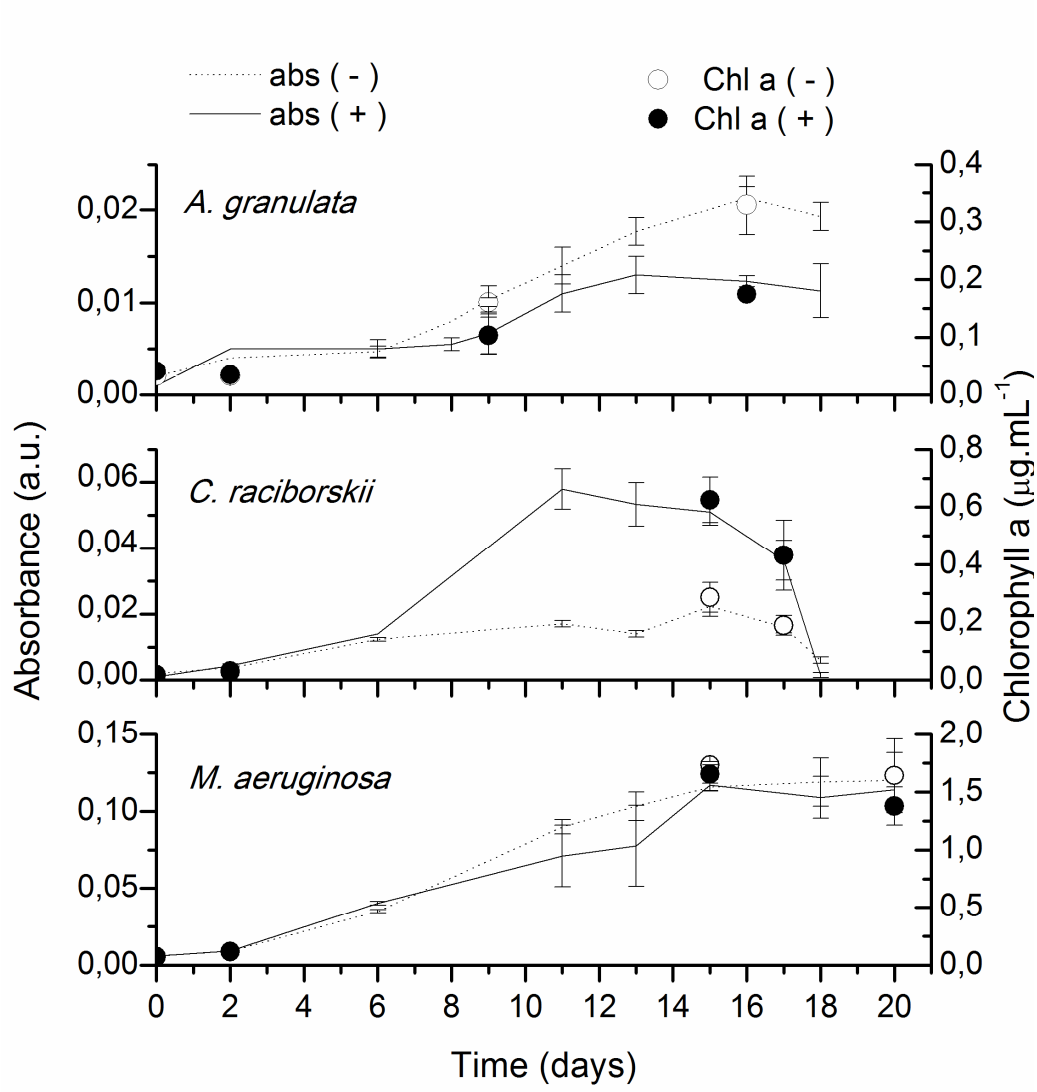

Figure 1.1 - Growth curves of *Aulacoseira granulata*, *Cylindrospermopsis raciborskii* and *Microcystis aeruginosa*. Lines represent absorbance and symbols represent chlorophyll a concentration. Dashed lines and open symbols are axenic* cultures (-), and solid lines and symbols are non-axenic cultures (+). Error bars represent standard deviation of 3 independent replicates. * C. raciborskii was not axenic. Differences in growth curves were significant to *Aulacoseira granulata* (ANCOVA, $p<0.001$, $F=31.82$) and *Cylindrospermopsis raciborskii* (ANCOVA, $p<0.001$, $F=16.34$). No significant effect was observed to *Microcystis aeruginosa* (ANCOVA, $p=0.11$, $F=2.62$).

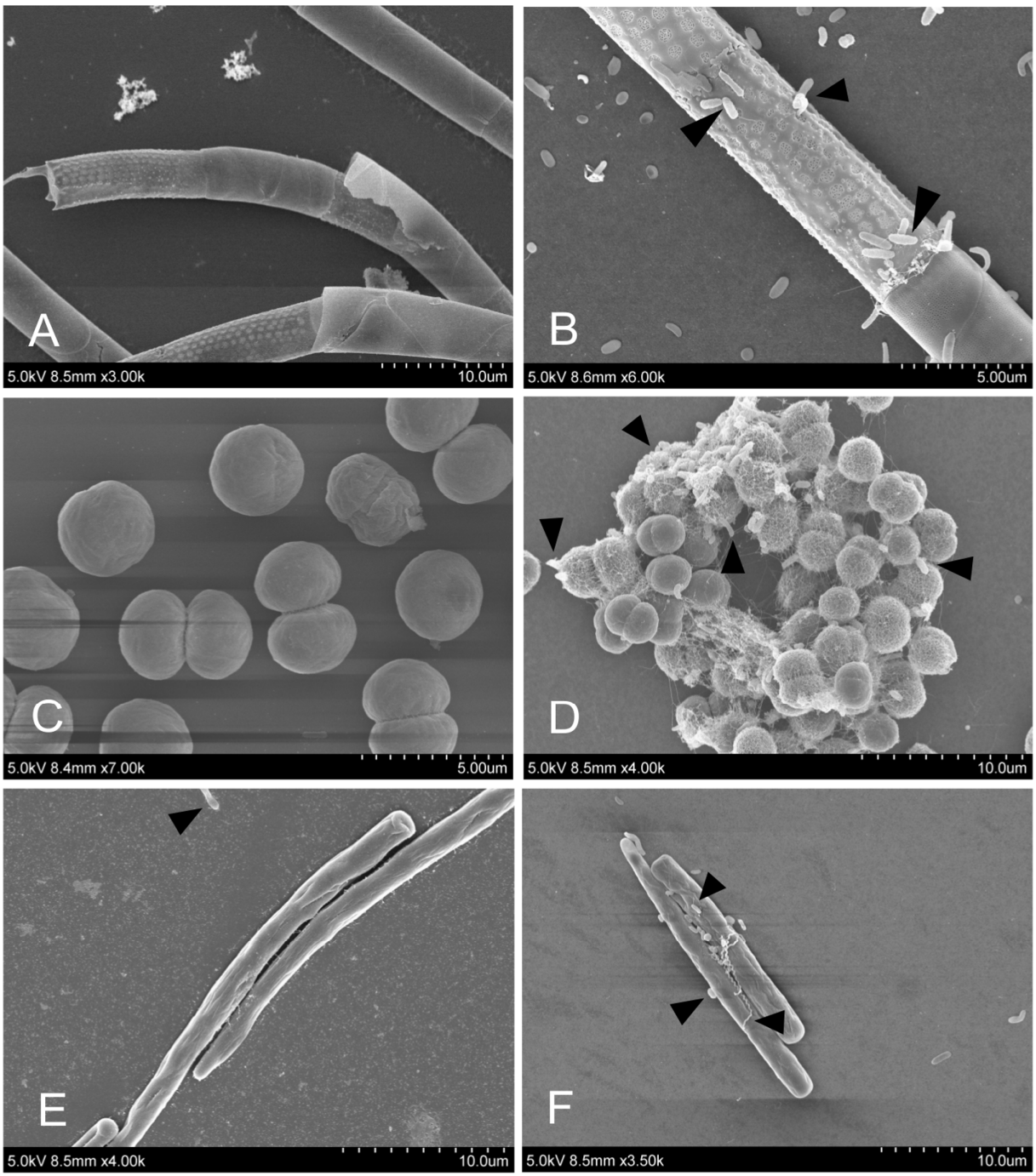

Figure 1.2 - Scanning electron microscopy photomicrograph of the microalgae without (left) and with (right) bacterial inoculum. *Aulacoseira granulata* (a,b), *Microcystis aeruginosa* (c,d) and *Cylindrospermopsis raciborskii* (e,f) cultures without (a, c, e) and with (b,d,f) environmental bacterial inoculum. Black arrows indicate some bacterial (non-phytoplankton) cells.

Reproducibility and specificity of OTU-phytoplankton association

Samples were pyrosequenced in triplicate, except for controls (see methods) and the bacterial inoculum. On average 3,753 bacterial 16S rRNA sequences were obtained for each of the 58 samples (range 86-10036 sequences, see Figure S1). Using a 97% similarity cutoff [47] a total of 4085 OTUs were obtained with approximately half (2187 OTUs) of them being singletons.

Considering the whole sampling period, for both attached and free-living bacteria, a total of 1609 OTUs were obtained for *A. granulata* (552 adhered and 1279 free-living), 1366 for *M. aeruginos*a (134 adhered and 1319 free-living), and 1419 for *C. raciborskii* (279 adhered and 1280 free-living). For the bacterial inoculum we obtained 668 OTUs, implying the presence of other rare OTUs that were not detected in our analysis of this bacterial source community.

Considering only OTUs that occurred in all 3 replicates for each treatment, *i.e.* by phytoplankton species, day and fraction (attached or free-living), we obtained 186 OTUs for *A. granulata* with 51 being phytoplankton-specific (see definition of specificity below); 140 for *C. raciborskii,* 25 being specific; and 131 for *M. aeruginosa*, 34 being specific. For the 100 most abundant OTUs for each phytoplankton species (corresponding to more than 92% or the reads of each sample), 79% were detected in each of the triplicates for *C. raciborskii*. The corresponding fraction was 81% for *A. granulata* and 84% for *M. aeruginosa.*

Only the OTUs that occurred in the 3 independent replicates of one phytoplankton species and that had a maximum of 2 reads in the treatments of each of the other two were considered specific. Phytoplankton-specific OTUs (Table 1.S4) were typically found in low abundance, each representing less than 1.5% of the total bacterial reads obtained for the sample, and were found in different fractions and

growth phases of the three phytoplankton species (Figure 1. 3). Within total specific OTUs (Table 1. S4) for *C. raciborskii*, 7 OTUs were found only in the attached fraction and 13 only in the free-living bacterial community. For *A. granulata* 13 OTUs were specific in the attached fraction and 32 specific free-living bacteria. All the specific OTUs for *M. aeruginosa* were found in free-living fraction.

Differences in bacterial community: succession and selectivity by host species and fraction

Bacterial communities differed between the 3 phytoplankton species (Figure 1.4) and this was also supported by PERMANOVA (p-value<0.001). The differences between phytoplankton cultures were significant for the total bacterial community as well as for the free-living or attached bacteria fractions (Table 1.1). There were also significant differences between free-living and particle-attached communities for each species (*M. aeruginosa* p<0.001, *A. granulata* p<0.002 and *C. raciborskii* p=0.003). Differences were also significant between growth phases (Table 1.1), suggesting a gradual shift in bacterial community composition over time, which is visualized in the NMDS plot (Figure 1.4).

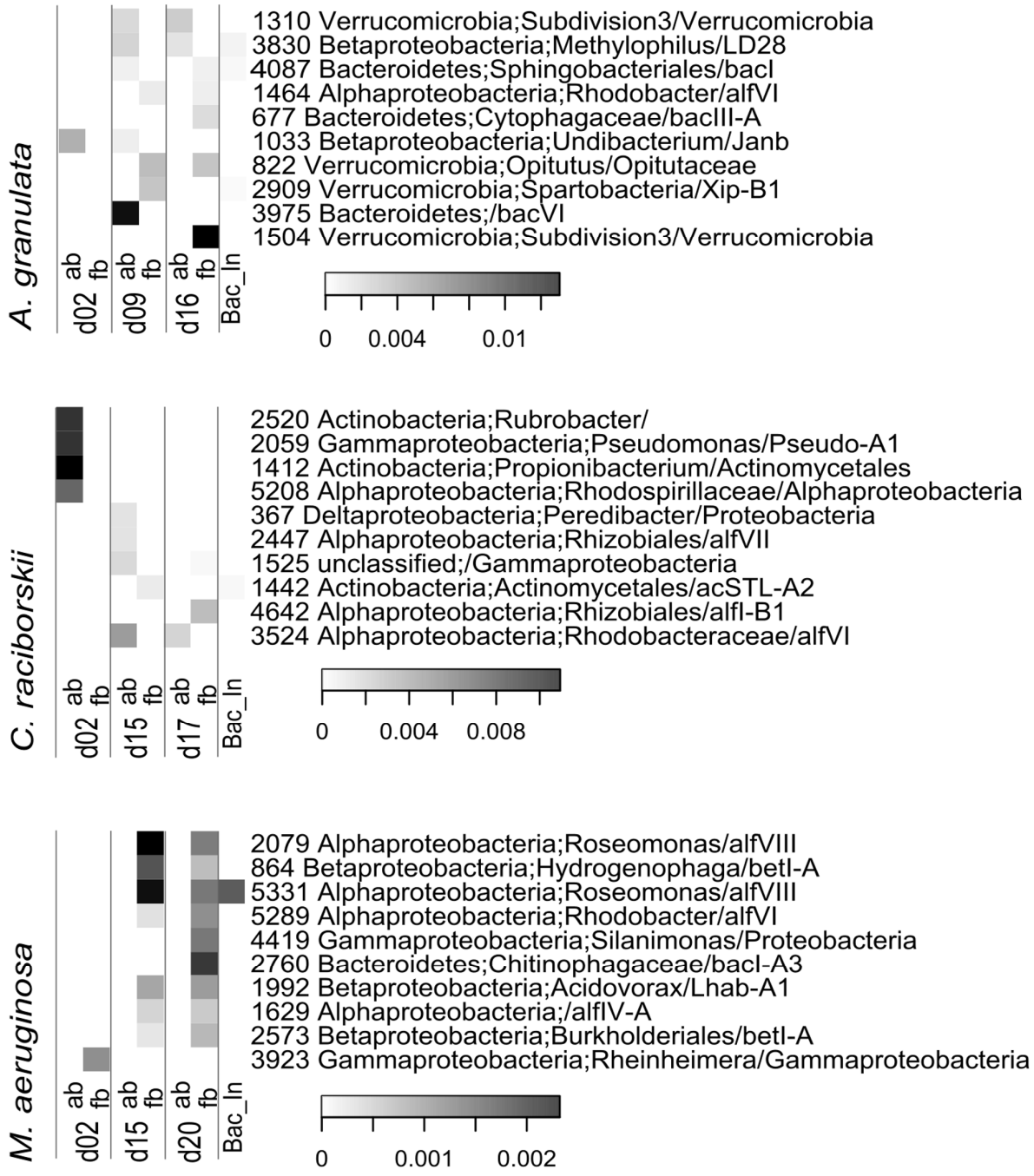

Figure 1.3 - Heatmap of the 10 most abundant specific OTUs associated with each studied phytoplankton species. The non-resampled data was used and only the OTUs that occurred in the 3 independent replicates of one phytoplankton species and that had less than 2 reads in the treatments of the other two were considered specific. Bac_In is the bacterial inoculum; ab indicates attached bacteria and fb, free-living bacteria; dxx indicates the day of sampling. Frequencies are given by relativizing OTUs against the total number of reads of the sample, showing their low proportion. Taxonomic affiliation of two classification databases is shown after identification number: rdp/FW

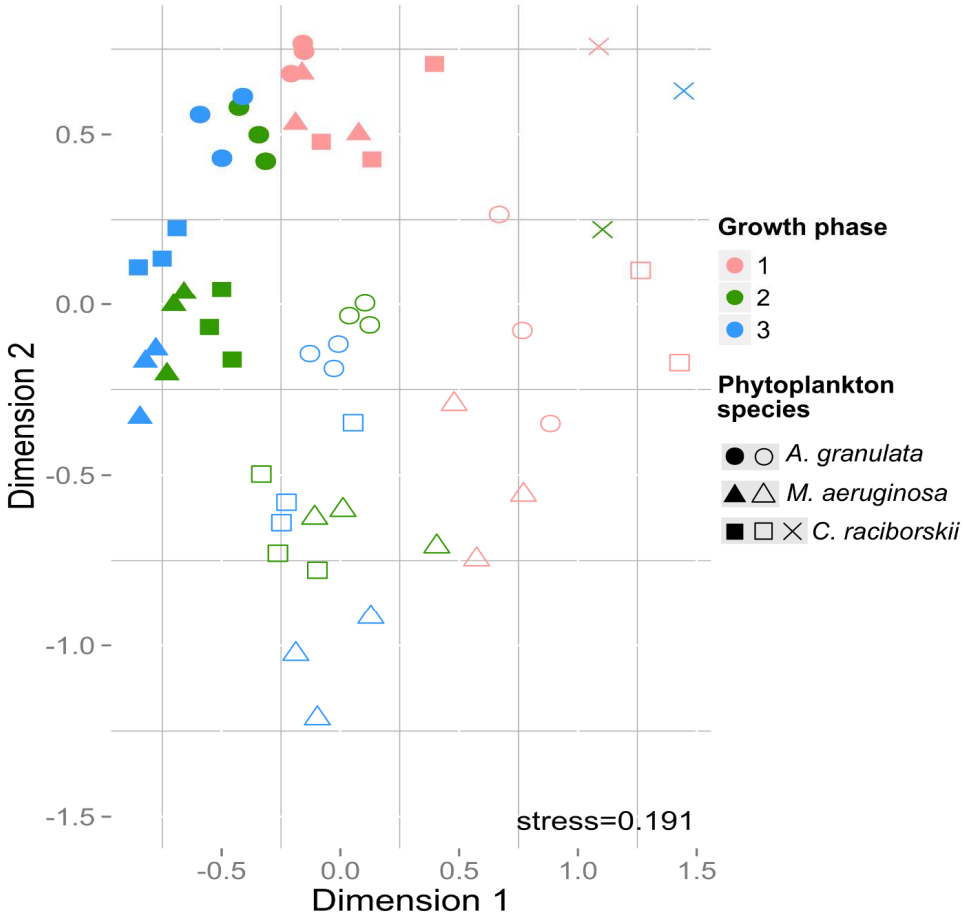

Figure 1.4 - Non-metric multidimensional scaling (NMDS) plot showing differences among bacterial communities by phytoplankton species, fraction and growth phase. Solid and open symbols represent, respectively, free-living and attached communities. The crosses represent *Cylindrospermopsis raciborskii* controls. 1 = lag or beginning of exponential growth phase, 2 *=exponential growth phase, 3* = stationary growth phase. * To *C. raciborskii* 2 and 3 were stationary and senescent phases, respectively.

Table 1.1 – Results of Permutational MANOVA comparing bacterial community composition between fraction (attached and free-living), growth phase (age), and phytoplankton species.

		Df	Fstats	R^2	p
Aulacoseira granulata (A)	fraction	1	15.04	0.31	0.002
	age	2	5.57	0.23	0.005
	fraction*age	2	5.32	0.22	0.002
Microcystis aeruginosa (M)	fraction	1	31.12	0.26	<0.001
	age	2	28.50	0.48	<0.001
	fraction*age	2	8.84	0.15	0.002
Cylindrospermopsis raciborskii (C)	fraction	1	13.70	0.13	0.003
	age	2	38.53	0.70	<0.001
	fraction*age	2	3.81	0.07	0.002
A x M x C	species	2	8.03	0.25	<0.001
A x M x C only attached	species	2	5.57	0.33	0.002
A x M x C only free-living	species	2	6.88	0.37	<0.001
A x M	species	1	9.15	0.22	<0.001
A x C	species	1	10.67	0.25	<0.001
M x C	species	1	4.56	0.12	0.006

Removing less abundant OTUs, by using distinct cutoffs of abundance and occurrence, we obtained higher R^2 values (Table 1.S5), reflecting an increase in the differences of BCC between phytoplankton species. Still at all cutoffs significant differences were maintained. PERMANOVA analysis using only the 6 most abundant OTUs (OTUs that represented more than 3% of total bacterial reads of the analyzed samples, i.e. 3% cutoff) or the 6 most frequent OTUs (OTUs that occurred in more than 40 samples) provided higher R^2 values: 0.33 (Fstats=12.449, p<0.001) and 0.32 (Fstas= 11.34, P<0.001), respectively. Also, the R^2 value of the PERMANOVA using a cutoff of 1% (18 OTUs) was higher compared to when OTUs with abundance below 1% were included (4067 OTUs). These differences using only most abundant OTUs reflects the greater differences in the proportion of some *taxa*, for example, the OTU #4985 (*Sphingobacteria*) made up more than 10% of the reads in *M. aeruginosa*

samples and less than 0.5% in the other phytoplankton species samples, whereas OTU #4954 (*Dechloromonas*-related) represented more than 30% of the reads in *A. granulata* and less than 5% in the cyanobacteria.

PERMANOVA analysis also indicated that bacterial communities from cyanobacterial cultures (*M. aeruginosa* x *C. raciborskii*) were more similar among themselves compared to diatom cultures (*A. granulata*), since the R^2 value was lower among the pairwise comparison of cyanobacterial treatments (Table 1.1).

Bacterial community composition

To visualize the effects of the treatments on OTUs composition while minimizing the influence of sampling depth (Figure 1.5), the data was resampled using daisychopper.pl. After this, 437 OTUs remained (366 OTUs considering only the treatments). To display differences in proportion of the main Classes (Figure 1.6), the non-resampled matrix was used.

Overall, *Betaproteobacteria* was the dominant Class in all incubations (Table 1. 2), but its relative contribution decreased significantly from the first to the last sampling in all treatments ($p<0.05$) (Table 1.S6). Despite their dominance in all cultures, there were differences in the main OTUs in different phytoplankton species (Figure 1.5). In contrast, the relative contribution *of Alphaproteobacteria* as the second most abundant class, increased over time, but the significance was variable (Table 1.S6).

The dominance of the Phylum *Proteobacteria* contrasted with the dominant Phylum in the inoculum, in which 70% of the reads were assigned to *Actinobacteria* (Figure 1.6). Among the 20 most abundant OTUs in the inoculum, 5 belonged to *Proteobacteria,* one belonged to *Planctomycetes,* and 14 belonged to *Actinobacteria*. Two OTUs #245 (*Agrococcus*-related/Luna1-A1) and #1429 (*Ilumatobacter*-

related/acIV-C), both *Actinobacteria* were among the most abundant only in *C. raciborskii* cultures. None of the 5 originally abundant *Proteobacteria* was among the 20 most abundant OTUs in the phytoplankton cultures.

A major difference at the class level was that the occurrence of *Gemmatimonadetes* was higher in cultures with either of the cyanobacterial species, whereas it was only detected early on the free-living portion of diatom cultures at relative abundances below 0.6% (Figure 1.6, Table 1.S7).

The proportion of the *Actinobacteria* and *Verrucomicrobia* increased over time in *C. raciborskii* and *A. granulata* cultures. Although they represented a small part of the total bacterial community in all cultures, we observed a difference among *M. aeruginosa* and the other phytoplankton species.

Despite the similarity in the occurrence of *Actinobacteria* and *Verrucomicrobia* between *A. granulata* and *C. raciborskii*, the most abundant OTUs affiliated with these classes were different between the diatom and the Cyanobacteria. The *Actinobacteria Agrococcus* (#245, Luna1-A1) and *Ilumatobacter*-related OTUs (#1429, acIV-C) were dominant in the free-living portion of *C. raciborskii* cultures, whereas a *Terracoccus*-related (#3682, acIC) was more abundant on diatom cultures (Figure 1.5).

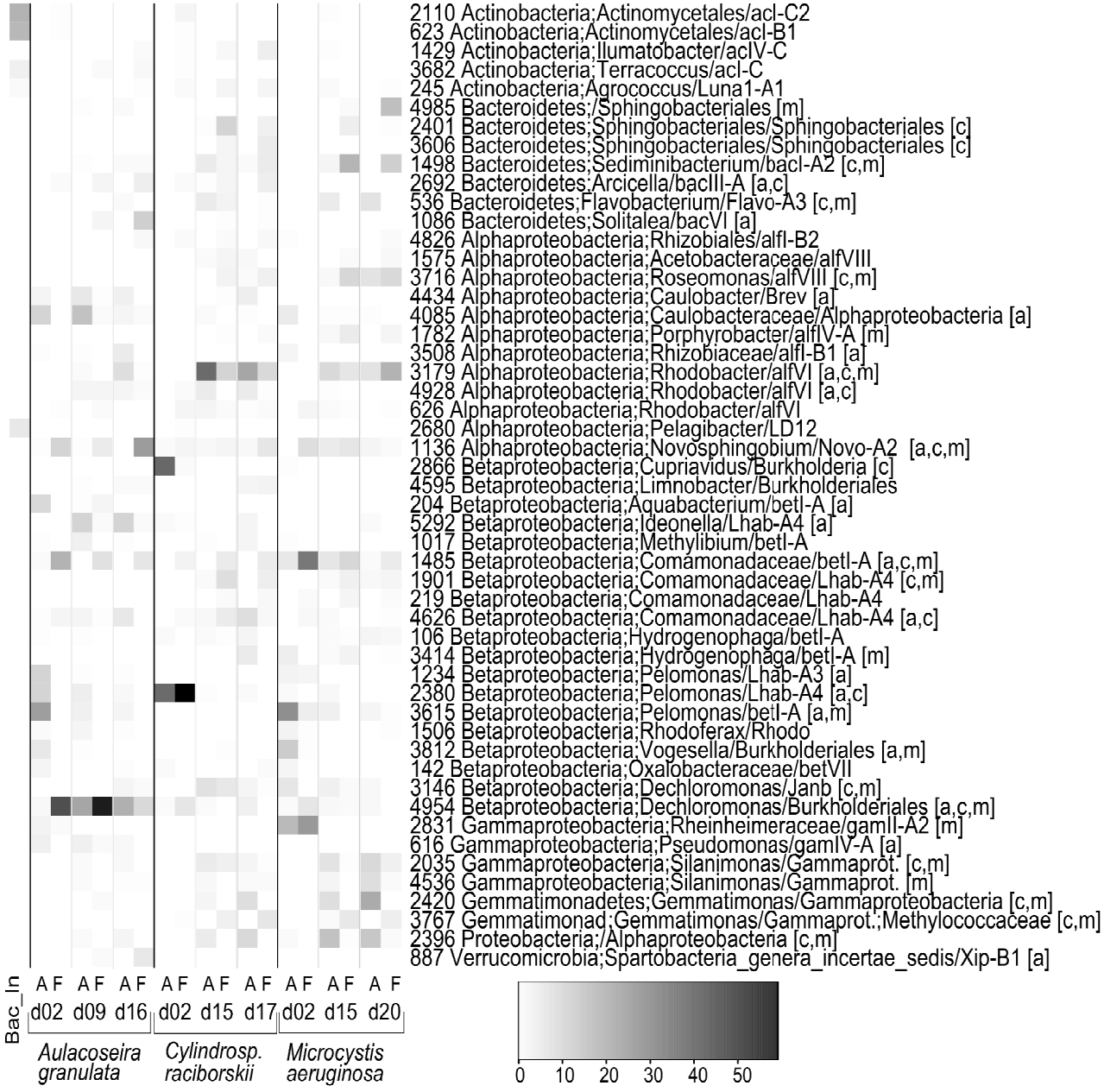

Figure 1.5 – Heatmap displaying the 51 most abundant OTUs after resampling. Taxonomic affiliation of two classification databases is shown after identification number: rdp/FW. A and F indicate attached and free-living communities, respectively, and dxx indicates day of sampling. Letters in brackets indicate the phytoplankton species in which that OTU was within the most abundant: a, *Aulacoseira granulata* (18 OTUs); c, *Cylindrospermopsis raciborskii* (20 OTUS)*;* m*, Microcystis aeruginosa* (20 OTUs)*.* All these OTUs, except #616, were present in triplicates in at least one treatment of the phytoplankton species where they occurred.

Table 1.2 – Relative abundances (%) of the main bacterial Classes in the 3 phytoplankton cultures in different sampling days (d). Mean ± pooled standard deviation of attached and free-living communities

	A. granulata			*C. raciborskii*			*M. aeruginosa*		
	d02	d09	d16	d02	d15	d17	d02	d15	d 20
Alphaproteobacteria	12 ± 3	22 ± 4	33 ± 15	6 ± 4	38 ± 0	36 ± 11	10 ± 3	28 ± 6	27 ± 4
Betaproteobacteria	82 ± 3	65 ± 7	38 ± 15	84 ± 7	22 ± 9	28 ± 10	63 ± 12	26 ± 2	15 ± 5
Gammaproteobacteria	5 ± 2	2 ± 3	4 ± 4	2 ± 1	5 ± 3	2 ± 01	24 ± 14	7 ± 4	12 ± 2
Flavobacteria	0 ± 0	1 ± 0	1 ± 0	0 ± 0	7 ± 6	2 ± 2	0 ± 0	2 ± 2	3 ± 3
Sphingobacteria	1 ± 0	5 ± 1	13 ± 7	1 ± 0	17 ± 0	14 ± 3	0 ± 0	18 ± 13	9 ± 5

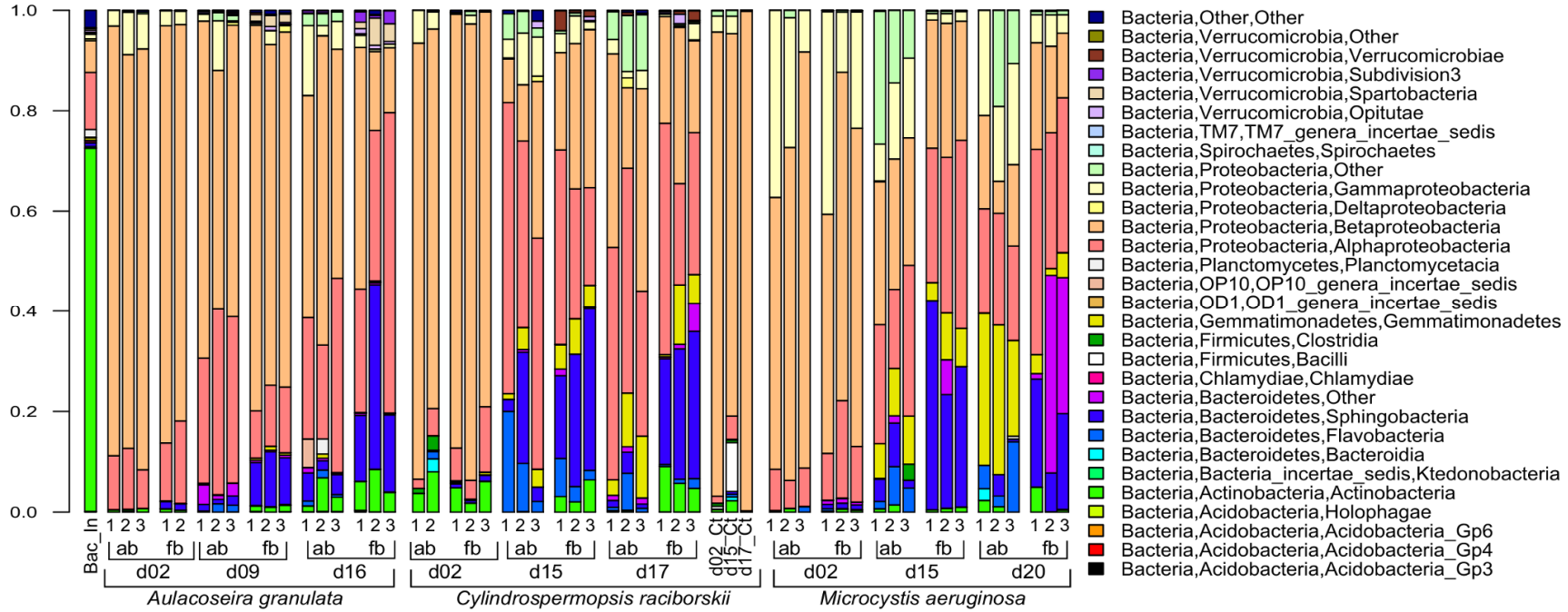

Figure 1.6 – Proportion of bacterial Classes in the replicates of each treatment of the 3 phytoplankton species. Naïve Bayesian classification was used. Bac_In is the bacterial inoculum. Numbers 1-3 represent the replicate, ab and fb indicate attached and free-living communities respectively, Ct is control and dxx is the sampling day.

Bacteroidetes was represented mainly by *Sphingobacteria*, which occurred in all cultures (Figure 1.6). However, the major OTUs differed between diatom and cyanobacteria. The genera *Arcicella* (*Cytophagaceae* #2692, bacIII-A) and *Solitalea* (*Sphingobacteriaceae* #1086, bacVI) were more abundant in the diatom culture, whereas the genera *Sediminibacterium* (*Chitinophagaceae* #1498 and #4889, bacI-A2) and two not identified *Sphingobacteriales* (#2401, #4985) were more abundant in the cyanobacterial cultures (Figure 1.5). The *Flavobacteria* (also *Bacteroidetes*) presented higher relative abundance in the attached fraction of cyanobacterial cultures.

Another difference between phytoplankton cultures was the higher proportion of *Gammaproteobacteria* in *M. aeruginosa* cultures. OTUs from this Class were among the 20 most abundant in *M. aeruginosa* cultures (Figure 1.5). A *Reinheimeraceae* (#2831, gamII-A2) was initially dominant, but was substituted by other *Gammaproteobacteria* as *Silanimonas lenta* (#2035), which was found mainly in the attached fraction of *M. aeruginosa* cultures (Figure 1.5).

Among the other dominant taxa of other Classes occurring associated with *M. aeruginosa* in attached portion were the OTUs #2420 (*Gemmatimonas*-related), #2396 (*Alphaproteobacteria*) and the *Dechloromonas*-related (#3146), whereas #1498 (*Sediminibacterium*, bacI-A2), #3179 (*Rhodobacter*-related, alfVI) and #3716 (*Roseomonas,* alfVIII) were more abundant in the free-living fraction.

In *C. raciborskii* the two most abundant OTUs in both fractions were #2380 and #3179. The OTU #2380, a *Pelomonas*, was the main contaminant in the control cultures of *C. raciborskii* (corresponding to more than 84% of total reads for the 3 controls), and its dominance was likely due to its abundance at the first sampling day

2nd cultivation day), but it was overwhelmed by other OTUs over time, mainly by *Rhodobacter* (alfVI,

#3179), predominant in the attached fraction and by some *Sphingobacteriales* (#2401) and *Comamonadaceae* (#1901, Lhab-A4) in the free-living fraction of exponential growth phase (Figure 1.5)

With regard to other differences in attached and free-living bacterial OTUs, the two most abundant OTUs related to the genus *Gemmatimonas* in both cyanobacterial cultures occurred in different fractions: one more abundant in the cyanobacterial-attached (#2420) community and another one in the free-living community (#3767) (Figure 1.5).

In *A. granulata* cultures, the genera *Arcicella* (*Cytophagaceae* #2692, bacIII-A) and *Solitalea* (*Sphingobacteriaceae* #1086, bacVI) were more abundant in free-living portion, whereas the *Caulobacteraceae* (#4085, #4434) and the genus *Ideonella* (#5292, Lhab-A4) were more abundant in the attached fractions. The most abundant OTU for *A. granulata* was a *Dechloromonas*-related (#4954, a *Rhodocyclales*), which predominated in both fractions (particle-attached and free-living) at the exponential growth of the alga

1.5. Discussion

Phytoplankton-specific bacterial community composition

Previous work suggests that the composition of phytoplankton-associated bacterial communities vary between phytoplankton species [12,17,27,58]. Here we characterized the specific taxonomic groups of bacteria that emerge during different growth stages of bloom forming freshwater phytoplankton species and did so in much greater detail than was previously possible. This deep characterization showed that the BCCs emerging from the same inoculum were characteristic for each phytoplankton species and their growth phases. The observed differences were mainly due to variations in the proportion of the OTUs, and not due to the presence or absence of specific taxa.

None of the more abundant bacterial phylotypes in the cultures appeared to feature specific associations to any of the studied phytoplankton species during the culturing time used in the experiments. This observation likely would not be possible using less sensitive techniques commonly used in previous studies, as DGGE [12,27] since we could have found, for example, that the OTU #4985 was specific to *M. aeruginosa,* once it occurred in less than 0.5% in the other phytoplankton cultures.

Although we only followed single life cycle of each phytoplankton species the variation in the OTUs abundance was strong enough to drive differences in betadiversity. Furthermore, our results suggest that the observed associations are robust and not merely due to chance, since more than 78% of the most abundant OTUs were present in each replicate of a specific treatment. This is in agreement with Grossart et al. [12] and Schäfer et al. [17] who both found that the community composition in different algal-bacterial co-cultures were reproducible if the same bacterial inoculum was used.

Some degree of specific association can be expected between bacteria and phytoplankton since there is a considerable variation in the contribution of different organic metabolites to the total biomass of different phytoplankton species [16]. Furthermore, different bacteria have distinct capacities to degrading and metabolizing phytoplankton-derived compounds [16]. Differential resistance to antibiotics produced by the phytoplankton has also been demonstrated in *M. aeruginosa*-associated bacteria and this may also foster specific heterotroph-phytoplankton interactions [20]. Still, we cannot rule out additional effects resulting from nutrient competition between the phytoplankton species and bacteria, as phytoplankton species are known to differ in their nutrient requirements and uptake kinetics [e.g. 60] and final concentrations of N and P differed between the phytoplankton treatments (Table 1.S2).

The expected variation in composition and proportion of organic matter released from viable cells (mainly polysaccharides [3,16]), is quite likely also reflected in the associated heterotrophic bacterial community, especially when using the same conditions for phytoplankton growth. Therefore, as many taxa are shared across our experimental incubations, we speculate that some excreted compounds may overlap between the phytoplankton species and merely vary in abundance. Gouvêa et al. [59] have shown that the high molecular weight material from *A. granulata* and *M. aeruginosa* strains isolated from Barra Bonita Reservoir share most of the monosaccharides, except the acid ones, although in different proportion.

Additionally, polymeric phytoplankton-derived organic matter will not only be processed by the actively hydrolyzing bacteria, but also a wider range of opportunistic bacteria capable of quenching the hydrolysis products will profit [18]. Thus, explaining the observation that differences in BCC between the treatments in

the culturing time used in our experiment will be in the proportion rather than in occurrence of OTUs.

More detailed analyses of the bacterial communities revealed a few less abundant phytoplankton-specific OTUs in all treatments (Figure 1.3 and table 1.S4), with exception for the attached *M. aeruginosa* community. The absence of specific OTUs in *M. aeruginosa* attached community is likely the result of the dominance of cyanobacterial 16S rRNA reads in these samples and the resulting superficial characterization of specific bacteria attached to this *Cyanobacteria* (Figure 1.S1). These few and less abundant specific OTUs may represent the heterotrophic taxa thriving on more species-specific phytoplankton metabolites. However, using a rigorous definition of specificity, we cannot exclude that sequencing depth influenced the classification into “specific OTU", as non-resampled data were used for this comparison.

Moreover, we showed that despite major differences in proportion of taxonomic groups, the emerging bacterial community had different effect on cyanobacterial and diatom growth (Figure 1.1), suggesting that local bacterial community may play an important role on the dominance of the different phytoplankton species. Although the emerging bacterial community from Barra Bonita reservoir enhanced the growth of *C. raciborskii*, it is not possible to say whether the inoculated bacteria from the lake directly stimulated the cyanobacterial growth or whether this effect was because they outcompeted the contaminants (mainly genus *Pelomonas*/Lhab-A4) which may have had a negative effect on phytoplankton growth in the control cultures.

Differences of Groups and OTUs by phytoplankton species

Both differences in OTUs proportions and the analysis of higher taxonomic levels revealed differences in associated bacterial communities among phytoplankton species.

Similar proportions (except for *Actinobacteria*) of the dominant groups found in our work associated with *M. aeruginosa* were also found by Eiler and Bertilsson [25] in a naturally occurring *Microcystis*-dominated bloom in eutrophic lake Ekoln. *Proteobacteria* (*Alpha*, *Beta* and to a lesser extent *Gammaproteobacteria*) dominated the associated bacterial communities which also featured an abundance of *Bacteroidetes* and *Actinobacteria.*

To the best of our knowledge, little data about the proportion of bacterial groups directly associated with *A. granulata* or *Cylindrospermopsis* blooms are available in the literature. Pope and Patel [28] reported the occurrence of the main Classes found in this work during a cyanobacterial bloom dominated by *Aphanizomenon* and *Cylindrospermopsis*, with the exception of *Gemmatimonadetes,* but they sequenced only 44 bacterial 16S rRNA clones. Shi et al [27] reported four bacterial strains associated with *C. raciborskii* maintained in culture, 3 *Burkholderiaceae* (*Betaproteobacteria*) and one *Sphingobacteriales* (*Bacteroidetes*).

One of the groups found in abundance in cyanobacterial cultures, the *Gemmatimonadetes,* is a minor component among freshwater lake bacteria [51] and scarce reports are available on its occurrence associated with phytoplankton. Shi et al. [26,61] also found *Gemmatimonadetes* associated with *Microcystis* colonies, but most encounters of this phylum is from soil microbial communities [62] where its abundance seems to be negatively correlated to pH [63].

Another phylum that varied between phytoplankton species was the *Actinobacteria*. This group was very abundant in the inoculum, but made up less than 10% of the emerging bacterial communities in cultures and being less abundant in *M. aeruginosa* cultures. Based on the proportion of the reads of free-living *Actinobacteria* and on the free-living bacterial density, there was a reduction in number of actinobacterial cells between the day of inoculation and the second day of incubation (Figure 1. S2). However, this was followed by an increase in abundance during the stationary growth phase, mainly in *C. raciborskii* and in *A. granulata* cultures, possibly indicating that this group profits more from stressed phytoplankton cells. The most abundant genus of this group was *Ilumatobacter*, from clade acIV-C (Iluma-C1 is also an acIV-C). The tribe Iluma-C1 is known to compete successfully when phosphorus levels are elevated [45], and phosphorous was indeed not limiting in the cultures of either of these two phytoplankton species (Table 1.S2). Despite some reported occurrences of *Actinobacteria* in heterotrophic communities associated with cyanobacterial blooms [25,31], *Actinobacteria* were not found attached to other members of the *Nostocales* (*Gloeotrichia echinulata*) [10], but were observed in the total fraction of associated bacteria during *Cylindrospermopsis* and *Aphanizomenon* blooms [28].

Verrucomicrobia, present in lower proportion in *C. raciborskii* and *A. granulata* cultures, are generally observed in abundance during cyanobacterial blooms [25,28,42], with less studies reporting their association with diatoms [64,65]. Members of the Phylum *Bacteroidetes* are usually found in association to phytoplankton blooms [51], but the classes and OTUs of this group seems to vary greatly between different phytoplankton species in our work (diatom and

cyanobacteria), possibly indicating ecological specialization and adaptation within this diverse phylum.

Comparison of cyanobacterial and diatom BCC

Despite significant differences, BCC associated with the two *Cyanobacteria* were more similar among themselves compared to the diatom culture, suggesting that compounds produced by the phytoplankton species may be a more important selection factor for the heterotrophic community than nitrate and phosphate availability, as these nutrients were depleted in the *M. aeruginosa* cultures, but not in the *C. raciborskii* and *A. granulata* cultures (Table 1.S2). Shi et al. [27] found that BCC in cyanobacterial cultures showed higher similarity values among *M. aeruginosa* cultures than when compared to other *Microcystis* spp, or among non-*Microcystis* cyanobacterial cultures. This could be related to the expectation that closely related phytoplankton taxa produce more similar organic compounds [66]. Since the range of organic molecules produced may select for well adapted bacterial populations [67], we could also expect more similarity among BCC of *Cyanobacteria*.

Bacterial community changes with phytoplankton growth phases

There were significant differences in bacterial populations in the different growth phases in both attached and free-living bacterial communities for the 3 phytoplankton species. Changes in bacterial community composition during blooms or over time in cultures have already been reported for diatoms [12] and cyanobacteria [68]. Grossart et al. [12] also reported distinct successions of bacterial communities in the surrounding water (free-living) and attached onto algal particles of diatoms cultures. These studies conclude that succession likely take place because

of differences in the ecophysiology of the phytoplankton, also influenced by differences in nutrient concentration in the cultures.

Shi et al. [27] reported no difference in the dominant bacterial 16S rRNA DGGE bands in different growth phases of 12 cyanobacterial strains (among them *M. aeruginosa* and *C. raciborskii*) in cultures, but this study was only qualitative and not quantitative. Also, Schäfer et al. [17] did not observe any differences in BCC when DGGE profiling of 16S rRNA genes was applied to diatom cultures in exponential and stationary growth, but Grossart el al. [12] argue that this apparent stability could be due to missing samples from the initial exponential growth phase. As seen in Figures 1.4-1.6, exponential and senescent growth phases are more similar than the initial exponential phase, with higher variability in proportion of groups than occurrence of distinct groups.

The most obvious differences in bacterial classes over time in the cyanobacterial cultures were the increasing proportion of *Gemmatimonadetes* in the adhered fraction. The only isolated member of this phylum, *Gemmatimonas aurantiaca,* is a slow growing and phosphate accumulating bacteria [69]. The phosphate accumulation could explain its gradually increasing representations in the later stages of the *M. aeruginosa* cultures where nutrients were depleted, but this is most likely not the case for *C. raciborskii* cultures, where phosphate was still available. Thus some compound(s) released by *Cyanobacteria* could have selected for members of this group.

The proportion of *Bacteroidetes* increased towards the end of the experimental incubations. This is not surprising considering that members of the *Sphingobacteria* and *Flavobacteria* (both affiliated with *Bacteroidetes*) are often found in high abundance during periods following phytoplankton blooms [12,51], either in the

surrounding water or attached to algae, which are distinct microenvironments each with its own specific organic matter signature [12].

Among the most abundant OTUs during the first sampling were several *Comamonadaceae* (*Betaproteobacteria*). As OTUs of this Family were rare in the inoculum, they likely feature rapid growth in response to the experimental manipulation. The clade betI-A (from this Family) is known for their ability to grow fast and can respond rapidly to changing environmental conditions, while their abundance also seem to be positively associated with low-molecular weight alga-derived substrates (see review [51]).

Differences between attached and free-living bacterial communities

In agreement with previous studies, differences were also observed between attached and free-living communities [12,58]. The comparison between the phytoplankton-attached and the free-living bacterial communities may to some extent have been influenced by the high density of Cyanobacteria/Chloroplast fragments in the phytoplankton-associated fraction; i.e. although we had at least 7000 sequences for each attached community, some heterotroph communities were only represented by less than 100 heterotroph 16S rRNA sequences. However, the observed differences were significant and we could show variations in the relative abundances of some groups between attached and free-living portions. One plausible explanation for this is that the colony and aggregate formation provide a distinct micro-habitat for particle attached bacterial communities [70], which might select for different functions and different enzymes compared to free-living communities [71].

The genus *Flavobacterium* (*Bacteroidetes*) was mainly found attached to the *Cyanobacteria*. The *Flavobacteria* seem to play a particularly important role in

degradation of complex biopolymers [72,73] and are also known to feature a copiotroph lifestyle [74], explaining their higher proportion in the attached fraction. Studying marine diatoms, Grossart et al. [12] found that *Flavobacteria* and *Sphingobacteria* dominated the attached community, but in our study, *Sphingobacteria* were instead found in higher proportions in the free-living bacterial community.

The *Microcystis*-attached community carried a particularly high proportion of *Gemmatimonadetes*. The dominant *Gemmatimonas*-related OTU in this attached community was different from the most abundant OTU in the free-living fraction of both *Cyanobacteria*. In soil, clones affiliated with *Gemmatimonadetes* were overwhelmingly obtained from inner-aggregate fractions [75] and in the biofilm core in stream biofilms [76]. The dominance of different *Gemmatimonas*-related OTUs in different fractions may suggest ecological diversification with some OTUs being more common in aggregates and colonies.

In the diatom cultures, a *Caulobacteraceae* (#4085, *Alphaproteobacteria*) was mainly found in the attached community. This family is often found attached to surfaces [77]. A *Caulobacter* has previously been reported to occur in close association with *Cryptomonas*, but did not have any adverse affect on phytoplankton growth [78].

Our results showed the dynamic development of changing bacterial communities along phytoplankton growth phases and also differences between attached and free-living communities. Using a single bacterial inoculum, we showed that a characteristic bacterial community emerges in response to each type of phytoplankton. Some of these differences even appear at the class level. Different phytoplankton species and microhabitats clearly favor contrasting subsets of the

heterotrophic bacterial community and since different taxa likely represent particular microbial functions, this may have important consequences for the carbon flow in aquatic environments after a phytoplankton bloom. The new in depth information on bacterial taxa co-occurring with bloom forming phytoplankton species in attached and free-living portions are only a first step in the understanding of phytoplankton bloom development and dynamics. Future studies should now focus on revealing the mechanisms on how associated bacteria can affect the growth of different phytoplankton species and their bloom formation.

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1.8. Supporting figures

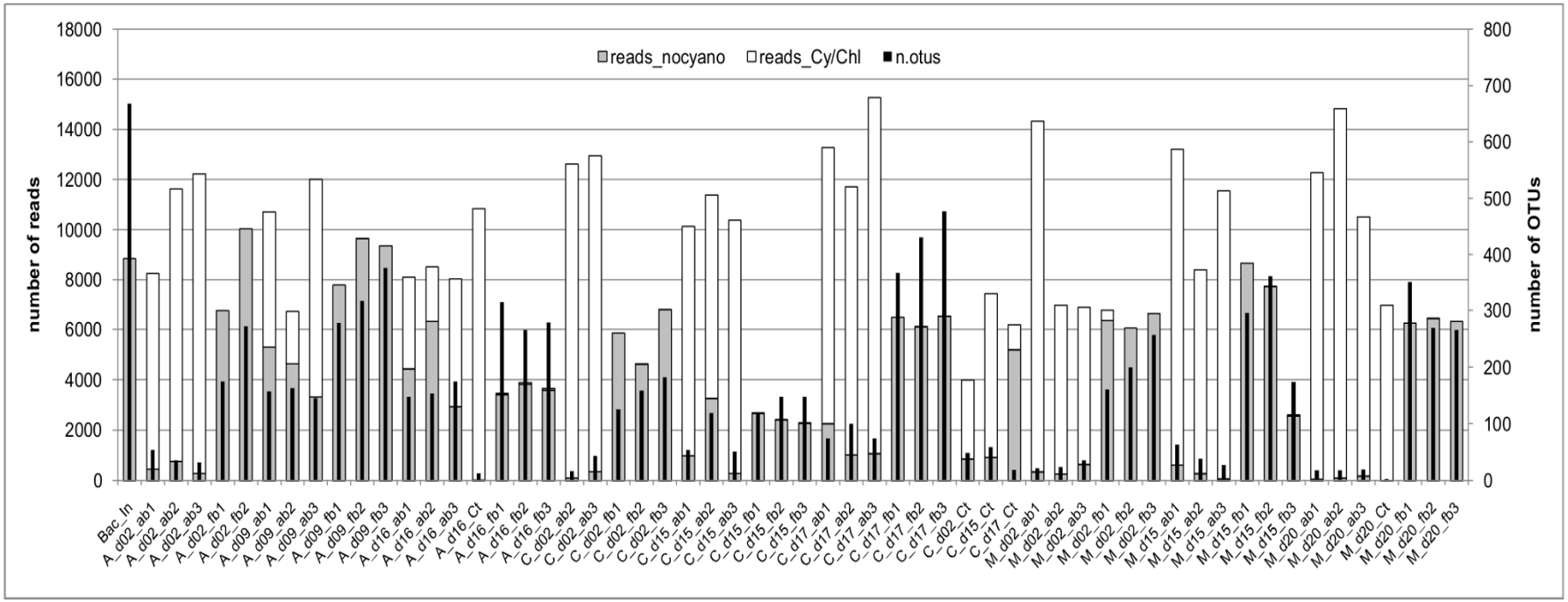

Figure 1.S1 – Numbers of non-cyano/chloroplast and of cyano/chloroplast reads, and number of OTUs per sample. *A. granulata* (A), *C. raciborskii* (C), and *M. aeruginosa* (M) cultures, in different days (dxx) and fractions (ab, adhered bacteria; fb, free-living bacteria).

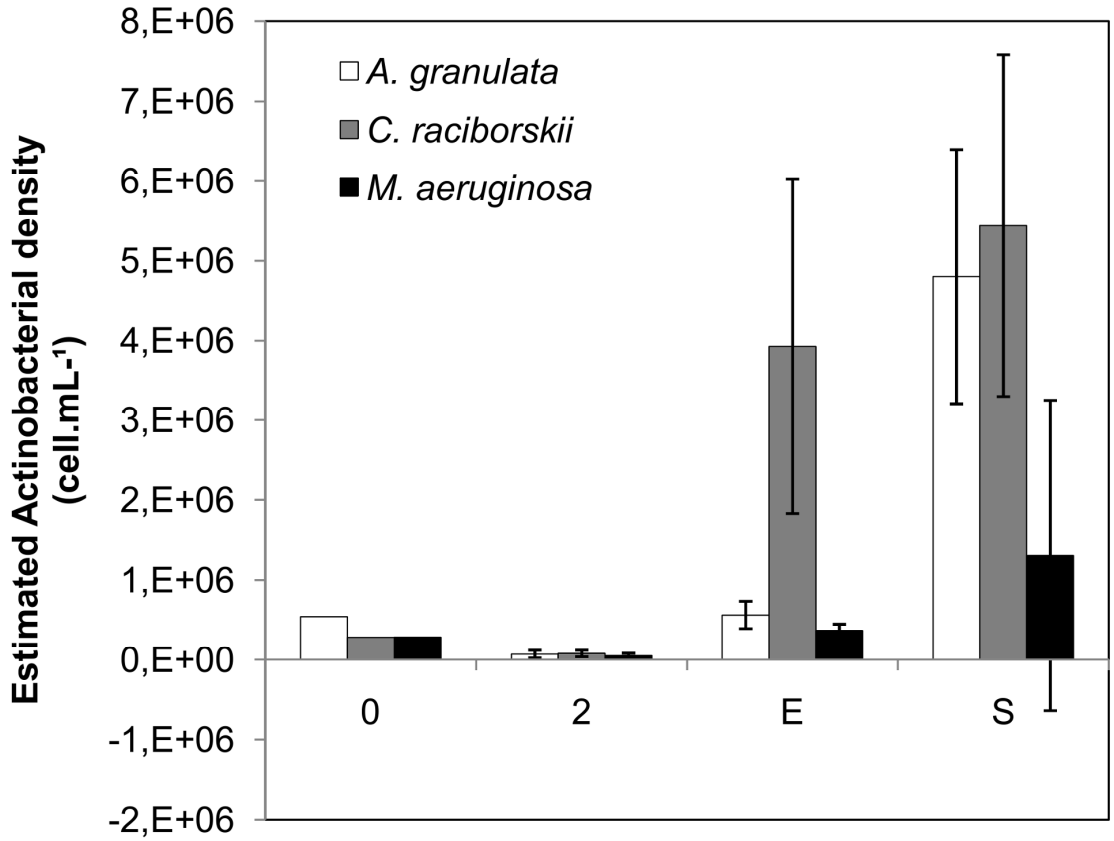

Figure 1.S2 – Estimated cell density (cell.mL-1) of Actinobacteria in phytoplankton cultures. 0 and 2 are initial and second days of sampling. 'E' is exponential growth phase to *A. granulata* and *M. aeruginosa,* and stationary to *C. raciborskii.* 'S' is stationary growth phase *A. granulata* and *M. aeruginosa,* and Senescent to *C. raciborskii.* Error bars are standard deviation of n=3. For the initial sampling (day 0), n=1.

1.9. Supporting Tables

Table 1.S1. Barcode sequences for each sample. Bac_In, bacterial inoculum. Names of samples are comprised by the first letter of the alga name (A, *Aulacoseira granulata;* C, *Cylindrospermopsis raciborskii,* M, *Microcystis aeruginosa*), day of sampling (dxx), fraction (ab, adhered bacteria; fb, free-living bacteria) and replicate number.

Sample name	Barcode	Sample name	Barcode	Sample name	Barcode
Bac_In	TACTCTC	C_d02_ab2	TAGCACT	M_d02_ab1	TACGCTA
A_d02_ab1	TACTCGA	C_d02_ab3	TAGCAGA	M_d02_ab2	TAGTCAC
A_d02_ab2	TACTGAC	C_d02_fb1	TAGCGTA	M_d02_ab3	TAGACTC
A_d02_ab3	TACTGCA	C_d02_fb2	TCTACTC	M_d02_fb1	TAGACGA
A_d02_fb1	TACGTCA	C_d02_fb3	TCTCTCA	M_d02_fb2	TAGAGAC
A_d02_fb2	TACGAGT	C_d15_ab1	TCACAGT	M_d02_fb3	TAGAGCA
A_d09_ab1	TCTCATC	C_d15_ab2	TCACGTA	M_d15_ab1	TCATCGA
A_d09_ab2	TCTCACT	C_d15_ab3	TCACGAT	M_d15_ab2	TCATGAC
A_d09_ab3	TCTCAGA	C_d15_fb1	TCAGTCA	M_d15_ab3	TCATGCA
A_d09_fb1	TCTGAGT	C_d15_fb2	TCAGATC	M_d15_fb1	TCACTAC
A_d09_fb2	TCATAGC	C_d15_fb3	TCAGAGA	M_d15_fb2	TCACTCT
A_d09_fb3	TCATCTC	C_d17_ab1	TGTCACA	M_d15_fb3	TCACTGA
A_d16_ab1	TCGTAGA	C_d17_ab2	TGTCGTA	M_d20_ab1	TGAGTAC
A_d16_ab2	TCGTGTA	C_d17_ab3	TGTGTCA	M_d20_ab2	TGAGTCT
A_d16_ab3	TCGATCA	C_d17_fb1	TGTGCTA	M_d20_ab3	TGAGTGA
A_d16_Ct	TCAGCTA	C_d17_fb2	TGATCAC	M_d20_fb1	TGAGCAT
A_d16_fb1	TCGACTA	C_d17_fb3	TGACTCA	M_d20_fb2	TGCTAGA
A_d16_fb2	TCGCATA	C_d02_Ct	TAGCTCA	M_d20_fb3	TGCTGTA
A_d16_fb3	TGTACGA	C_d15_Ct	TCACACA	M_d20_Ct	TGACACT
		C_d17_Ct	TGTAGCA		

Table 1.S2. Concentration of dissolved nutrients (mg.L-1) in control and treatment cultures of the 3 host species (mean±SD)

		Control		**Treatment**	
	day	**Nitrate**	**Phosphate**	**Nitrate**	**Phosphate**
A. granulata	2	61.8±0.8	2.9±0.0	64.0±1.2	3.1±0.1
	9	67.5±0.6	3.2±0.0	63.1±5.8	2.9±0.3
	16	64.2±0.5	2.8±0.1	63±1.6	2.6±0.2
M. aeruginosa	2	64.1±0.1	3.2±0.0	66.3±0.2	3.3±0.1
	15	0.1±0.2	0.0	0.1±0.1	0.0
	20	0.1±0.1	0.0	0.0	0.0
C. raciborskii	2	66.0±0.4	3.0±0.1	66.1±0.7	3.0±0.1
	15	55.5±2.3	2.5±0.1	32.5±1.6	1.4±0.1
	17	61.9±1.0	2.7±0.2	40.2±3.6	1.7±0.1

Table 1.S3 – Number of reads of contaminant OTUs in control cultures of *Aulacoseira granulata* (16th cultivation day) and *Microcystis aeruginosa* (20th cultivation day).

#OTU	*A. granulata*	*M. aeruginosa*	Classification
1429	2	0	Bacteria;Actinobacteria;Actinobacteria;Ilumatobacter (acIV-C)
3665	1	1	Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Corynebacterium (acTH2;Myco)
2692	2	-	Bacteria;Bacteroidetes;Sphingobacteria;Sphingobacteriales;Arcicella (bacIII-A)
1415	-	1	Bacteria;Firmicutes;Clostridia;Clostridiales;Veillonellaceae (unclassified)
4051	1	-	Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales (alfI)
4044	1	-	Bacteria;Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacter (alfVI)
3179	1	-	Bacteria;Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacter (alfVI)
4928	1	-	Bacteria;Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacter (alfVI)
1136	4	-	Bacteria;Proteobacteria;Alphaproteobacteria;Sphingomonadales;Novosphingobium (Novo)
1506	-	1	Bacteria;Proteobacteria;Betaproteobacteria;Rhodoferax (betI)
4595	1	-	Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Limnobacter (Burkholderiales)
4954	5	-	Bacteria;Proteobacteria;Betaproteobacteria;Rhodocyclales;Dechloromonas (Burkholderiales)
1598	1	-	Bacteria;Proteobacteria;Gammaproteobacteria (LiUU-3-334)
110	1	-	Bacteria;Proteobacteria;Betaproteobacteria;Rhodocyclales;Dechloromonas (Burkholderiales)
854	2	-	Bacteria;Verrucomicrobia;Opitutae;Opitutales;Opitutus (Opitutaceae)
Total (contamination)	23	3	
Chloroplast/ cyanobacterial reads	10861	6944	

Table 1.S4 - Specific OTUs associated with each studied phytoplankton species. Taxonomic affiliation of two classification databases is shown after identification number: RDP (Ribosomal database Projetct) and FW (Freshwater database).

Aulacoseira granulata			
# OTU	RDP/FW	# OTU	RDP/FW
1033	Betaproteobacteria;Undibacterium/Janb	359	Alphaproteobacteria;/alfI-A1
1132	Betaproteobacteria;Rhodocyclaceae/Burkholderiales	3687	Betaproteobacteria;/betVI
1310	Verrucomicrobia;Subdivision3/Verrucomicrobia	380	Alphaproteobacteria;/M-L-85
1330	Betaproteobacteria;Inhella/Burkholderiales	3826	Betaproteobacteria;Rhodocyclaceae/PnecD
1464	Alphaproteobacteria;Rhodobacter/alfVI	3830	Betaproteobacteria;Methylophilus/LD28
1504	Verrucomicrobia;Subdivision3/Verrucomicrobia	3969	Actinobacteria;Actinomycetales/Actinomycetales
1889	Alphaproteobacteria;Blastomonas/alfIV	3975	Bacteroidetes;/bacVI
1914	Alphaproteobacteria;Rhodobacteraceae/alfVI	3998	Bacteroidetes;Flavobacteriales/bacVI
1988	Betaproteobacteria;Comamonadaceae/Lhab-A4	4053	Betaproteobacteria;Comamonadaceae/betI-A
235	Gammaproteobacteria/Gammaprot.;Methylococcaceae	4078	Alphaproteobacteria;Sphingomonadales/Novo-A2
2371	Bacteroidetes;Sphingobacteriales/bacVI	4087	Bacteroidetes;Sphingobacteriales/bacI
2410	Alphaproteobacteria;Rhodobacter/alfVI	4473	Bacteroidetes;Cytophagaceae/bacIII-A
2422	Betaproteobacteria;Comamonadaceae/Lhab-A4	4548	Alphaproteobacteria;Rhodobacteraceae/alfVI
2495	Betaproteobacteria;Pelomonas/Burkholderiales	4624	Gammaproteobacteria;Rheinheimera/Sphingomonadaceae
2560	Betaproteobacteria;Comamonadaceae/Lhab-A4	4698	Bacteroidetes;/bacVI
2610	Betaproteobacteria;Pseudacidovorax/betI-A	4868	Gammaproteobacteria;/Gammaprot;Methylococcaceae
2666	Alphaproteobacteria;Caulobacter/alfVI	4994	Alphaproteobacteria;Sphingomonadaceae/M-L-85
2881	Bacteroidetes;Chitinophagaceae/bacI-B1	5171	Betaproteobacteria;Ideonella/Lhab-A4
2909	Verrucomicrobia;Spartobacteria/Xip-B1	5199	Gammaproteobacteria;Rheinheimera/Gammaproteobacteria
2976	Verrucomicrobia;Subdivision3/Verrucomicrobia	666	Bacteroidetes;Sphingobacteriales/Sphingobacteriales
3037	Alphaproteobacteria;Blastomonas/alfIV-B	677	Bacteroidetes;Cytophagaceae/bacIII-A
3056	Bacteroidetes;Cytophagaceae/bacIII-A1	694	Alphaproteobacteria;Rhizobiales/alfI-A1
3100	Betaproteobacteria;Vogesella/Burkholderiales	765	Betaproteobacteria;Herbaspirillum/betVII-B1
3228	Betaproteobacteria;Vogesella/Burkholderiales	822	Verrucomicrobia;Opitutus/Opitutaceae
3357	Alphaproteobacteria;/Pyxis	966	Betaproteobacteria;Comamonadaceae/alfIV
3576	Bacteroidetes;Chitinophagaceae/bacI-A2		

Microcystis aeruginosa			
# OTU	RDP/FW	# OTU	RDP/FW
1227	Alphaproteobacteria;Acetobacteraceae/alfVIII	4419	Gammaproteobacteria;Silanimonas/Proteobacteria
1550	Bacteroidetes;Cloacibacterium/bacVI	450	Bacteroidetes;Sphingobacteriales/bacI
1629	Alphaproteobacteria;/alfIV-A	4507	Proteobacteria;/Burkholderiales
1673	Alphaproteobacteria;Sphingomonadales/Novo-A2	4585	Alphaproteobacteria;Acetobacteraceae/alfVIII
1992	Betaproteobacteria;Acidovorax/Lhab-A1	4810	Betaproteobacteria;Rhodocyclaceae/Burkholderiales
2079	Alphaproteobacteria;Roseomonas/alfVIII	4998	Betaproteobacteria;Comamonadaceae/betI-A
2123	Proteobacteria;/alfVI	5109	Betaproteobacteria;Comamonadaceae/betI
2573	Betaproteobacteria;Burkholderiales/betI-A	5250	Alphaproteobacteria;/alfVI
2760	Bacteroidetes;Chitinophagaceae/bacI-A3	5289	Alphaproteobacteria;Rhodobacter/alfVI
2886	Betaproteobacteria;Burkholderiales/Betaproteobacteria	5301	Alphaproteobacteria;Erythrobacteraceae/Novo-A1
3013	Betaproteobacteria;Burkholderiales/Lhab-A4	5331	Alphaproteobacteria;Roseomonas/alfVIII
3105	Gammaproteobacteria;Rheinheimera/Proteobacteria	5359	Alphaproteobacteria;Acetobacteraceae/alfVIII
355	Gemmatimonadetes;Gemmatimonas/Proteobacteria	5395	Alphaproteobacteria;Porphyrobacter/alfIV-A
3923	Gammaproteobacteria;Rheinheimera/Gammaproteobacteria	639	Gemmatimonadetes;Gemmatimonas/Gammaproteobacteria
4149	Alphaproteobacteria;Erythromicrobium/Novo-A1	717	Gemmatimonadetes;Gemmatimonas/Gammaproteobacteria
423	Gammaproteobacteria;Silanimonas/Proteobacteria	864	Betaproteobacteria;Hydrogenophaga/betI-A
4393	Alphaproteobacteria;Rhodobacteraceae/alfVI	925	Alphaproteobacteria;Acetobacteraceae/alfVIII
Cylindrospermopsis raciborskii			
# OTU	RDP/FW	# OTU	RDP/FW
1182	Bacteroidetes;Algoriphagus/Algor	3368	Betaproteobacteria;Burkholderiales/Lhab-A4
1272	Betaproteobacteria;Oxalobacteraceae/betVII-B1	3484	Alphaproteobacteria;Rhodobacteraceae/M-L-85
1412	Actinobacteria;Propionibacterium/Actinomycetales	3524	Alphaproteobacteria;Rhodobacteraceae/alfVI
1442	Actinobacteria;Actinomycetales/acSTL-A2	3632	Bacteroidetes;Cytophagaceae/bacIII-A
1525	unclassified;/Gammaproteobacteria	367	Deltaproteobacteria;Peredibacter/Proteobacteria
1936	Bacteroidetes;Sphingobacteriales/Algor	3993	Gammaproteobacteria;Alteromonadales/gamII-A2
2059	Gammaproteobacteria;Pseudomonas/Pseudo-A1	4642	Alphaproteobacteria;Rhizobiales/alfI-B1
2168	Alphaproteobacteria;Bosea/alfI-A1	4758	Actinobacteria;Actinomycetales/Luna1-A1
2190	Betaproteobacteria;Aquabacterium/Burkholderiales	4949	Actinobacteria;/Actinobacteria
2447	Alphaproteobacteria;Rhizobiales/alfVII	4965	Bacteroidetes;Sphingobacteriales/Sphingobacteriales
2520	Actinobacteria;Rubrobacter/	5208	Alphaproteobacteria;Rhodospirillaceae/Alphaproteobacteria
2558	Alphaproteobacteria;Rhodobacteraceae/alfVI	687	Bacteroidetes;Flavisolibacter/bacI-A
3165	Bacteroidetes;Cytophagaceae/bacIII-A		

Table 1.S5 – Partial Regression Coefficients (R^2) from Permutational MANOVA comparing bacterial community compositions between the three phytoplankton host species using different cutoffs to exclude less abundant (based on proportion of reads of each OTU in relation of total bacterial reads of analyzed samples) or less frequent OTUs (frequency of presence in the samples). Numbers of remained OTUs after the cutoff are between brackets. All the p-values were significant (<0.001).

Cutoff proportion (remained OTUs)	**R^2**
>0,001% (1099)	0.253
>0,01% (265)	0.254
>0,1% (81)	0.255
>0,5% (33)	0.264
>1% (18)	0.276
>2% (9)	0.299
>3% (6)	0.333
Cutoff frequency (remained OTUs)	
>3 samples (554)	0.254
>10 samples (125)	0.256
>15 samples (75)	0.258
>20 samples (51)	0.257
>25 samples (29)	0.271
>40 samples (6)	0.318

Table 1.S6 – *P-values* from Welch Two Sample t-test of *Alphaproteobacteria* and *Betaproteobacteria* proportions between sampling days. Bold values indicate samples where the mean proportion decreased with time. 1 = lag or beginning of exponential growth phase, 2 *=exponential growth phase, 3* = stationary growth phase. * To *Cylindrospermopsis raciborskii* 2 and 3 were stationary and senescent phases, respectively.

		Alphaproteobacteria			**Betaproteobacteria**		
		1-2	2-3	1-3	1-2	2-3	1-3
Attached	*A. granulata*	0.017	**Ns**.	Ns.	**0.035**	**Ns.**	**0.018**
	M. aeruginosa	Ns.	**Ns.**	<0.001	**0.037**	**Ns.**	**0.012**
	C. raciborskii	0.013	Ns.	0.016	**0.006**	Ns.	**0.014**
Free-living	*A. granulata*	**Ns.**	Ns.	Ns.	**Ns.**	**0.049**	**0.038**
	M. aeruginosa	0.014	Ns.	0.022	**0.025**	**Ns.**	**0.009**
	C. raciborskii	Ns.	Ns.	Ns.	**<0.001**	**Ns.**	**<0.001**

Table 1.S7 - Relative abundances (% of reads per sample) of Classes in the replicates of each treatment. Naïve bayesian classification was used. Bac_In, bacterial inoculum. Names of samples are comprised by the first letter of the alga name (A, *A. granulata;* C, *C. raciborskii,* M, *Mi. aeruginosa*), day of sampling (dxx), fraction (ab, adhered bacteria; fb, free-living bacteria). Mean ±SD, n = 3. – denotes not detected.

Taxon	Bac_In	A_d02_ab	A_d02_fb	A_d09_ab	A_d09_fb	A_d16_ab	A_d16_fb
Bacteria,Acidobacteria,Acidobacteria_Gp3	-	-	-	-	0.06±0.05	0.09±0.04	0.13±0.17
Bacteria,Acidobacteria,Acidobacteria_Gp4	0.11	-	-	-	-	-	-
Bacteria,Acidobacteria,Acidobacteria_Gp6	-	-	-	-	0±0.01	-	0.01±0.02
Bacteria,Acidobacteria,Holophagae	0.01	-	-	-	-	-	-
Bacteria,Actinobacteria,Actinobacteria	72.4	0.43±0.28	0.48±0.14	0.04±0.04	1.11±0.16	3.58±2.83	6.02±2.3 1
Bacteria,Bacteria_incertae_sedis,Ktedonobacteria	0.02	-	-	-	-	-	0.01±0.02
Bacteria,Bacteroidetes,Bacteroidia	-	-	-	-	-	-	-
Bacteria,Bacteroidetes,Flavobacteria	0.33	-	0.02±0.01	1.03±0.8	0.13±0.05	1±0.51	0.05±0.04
Bacteria,Bacteroidetes,Sphingobacteria	0.68	-	1.24±0.13	1.34±0.49	9.57±1.21	3.8±1.86	21.74±13.1
Bacteria,Bacteroidetes,Other	0.05	-	0.09±0.03	2.42±1.53	0.43±0.1	0.62±0.39	0.38±0.09
Bacteria,Chlamydiae,Chlamydiae	0.38	-	0.01±0.01	-	-	-	-
Bacteria,Firmicutes,Bacilli	0.07	-	-	0.01±0.01	-	-	-
Bacteria,Firmicutes,Clostridia	-	0.13±0.23	-	-	-	-	-
Bacteria,Gemmatimonadetes,Gemmatimonadetes	0.49	-	0.02±0.02	0.04±0.02	0.55±0.2	0.3±0.47	0.16±0.11
Bacteria,OD1,OD1_genera_incertae_sedis	0.15	-	-	-	-	-	-
Bacteria,OP10,OP10_genera_incertae_sedis	-	-	-	0.08±0.14	-	1.9±3.23	0.01±0.02
Bacteria,Planctomycetes,Planctomycetacia	1.53	-	-	-	-	1.01±1.72	0.01±0.02
Bacteria,Proteobacteria,Alphaproteobacteria	11.38	10.18±2.27	14.74±2.8	31.65±6.17	11.53±1.9	27.18±10.4	38.22±19.0
Bacteria,Proteobacteria,Betaproteobacteria	6.31	82.71±3.73	80.43±2.4	57.68±9.8	71.89±4.5	50.56±9.69	25 .58±19.6
Bacteria,Proteobacteria,Deltaproteobacteria	0.4	-	-	0.21±0.34	0.46±0.71	0.03±0.03	-
Bacteria,Proteobacteria,Gammaproteobacteria	0.9	6.19±2.74	2.8±0.18	3.84±5.27	1.09±1.37	7.16±6.12	1.18± 1.07
Bacteria,Proteobacteria,Other	0.26	0.16±0.18	0.13±0.05	0.95±0.78	0.06±0.02	2.2±0.25	0.12±0.12
Bacteria,Spirochaetes,Spirochaetes	0.01	-	-	-	-	0.01±0.02	0.01±0.02
Bacteria,TM7,TM7_genera_incertae_sedis	0.09	-	-	-	0±0.01	0.02±0.03	-
Bacteria,Verrucomicrobia,Opitutae	0.41	-	-	0.27±0.13	0.51±0.27	0.07±0.01	0.78±0.26
Bacteria,Verrucomicrobia,Spartobacteria	0.34	-	-	0.01±0.01	1.72±0.44	0.03±0.04	3.41±2.06
Bacteria,Verrucomicrobia,Subdivision3	-	-	-	0.2±0.11	0.05±0.03	0.32±0.23	1.73±1.06
Bacteria,Verrucomicrobia,Verrucomicrobiae	0.21	-	-	0.11±0.09	0.25±0.12	-	0.02±0.02
Bacteria,Verrucomicrobia,Other	0.01	-	-	-	0.01±0.01	-	0.04±0.08
Bacteria,Other,Other	3.43	0.2±0.18	0.04±0.01	0.13±0 .15	0.57±0.17	0.1±0.05	0.39±0.39

Cont. Table 1.S7

Taxon	C_d02_ab	C_d02_fb	C_d15_ab	C_d15_fb	C_d17_ab	C_d17_fb	C_Ct
Bacteria,Acidobacteria,Acidobacteria_Gp3	-	-	-	-	0.03±0.05	0.03±0.01	-
Bacteria,Acidobacteria,Acidobacteria_Gp4	-	-	-	-	-	-	-
Bacteria,Acidobacteria,Acidobacteria_Gp6	-	-	-	-	-	-	-
Bacteria,Acidobacteria,Holophagae	-	-	-	-	-	-	-
Bacteria,Actinobacteria,Actinobacteria	5.87±3.01	4.25±2.21	0.03±0.05	3.83±2.27	0.16±0.15	6.47±2.28	0.9 2±1.18
Bacteria,Bacteria_incertae_sedis,Ktedonobacteria	-	-	-	-	0.05±0.05	0.01±0.01	-
Bacteria,Bacteroidetes,Bacteroidia	1.29±1.82	-	-	-	-	-	0.28±0.42
Bacteria,Bacteroidetes,Flavobacteria	0.71±1.01	0.01±0.02	10.57±8.98	4.17 ±3	2.91±3.82	1.05±0.8	0.22±0. 28
Bacteria,Bacteroidetes,Sphingobacteria	0.14±0.2	0.83±0.33	9.09±11.27	24.96±7.94	2.12±1.81	25.37±4.18	0 .18±0.31
Bacteria,Bacteroidetes,Other	-	0.09±0.04	0.22±0.29	0.56±0.67	1.1±0.1	2.34±2.91	-
Bacteria,Chlamydiae,Chlamydiae	-	0.02±0.02	-	-	-	-	-
Bacteria,Firmicutes,Bacilli	-	0.01±0.01	-	-	-	-	3.43±5.44
Bacteria,Firmicutes,Clostridia	1.9±1.36	0.07±0.07	-	-	-	-	0.41±0.26
Bacteria,Gemmatimonadetes,Gemmatimonadetes	-	0.26±0.23	3.01±1.69	5.42±1.39	8.66±4.91	6.03±5.74	-
Bacteria,OD1,OD1_genera_incertae_sedis	-	0.02±0.01	-	-	-	-	-
Bacteria,OP10,OP10_genera_incertae_sedis	-	-	-	0.03±0.04	-	0.01±0.01	0.04±0.06
Bacteria,Planctomycetes,Planctomycetacia	-	0.02±0.02	-	0.01±0.02	-	-	-
Bacteria,Proteobacteria,Alphaproteobacteria	3.65±2.52	7.74±4.75	47.16±10.4	28.12±9.84	40.08±9.68	31.58 ±13.3	2.01±2.38
Bacteria,Proteobacteria,Betaproteobacteria	81.32±7.92	85.39±6.2	17.02±12.4	26.56±6.37	31.64±13.56	23.5 3±6.7	89.44±11.91
Bacteria,Proteobacteria,Deltaproteobacteria	-	0.02±0.02	0.43±0.55	0.04±0.04	0.65±1.13	0.1±0.1	0.01±0.0 1
Bacteria,Proteobacteria,Gammaproteobacteria	4.99±2.2	0.7±0.88	7.27±3.3	3.61±2.01	2.6±1.22	1.33±1.67	2. 27±1.79
Bacteria,Proteobacteria,Other	-	0.31±0.44	3.73±1.73	0.46±0.21	9.31±3.26	0.27±0.15	0.75±0.64
Bacteria,Spirochaetes,Spirochaetes	-	-	-	-	-	-	-
Bacteria,TM7,TM7_genera_incertae_sedis	-	0±0.01	-	-	-	-	-
Bacteria,Verrucomicrobia,Opitutae	0.14±0.2	0.04±0.03	0.47±0.82	0.32±0.45	-	0.87±0.91	-
Bacteria,Verrucomicrobia,Spartobacteria	-	0.02±0.03	-	-	-	-	-
Bacteria,Verrucomicrobia,Subdivision3	-	-	-	-	-	0.01±0.02	-
Bacteria,Verrucomicrobia,Verrucomicrobiae	-	0.02±0.03	-	1.87±1.84	0.32±0.25	0.88±0.89	-
Bacteria,Verrucomicrobia,Other	-	0±0.01	-	-	-	-	-
Bacteria,Other,Other	-	0.17±0.17	1.01±1.01	0.04±0.0 4	0.38±0.19	0.13±0.04	0.04±0.07

Cont. Table 1.S7

Taxon	M_d02_ab	M_d02_fb	M_d15_ab	M_d15_fb	M_d20_ab	M_d20_fb
Bacteria,Acidobacteria,Acidobacteria_Gp3	-	-	-	-	-	-
Bacteria,Acidobacteria,Acidobacteria_Gp4	-	-	-	-	-	-
Bacteria,Acidobacteria,Acidobacteria_Gp6	-	-	-	0±0.01	-	-
Bacteria,Acidobacteria,Holophagae	-	-	-	-	-	-
Bacteria,Actinobacteria,Actinobacteria	0.25±0.43	0.4±0.19	0.7±0.72	0.72±0.27	1.13±1.16	1.79±2.71
Bacteria,Bacteria_incertae_sedis,Ktedonobacteria	-	-	-	-	-	-
Bacteria,Bacteroidetes,Bacteroidia	-	-	-	-	0.78±1.34	-
Bacteria,Bacteroidetes,Flavobacteria	0.46±0.57	0.22±0.2	4.6±3.06	0.03±0.01	6.92±6.24	0.06±0.06
Bacteria,Bacteroidetes,Sphingobacteria	-	0.96±0.1	4.89±3.56	30.7±9.86	1.6±2.31	16.07±7.33
Bacteria,Bacteroidetes,Other	-	0.75±0.21	0.48±0.83	2.33±3.98	-	22.56±19.58
Bacteria,Chlamydiae,Chlamydiae	-	0.01±0.01	-	-	-	-
Bacteria,Firmicutes,Bacilli	-	-	-	-	0.19±0.32	-
Bacteria,Firmicutes,Clostridia	-	-	1.11±1.79	-	-	-
Bacteria,Gemmatimonadetes,Gemmatimonadetes	-	0.02±0.01	8.59±1.5	6.82±2.93	26.34±6.36	3.37±1.85
Bacteria,OD1,OD1_genera_incertae_sedis	-	-	-	-	-	-
Bacteria,OP10,OP10_genera_incertae_sedis	-	-	-	-	-	-
Bacteria,Planctomycetes,Planctomycetacia	-	0.01±0.01	-	0±0.01	-	-
Bacteria,Proteobacteria,Alphaproteobacteria	7.13±1.41	13.24±5.39	23.25±7.15	31.84±5.43	20.76±1.68	32.9 7±7.21
Bacteria,Proteobacteria,Betaproteobacteria	67.88±14.41	58.9±9.74	26.66±1.7	25.29±1.48	13.73±6.47	17.11 ±4.21
Bacteria,Proteobacteria,Deltaproteobacteria	-	-	0.05±0.09	0.01±0.01	-	0.01±0.01
Bacteria,Proteobacteria,Gammaproteobacteria	23.78±14.59	25.16±14.28	12.8±4.73	1.71±0.35	18.65±3.27	5.1 6±1.31
Bacteria,Proteobacteria,Other	0.49±0.85	0.31±0.06	16.81±8.72	0.37±0.19	9.92±9.59	0.76±0.1
Bacteria,Spirochaetes,Spirochaetes	-	-	-	-	-	0.01±0.01
Bacteria,TM7,TM7_genera_incertae_sedis	-	-	-	0±0.01	-	-
Bacteria,Verrucomicrobia,Opitutae	-	-	-	0.01±0.01	-	-
Bacteria,Verrucomicrobia,Spartobacteria	-	-	-	-	-	0.01±0.01
Bacteria,Verrucomicrobia,Subdivision3	-	-	-	-	-	-
Bacteria,Verrucomicrobia,Verrucomicrobiae	-	-	-	0.01±0.01	-	0.04±0.06
Bacteria,Verrucomicrobia,Other	-	-	-	-	-	-
Bacteria,Other,Other	-	0.03±0.03	0.05±0.09	0.14±0.03	-	0.09±0.07

CAPÍTULO 2:

EFFECT OF BACTERIAL SOURCE AND GROWTH CONDITIONS ON THE SPECIFICITY OF PHYTOPLANKTON- BACTERIA ASSOCIATION

2.1 . Abstract

The occurrence of nuisance phytoplankton blooms is a worldwide problem where the phytoplankton-bacteria interactions may play a central role. One important aspect to elucidate is the strength of these interactions with varying factors. Using the 454-Pyrosequencing of 16S rRNA gene amplicons we characterized bacterial communities associated with two bloom forming phytoplankton species, the *Cyanobacteria Microcystis aeruginosa* and the diatom *Aulacoseira granulata* in two different experiments with distinct bacterial inocula. We found few but relatively abundant recurrent OTUs in both experiments associated with each phytoplankton species, and some OTUs with putative tight association, as one *Silanimonas* associated with *M. aeruginosa* and one *Dechloromonas*-related associated with the diatom. The proportion of some bacterial families differentiated both phytoplankton species: *Rhodocyclaceae* and one family of *Spartobacteria* was more abundant in *A.granulata* cultures, whereas a higher relative abundance of *Xanthomonadaceae, Rhodobacteraceae* and *Acetobacteraceae* was found associated with the cyanobacterium. The total bacterial community was significantly different between the phytoplankton species in the same experiment, and also between experiments for the same phytoplankton species. However, the variation in bacterial community composition was better explained by phytoplankton species than by the differences in the experiments, evidencing the importance of the organic compounds released by each phytoplankton species in selecting bacterial communities. Furthermore, using higher taxonomic levels to compare bacterial communities increased the variation explained by phytoplankton species and reduced the variation explained by different experiments, what may suggest ecological redundancy of closely-related bacterial OTUs. Thus, the studies on the specificity of phytoplankton-bacteria association should also consider the ecological function of the associated bacteria.

2.2 . Introduction

The occurrence of nuisance phytoplankton blooms is a worldwide problem that may cause deterioration of water quality due to oxygen depletion, surface scums and toxin production, and can greatly impact the energy flux in aquatic environments due to the high amount of organic matter produced (Carmichael 2001, Paerl et al. 2001). Among the common bloom forming freshwater phytoplankton species, the cyanobacterium *Microcystis aeruginosa* and the diatom *A. granulata* form frequent blooms in Barra Bonita Reservoir and in aquatic environments around the world (Carmichael 2001, Paerl et al. 2001, Vieira et al. 2008).

Many abiotic and biotic factors can interfere with the dynamic and occurrence of phytoplankton blooms (Paerl 1996, Huisman et al. 1999), among them the interaction with the local bacterial community may play an important role (Doucette 1995). The bacteria may improve the phytoplankton growth by remineralization and transference of inorganic nutrients and vitamins (Croft et al. 2005, Jiang et al. 2007), or inhibit it by antibiotic production (Mayali and Azam 2004) or competition for nutrients (Danger et al. 2007). Nevertheless these interactions are of two-way, at the same time that bacterial communities influence phytoplankton blooms, they are also selected by the kind of organic matter released by the phytoplankton, either by the compounds that they are able to metabolize (Van Hannen et al. 1999, Schäfer et al. 2002, Teeling et al. 2012) or by compounds with antimicrobial effects (Kreitlow et al. 1999, Casamatta and Wickstrom 2000, Grossart and Simon 2007). Thus, even to some phytoplankton species to which species-specificity may be less tight, we could expect some specificity of functional groups.

Despite the possible co-selection between phytoplankton and bacteria, we have little and contrasting information about specificity and reproducibility of these associations (Grossart et al. 2005, Sapp et al. 2007, Dziallas and Grossart 2011, Eigemann et al. 2012). One important step to understand phytoplankton-bacteria interactions concerns the taxonomic composition and diversity of associated bacterial communities (Grossart et al. 2005). Determine the taxonomic composition of bacterial communities associated with isolated phytoplankton species with distinct bacterial source could help understanding the strength of these interactions, which can have a major influence on the fate of photosynthetically produced organic material, at least locally (Bertilsson and Jones 2003). Furthermore, it may also be important to

understand the occurrence of phytoplankton blooms, since some bacterial groups are known to present phytoplankton-lysing effects (Rashidan and Bird 2001) or enhance phytoplankton growth (Shi et al. 2009a, Sher et al. 2011).

The phytoplankton supports free-living bacteria by releasing dissolved organic matter, mainly carbohydrates (Giroldo et al. 2003, Teeling et al. 2012), but some species can also hold an attached bacterial community on their surface, and such communities may differ significantly in composition (Grossart et al. 2005, Rooney-Varga et al. 2005). It is known that the proximity with bacterial cells can have a direct influence on the phytoplankton-bacteria interaction and on the growth of the phytoplankton species (Mayali and Azam 2004, Jiang et al. 2007, Sher et al. 2011), and that the colony or aggregate formation by the phytoplankton can create micro-habitat with specific characteristics (Worm and Sondergaard 1998). Thus, physical attachment of bacteria may suggest a tighter association (Grossart et al. 2005, Sigee 2005, Malfatti and Azam 2009), and verify if specific bacterial taxa occur in different fractions is an important information to understand the ecology and physiology of phytoplankton (Grossart et al. 2005, Jiang et al. 2007) and the carbon cycling in freshwater environments (Mayali and Azam 2004).

Previous studies have reported bacterial communities accompanying *M. aeruginosa* (e.g. Eiler and Bertilsson 2004, Shi et al. 2009b, Shi et al. 2010) and other diatom species (Grossart et al. 2005, Eigemann et al. 2012) in cultures or natural blooms, but no data is available for *A. granulata*. Some recurrent groups are often found associated with *Microcystis*, for example *Burkholderiales, Sphingomonadales* and *Xanthomonadaceae* (Dziallas and Grossart 2011, Li et al. 2011, Shi et al. 2012). However, the dominance of some groups seems to vary among global distributed reports (Eiler and Bertilsson 2004, Shi et al. 2009b, Kormas et al. 2010, Dziallas and Grossart 2011, Shi et al. 2011, Shi et al. 2012).

One of the reasons for the variability is likely the bacterial source, since it can be the driving force to determine bacterial community composition in environments with varying abiotic factors (Langenheder et al. 2006). However, only few studies analyzed the influence of source inoculum in the phytoplankton-associated bacterial community (Dziallas and Grossart 2011, Eigemann et al. 2012, Paver et al. 2013). Dziallas and Grossart (2011) showed that the bacterial inoculum is an important factor influencing bacterial community composition (BCC) associated with *M. aeruginosa*, but, to our knowledge, there is no previous study about specificity of

bacteria-phytoplankton association using high-throughput sequencing, which can provide much more information, specially by allowing detection of less abundant OTUs (operational taxonomic units).

A deeper sequencing can provide more robust data to compare phylogenetic proximity of the OTUs co-occurring with phytoplankton species and may help elucidate some questions about this variability found in the groups associated with the same phytoplankton species in different studies.

Using a high throughput sequencing technology we initially aimed to identify the recurrent phytoplankton-associated bacterial taxa in cultures of each phytoplankton species inoculated with distinct bacterial inocula (and to *M. aeruginosa* we also used different growth medium). Besides, we aimed to determine if the BCC associated with each phytoplankton species would be similar using the different experimental designs (specially different inocula), and if differences in experiments would be more important to lead changes in BCC than phytoplankton species, thus, testing the strength of phytoplankton-bacteria interaction.

2.3 . Material and Methods

To study the strength of phytoplankton-bacteria interaction and determine some putative specific bacterial taxa associated with the diatom *Aulacoseira granulata* and the cyanobacterium *Microcystis aeruginosa*, we performed two experiments, varying the bacterial inoculum. However, for *M. aeruginosa* we also varied the growth medium and fractionation between experiments (Figure 2.1).

Furthermore, some filaments of *A. granulata* and colonies of *M. aeruginosa* were isolated from the same reservoir where we collected the bacterial inoculum, the Barra Bonita Reservoir, to determine the co-occurring bacteria in the natural environment. The detailed experimental setup is described below.

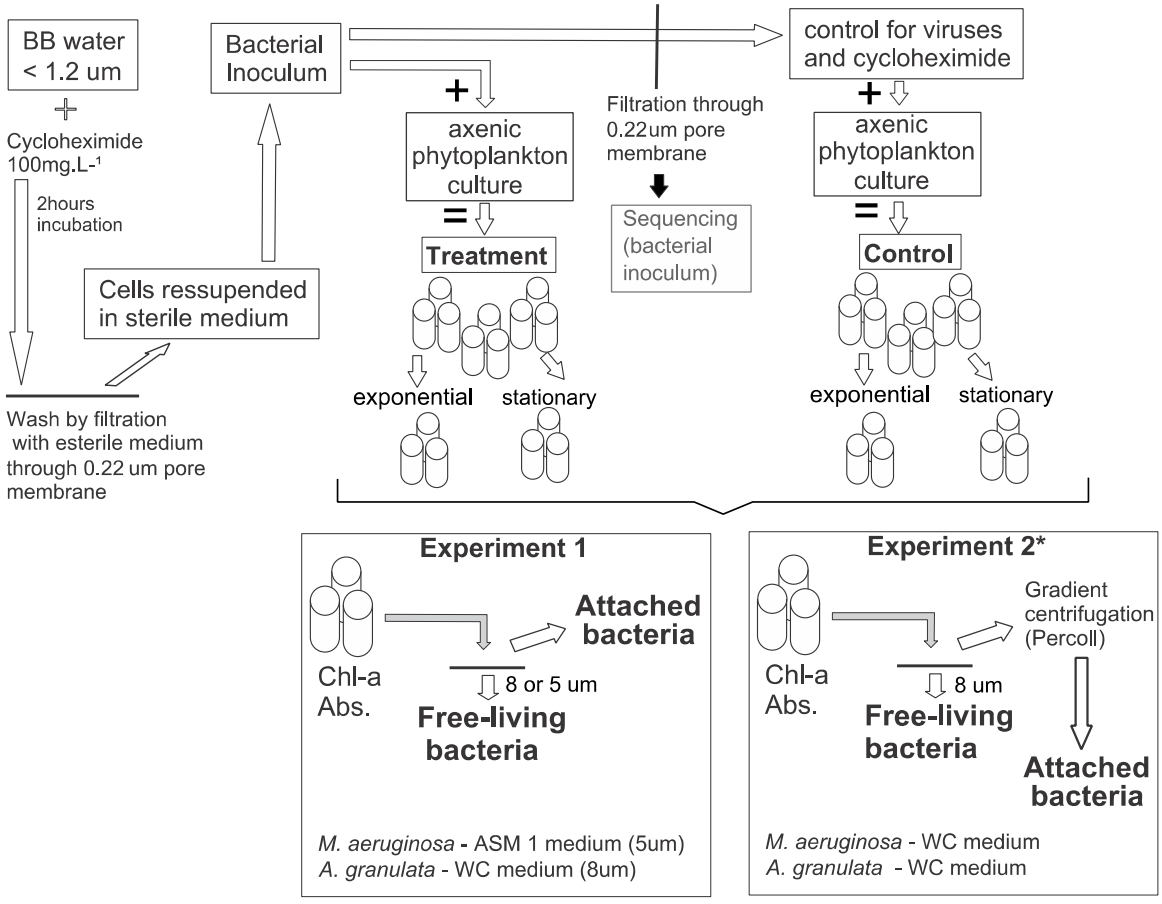

Figure 2.1 – Experimental design of both experiments. Experiment 1 and Experiment 2 differs in the bacterial inoculum, in fractionation of attached and free-living bacteria and for *M. aeruginosa* the growth medium also differed between experiments. *Already presented in 'Capítulo 1'. BB = Barra Bonita Reservoir.

Phytoplankton species studied

The Cyanobacteria *Microcystis aeruginosa* (Kütz) Kützing (BB005) and the diatom *Aulacoseira granulata* var. *granulata* (Ehrenberg) Simonsen (BB001) were isolated from the hypereutrophic Barra Bonita Reservoir in São Paulo State, Brazil. They were made axenic and maintained in the culture collection of the Botany Department at Federal University of São Carlos (World Data Center for Microorganisms No. 835).

The algal inocula were checked for axenic conditions before both experiments in WC medium supplemented with peptone and glucose (250 $mg.L^{-1}$ of each, WC p+g) and in CPS (casein, peptone and starch) broth and agar media. No specific permission was required for sampling the phytoplankton species and the bacteria studied.

Bacterial inocula

In both experiments the bacterial inoculum was obtained from the same reservoir and using the same sampling method. The bacterial inoculum for the first experiment was obtained at the end of winter season in Brazil, on 12th of September-2011, during a bloom of *M. aeruginosa* and *Anabaena* c.f. spiroides. The bacterial inoculum for the second experiment was obtained at the end of summer season in Brazil, on 06th of March-2012 during a bloom of *Ceratium* sp. and *Anabaena* c.f. *spiroides*.

The sampling method and preparation of bacterial inocula for both experiments are described in 'Capítulo 1'. In summary, each inoculum consisted of a mixture of equal proportion of the water collected at different depths (0.5, 1, 5, 10 and 15m) in Barra Bonita Reservoir. In the laboratory the collected water was filtered through 1.2 µm glass fiber filter, incubated with cycloheximide solution (to kill nanoflagellates that could have passed through 1.2 µm glass fiber filter) and washed with sterile WC medium by filtration. The cells were resuspended and used as bacterial inoculum. A fraction of this inoculum was filtered again through 0.22 µm polycarbonate membrane filters and the filtrate was inoculated in the axenic cyanobacterial and diatom cultures as control for viruses and potential cycloheximide effect ('Capítulo 1', section 1.3.). The bacteria captured on the polycarbonate membrane were resuspended in WC medium, centrifuged (16000 *xg*, 25 min) and

stored at -20 °C for DNA extraction and sequencing of partial 16S rRNA gene from the bacterial inoculum.

Experimental design

Part of the data presented in this work, regarding to the experiment 2, was obtained in the same experiment presented in 'Capítulo 1'.

In the experiment 1, *M. aeruginosa* and *A.granulata* in exponential growth were inoculated, respectively, in 7.5 L of ASM-1 medium, pH 7.0 (Gorham et al. 1964) and 7.5 L of WC medium pH 7.0 (Guillard and Lorenzen 1972) supplemented with silica (final $Na_2SiO_3.9\ H_2O$ concentration 56.84 mg L^{-1}).

In the second experiment, each phytoplankton species were inoculated separately in 4 L WC medium pH 7.0 (Guillard and Lorenzen 1972) supplemented with silica for the diatom.

The preparation of the treatments and sampling procedure were the same in both experiments (Figure 2.1). All the procedures were done under aseptic conditions. In a few words, *M. aeruginosa* and *A. granulata* cultures in each experiment were immediately divided in 2 flasks, and inoculated with bacteria (treatment, non-axenic) or with the water from the bacterial inoculum filtered through 0.22 µm polycarbonate membrane filter (control, axenic), as described above. The volume of the bacterial and control inocula were 5% of the final volume of the culture. The cultures were then homogenized, divided in volumes of 150 mL in 250 mL culture flasks and incubated at 23 ± 2 °C under illumination of 90±10 µmol photons m^{-2} s^{-1} in 12h:12h light-dark cycle. For each sampling, 3 flasks (independent replicates) of the control cultures and 3 of the treatment cultures were analyzed.

Sampling days for A. granulata

The diatom was inoculated in the same medium in both experiments and sampled in the exponential and stationary/senescent growth phases. In experiment 1, algal growth parameters were measured on the 1st, 4th, 10th, 15th, 18th and 21st days, and bacterial diversity was analyzed on days 10 and 15. In the second experiment chlorophyll *a* was quantified on days 2, 9 and 16, and on these last samplings, exponential and stationary growth phases, bacterial metagenome was also analyzed (Figure 2.2). Absorbance was measured on 5 more days.

Sampling days for M. aeruginosa

Since ASM-1 has more nitrate and phosphate than WC medium, *M. aeruginosa* growth curves were longer in the first experiment, resulting in very distinct sampling days compared to the second experiment. Samples for metagenomic analyses of experiment 1 (ASM-1 medium) were collected in 17th and 34th culturing days, whereas in experiment 2 (WC medium) cultures were sampled in the 15th and 20th culturing days (Figure 2.3). Controls were checked for axenic condition in WC p+g medium on these same days.

In experiment 1, the chlorophyll *a* analyses were carried out on days 1, 8, 17, 28 and 34. In experiment 2, the analyses of chlorophyll *a* were carried out on the initial and 2nd, 15th, 20th days, and absorbance was measured on the same days and on 4 more occasions during the culture growth.

Phytoplankton growth parameters

Chlorophyll a concentration: chlorophyll-a was extracted with hot 90% ethanol (Nusch 1980), measured spectrophotometrically and quantified using Lorenzen (1967) equations.

Absorbance: In the 1st experiment the absorbance was measured from 250-750 nm and the wavelengths with better correlations with chlorophyll *a* were chosen and used in the 2nd experiment. The values chosen for population growth were the absorbance in 680 nm corrected for the absorbance at 730 nm for *M. aeruginosa* (R^2=0.98) and corrected for the absorbance at 750 nm for *A. granulata* (R^2= 0.91).

Fractions separation: free-living and attached bacteria

The procedure to fraction separation was different between experiments (Figure 2.1):

First experiment

The attached and free-living bacteria of *M. aeruginosa* cultures were separated by filtration through 5 µm cellulose acetate membrane, whereas *A. granulata* was separated by filtration through 8 µm cellulose acetate membrane. A fraction of 30-40 mL of each culture was filtered and the cells that remained in the filters were washed twice with 30-40 mL of sterile WC medium and resuspended in

the same medium using a Pasteur pipete. Both fractions, free-living (<5 or 8µm) and attached (>5 or 8µm), were centrifuged for 25 min at 16000 *x*g and the pellet stored for ~ 7 months at -20°C until DNA extraction.

An aliquot of the total culture of one replicate of the controls was sampled on the 32nd day for *M. aeruginosa* and on 15th day for *A. granulata,* centrifuged and also stored at -20°C until DNA extraction.

Second experiment

Samples for DNA based analysis of the second experiment are part of those already presented in 'Capítulo 1', and both phytoplankton species were fractionated using the same procedure, except the Percoll concentration (Ge-HealthCare). In summary, 30 to 40 mL of each culture were aseptically filtered through an 8 µm pore-size cellulose acetate membrane and washed once with 20 mL of sterile WC medium. The free-living bacteria were defined as the fraction < 8 µm and it was centrifuged for 25 min at 16000 *x*g. The attached bacteria (>8 µm) were processed as described in 'Capítulo 1' with Percoll gradient density centrifugation. The pellets with cells of both fractions were stored at -20°C until the DNA extraction. An aliquot of total fraction of one control culture for each phytoplankton species (20th day for *M. aeruginosa* and 16th day for *A.* granulata) was also centrifuged and stored at -20°C until the DNA extraction.

Isolation of M. aeruginosa *colonies and* A. granulata *filaments*

In the second experiment, 15 *M. aeruginosa* colonies and 20 *A. granulata* filaments were isolated from the water sample collected in Barra Bonita Reservoir for bacterial inoculum preparation. The colonies and filaments were isolated using capillary isolation technique (Vieira 1977) using aseptic procedures in a flow cabinet, and stored at -20°C until the DNA extraction. These colonies and filaments were isolated aiming to determine some occurring OTUs in natural environment. However, *A. granulata* filaments with attached bacteria did not provide sufficient DNA and the PCR failed, thus we present these data only for *M. aeruginosa*.

DNA extraction, PCR amplification

DNA extraction was performed as described in 'Capítulo 1'. Briefly, DNA was chemically extracted with proteinase K solution, 2% CTAB and 1.35 M NaCl, and

warm water bath; physical extraction was done in vortex with 0.2 g of glass beads (150-212 µm, Sigma-Aldrich) added to each 1.5 mL tube. The extracted DNA was purified with phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform. DNA was precipitated with cold ethanol and sodium acetate followed by a washing step with cold 70% ethanol. The DNA was resuspended to 25 µL in TE-4 buffer and its concentration was determined by absorbance at 260 nm on a NanoDrop 2000C spectrophotometer.

Bacterial 16S rRNA gene was amplified using universal bacterial primers 341F and 805R and amplicons were pooled for pyrosequencing as described in 'Capítulo 1'.

Pyrosequencing and sequence analyses

Amplicons from each sample were sequenced from adaptor A on a 454-FLX system using Titanium chemistry (454 Life Sciences, Brandford, CT) at the SNP/SEQ platform hosted by SciLife Lab, Uppsala, Sweden.

From 1,295,767 reads for the total run, sequences from samples not included in this study and reads with low quality or that did not carry the exact primer sequence were removed. After this quality control, sequences were denoised to remove PCR and 454 pyrosequencing noise as well as chimeras, as described in 'Capítulo 1', remaining 471,857 reads. The number of reads per sample ranged from 2,607 to 14,870, resulting in an average of 8,426 sequences. The reads from experiment 2 (except environmental colonies and filaments) was already presented in 'Capítulo 1' and made up 228,288 reads. The samples of treatment cultures were in triplicates, except one sample for *M. aeruginosa* in the 17th day of the first experiment (e1_M.d17), which only two replicates were sequenced for each fraction (attached and free-living). For the environmental samples (*Microcystis* colonies isolated from Barra Bonita reservoir) we have only one replicate.

The OTU (operational taxonomic unit) was defined using a 97% sequence similarity cutoff with UCLUST (Edgar 2010). The representative sequence from each OTU was classified using the naïve Bayesian classifier (Ribosomal Database Project – RDP classifier) (Wang et al. 2007) implemented in MOTHUR (Schloss et al. 2009) in combination with the greengenes database gg_OTU_97 and the taxonomy after Hugenholtz (McDonald et al. 2011). The reads were also annotated against a freshwater bacterial sequence database (FW) that included almost 12,000 sequence

entries (Newton et al. 2011). In the text we used the taxonomic placement assigned by naïve Bayesian classifier, followed by the number of the OTU and classification of FW in parentheses.

After removal of contaminations as inferred from sequenced blanks as well as phytoplankton reads (cyanobacterial and chloroplasts reads from the cultured species or from algae present in the *M. aeruginosa* colonies isolated from the reservoir), 258,098 reads remained with a minimum sequence length of 350 bp length. From those sequences, 92,458 were from *M. aeruginosa* samples, 155,783 from *A. granulata* samples, and 9,857 from both inocula. The sequences have been deposited in the NCBI Short Read Archive under accession number SRR873436 (Sequences also used in 'Capítulo 1') and accession number (still not deposited) (Sequences used only in this chapter).

Statistical analyses

All statistical analyses were conducted using R (R Core Team 2012). The effect of bacterial communities on phytoplankton growth was statistically tested by comparison of chlorophyll *a* concentrations of treatment and control cultures on each sampling day using two-sample t-test.

The bacterial OTUs that were common in both experiments (and in the isolated colonies in the case of *M. aeruginosa*) for each host phytoplankton species were visualized in heatmaps (Legendre and Legendre 1998). Only the OTUs that occurred in the 3 independent replicates of at least one treatment of each experiment were used. These recurrent OTUs were also matched with RDP database to check their occurrences in previous studies, and closely related OTUs (similarity score > 0.97) that have occurred in phytoplankton blooms were presented.

Non-metric multidimensional scaling (NMDS) of Morisita-Horn distance matrix was ordinated in 3 dimensions to display differences in community structure between experiments, phytoplankton species and fractions (attached and free-living) using the function metaMDS in R. Since the number of reads varied among samples, the Morisita-Horn metrics was chosen based on its robustness with samples of different sizes (Wolda 1981). Also, a NMDS ordinated in 2 dimensions was used to better visualize differences in community between experiments and phytoplankton species using reads of attached and free-living bacterial communities separately to reduce the stress of the ordination.

The differences in bacterial community composition between experiments and between the cyanobacteria and the diatom were statistically tested by ANOVA on the basis of the Morisita-Horn distance measure, using permutation methods (Anderson 2001) and the function Adonis in R. The p-values of PERMANOVA tests were adjusted using the function "p.adjust" and the false discovery rate method (FDR) described by Benjamini and Hochberg (Benjamini and Hochberg, 1995).

The same procedure described above was used in matrices with OTUs grouped in higher taxonomic levels (Class, Order, Family and Genus).

2.4 . Results

Part of the results presented, related to the second experiment, was already shown in 'Capítulo 1', as growth curve analysis and some occurring OTUs. Nevertheless, the aim of this chapter was different from the first one.

Phytoplankton growth

The bacterial communities had negative effect on *A. granulata* growth in both experiments. In experiment 1, the growth yield, although lower, was not significantly different from the control, but the senescent phase occurred earlier in the treatment cultures (Figure 2.2). However, in the experiment 2 the chlorophyll *a* concentration in the treatment cultures were significantly lower in exponential (two sample t-test: p=0.038) and stationary phases (two sample t-test: p=0.007) (Figure 2.2).

The chlorophyll *a* concentration of *M. aeruginosa* cultures in presence of natural bacterial community was significantly higher in the exponential growth phase in the first experiment (two sample t-test: day 08, p=0.039; day 17, p=0.003), but at the end of the growth, there was no difference (Figure 2.3). No significant difference was observed in *M. aeruginosa* growth in the experiment 2 (Figure 2.3).

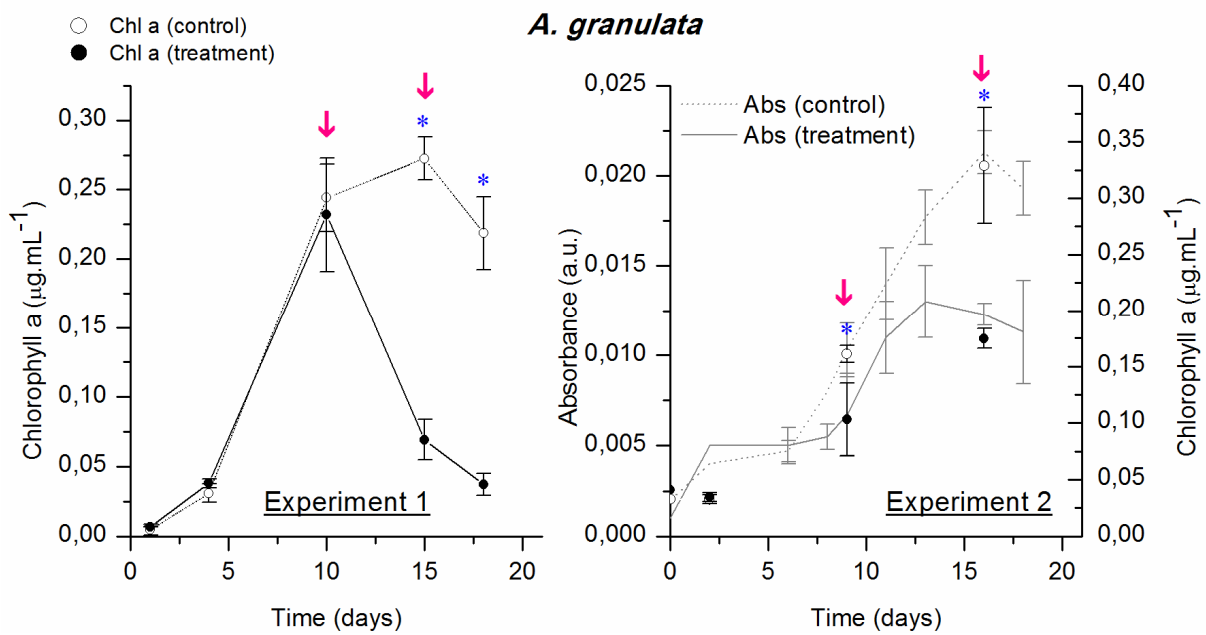

Figure 2.2 - Growth curves of *Aulacoseira granulata* in different experiments. Symbols represent chlorophyll a concentration in axenic (control, open symbols) and non-axenic (treatment, solid symbols). Gray lines in experiment 2 represent absorbance in control cultures (dashed lines) and treatment cultures (solid lines). Red arrows indicate sampling days for bacterial community analysis. Significant effects (two sample t-test, $p<0.05$) of treatment in the diatom growth are indicated by "*". Error bars represent standard deviation of 3 independent replicates.

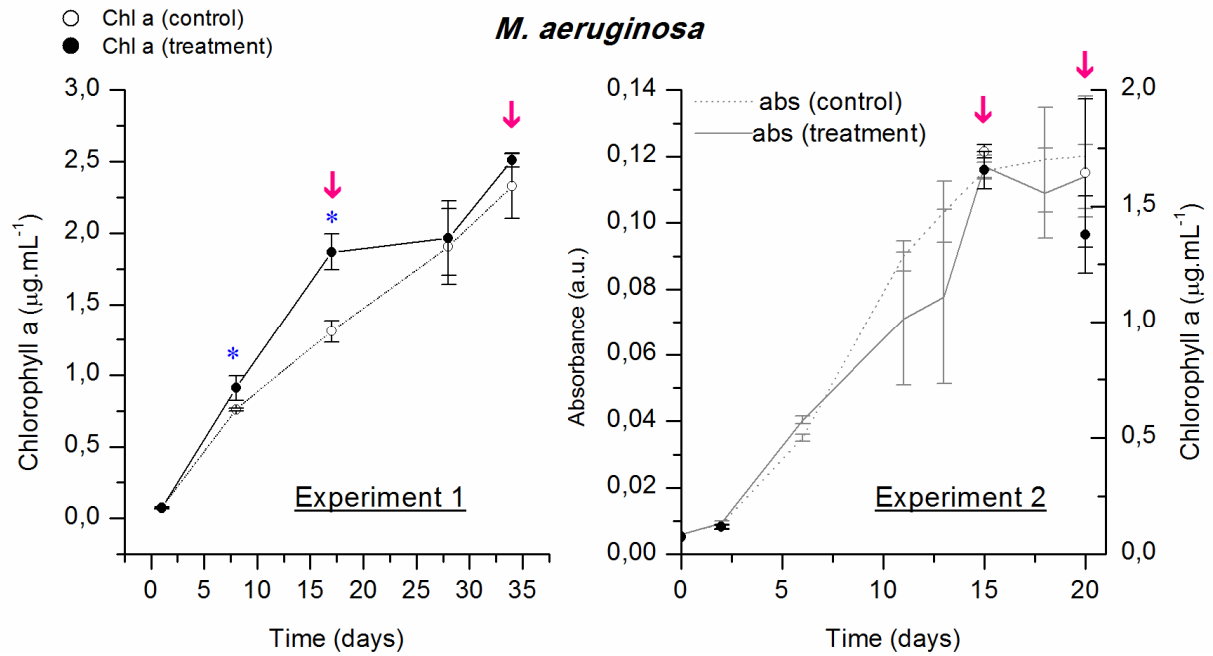

Figure 2.3 - Growth curves of *Microcystis aeruginosa* in different experiments. Symbols represent chlorophyll a concentration in axenic (control, open symbols) and non-axenic (treatment, solid symbols). Gray lines in experiment 2 represent absorbance in control cultures (dashed lines) and tratment cultures (solid lines). Red arrows indicate sampling days for bacterial community analysis. Significant effects (two sample t-test, $p<0.05$) of treatment in the cyanobacterial growth are indicated by "*". Error bars represent standard deviation of 3 independent replicates.

Effect of source community, growth condition and phytoplankton species in phytoplankton- associated bacterial community

The differences in bacterial communities were significant between phytoplankton species and between experiments considering total community or attached and free-living communities independently (Table 2.1), suggesting that host species and bacterial source are important driving forces to betadiversity of phytoplankton-associated bacterial communities. Due to the badness-of-fit (stress=0.22) of the ordination of all the samples using only two axis, a 3D plot was used to display differences among total community (Figure 2.4), as well as 2D plots with attached and free-living communities separately (Figure 2.S1, Figure 2.S2).

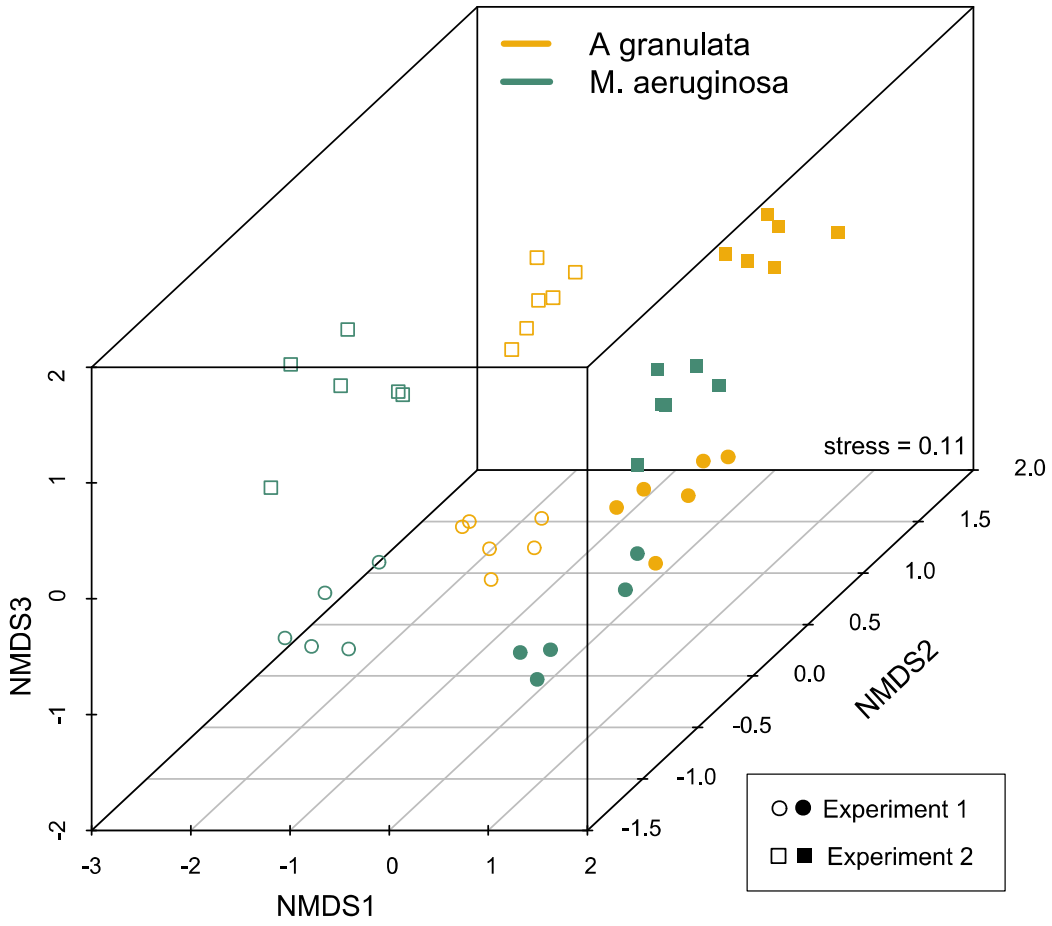

Figure 2.4 – Non-metric multidimensional scaling 3D plot showing differences between bacterial communities by phytoplankton species, fraction and experiment. Solid and open symbols represent, respectively, free-living and attached communities.

However, differences between phytoplankton species were greater than between experiments, for example, considering total bacterial community, phytoplankton species explained 27.1% of the variation (F-stats=22.97, p<0.001) whereas differences between experiments explained 13.5% of the variation (F-stats= 11.44, p<0.001). The same pattern was obtained in PERMANOVA tests comparing each phytoplankton species in different experiments (phytoplankton species exp1 x phytoplankton species exp2) and both phytoplankton species in the same experiment (*M. aeruginosa* x *A. granulata* exp1 or 2) (Table 2.1).

Comparing free-living and attached bacterial communities independently we also obtained that phytoplankton species explained a higher proportion of the variation in BCC than experiment, furthermore the variation in BCC between

phytoplankton species were greater for attached community (R^2=0.49 – Table 2.1) despite the differences in fractionation (pore size and/or centrifugation with Percoll) between experiments.

Table 2.1 – Results of Permutational MANOVA comparing total, attached and free-living bacterial community composition between phytoplankton species and experiment. M is *M. aeruginosa*; A is *A. granulata;* and exp1 and exp2 indicate experiment 1 and experiment 2, respectively.

Total community		**F-stats**	**R²**	**p**
Both experiments	Phytoplankton	22.97	0.27	<0.001
	experiment	11.44	0.14	<0.001
	phyto*exp	8.38	0.10	0.002
M *x* A exp1	phytoplankton	19.09	0.49	0.002
M *x* A exp2	phytoplankton	13.20	0.38	<0.001
M exp1 *x* M exp2	experiment	8.28	0.29	0.002
A exp1 *x* A exp2	experiment	11.74	0.35	0.002
Attached community		**F-stats**	**R²**	**p**
Both experiments	phytoplankton	36.30	0.49	<0.001
	experiment	10.67	0.14	0.003
	phyto*exp	8.41	0.11	0.004
Free-living community		**F-stats**	**R²**	**p**
Both experiments	phytoplankton	13.63	0.26	<0.001
	experiment	10.48	0.20	<0.001
	phyto*exp	8.99	0.17	<0.001

Recurrent phytoplankton-associated OTUs using different inocula

A total of 4627 OTUs were obtained in the present study, ranging from 3 to 668 per sample (Figure 2.S3). For the bacterial inoculum of the first experiment we found 125 OTUs, and in the second, 668. Regarding to the treatments, i.e. excluding controls and inocula, 3492 OTUs were obtained.

We obtained 806 OTUs in the first experiment and 1051 in the second experiment for *M. aeruginosa* and 245 OTUs in the *M. aeruginosa* colonies isolated from Barra Bonita Reservoir, making up 1861 OTUs, however 977 were singletons. Considering the 100 most abundant OTUs, which represented ~92% of the reads, 71% were in triplicates in at least one of the treatments of *M. aeruginosa.*

Regarding to *A. granulata,* 2119 OTUs were obtained considering only the treatments, being 1171 singletons. In experiment 1, we obtained 1090 OTUs associated with *A. granulata*, whereas 1400 were obtained in the second experiment. From the 100 most abundant OTUs (~92% of the reads), 74% were in triplicates in at least one treatment.

Despite the differences in the bacterial inocula (and in the growth medium for *M. aeruginosa*), we observed 38 recurrent OTUs (occurred in triplicates of at least one treatment in both experiments) for *A. granulata* (Figure 2.5) and 26 for *M. aeruginosa* (Figure 2.6). For the cyanobacterium, 9 OTUs were also found in the colonies isolated from Barra Bonita Reservoir (Figure 2.6). The recurrent OTUs represented, in number of OTUs, only 12% of the *M. aeruginosa* and 17% of the *A. granulata*-associated OTUs that occurred in triplicates. However, in proportion of reads per sample they were very important, representing on average 49.7% of the reads of *A. granulata* treatments (34.4 to 77.5% per sample) and on average 59.7% (from 20 to 77.8% per sample) in *M. aeruginosa* treatments (Figures 2.5 and 2.6).

From recurrent OTUs in the diatom cultures and in the cyanobacterial cultures, 14 (#60, #106, #626, #1084, #1136, #1485, #1506, #2396, #3179, #3615, #4481, #4626, #4826, #5391, Figures 2.5 and 2.6) occurred associated with both phytoplankton species.

Some recurrent OTUs represented a high proportion of total reads in the sample, as the OTUs #4954 (*Dechloromonas*-related) in *A. granulata* (Figure 2.5), being also important in the attached fraction. Interestingly this OTU was not abundant in the inocula. Another important OTU was the #1136 (*Novosphingobium*, Novo-A2), that presented higher relative abundance in the free-living portion. The Figure 2.5 shows that some OTUs presented higher relative abundances in attached fraction in both experiments in the diatom cultures, for example, the OTUs #3812 (*Vogesella*), #106 (*Hydrogenophaga,* betI-A), #4701 (*Rhizobiaceae*, alfI-B2), two *Pelomonas*-related (#3615, betI-A; and #2380, Lhab-A4), and #1506 (*Rhodoferax,*Rodo).

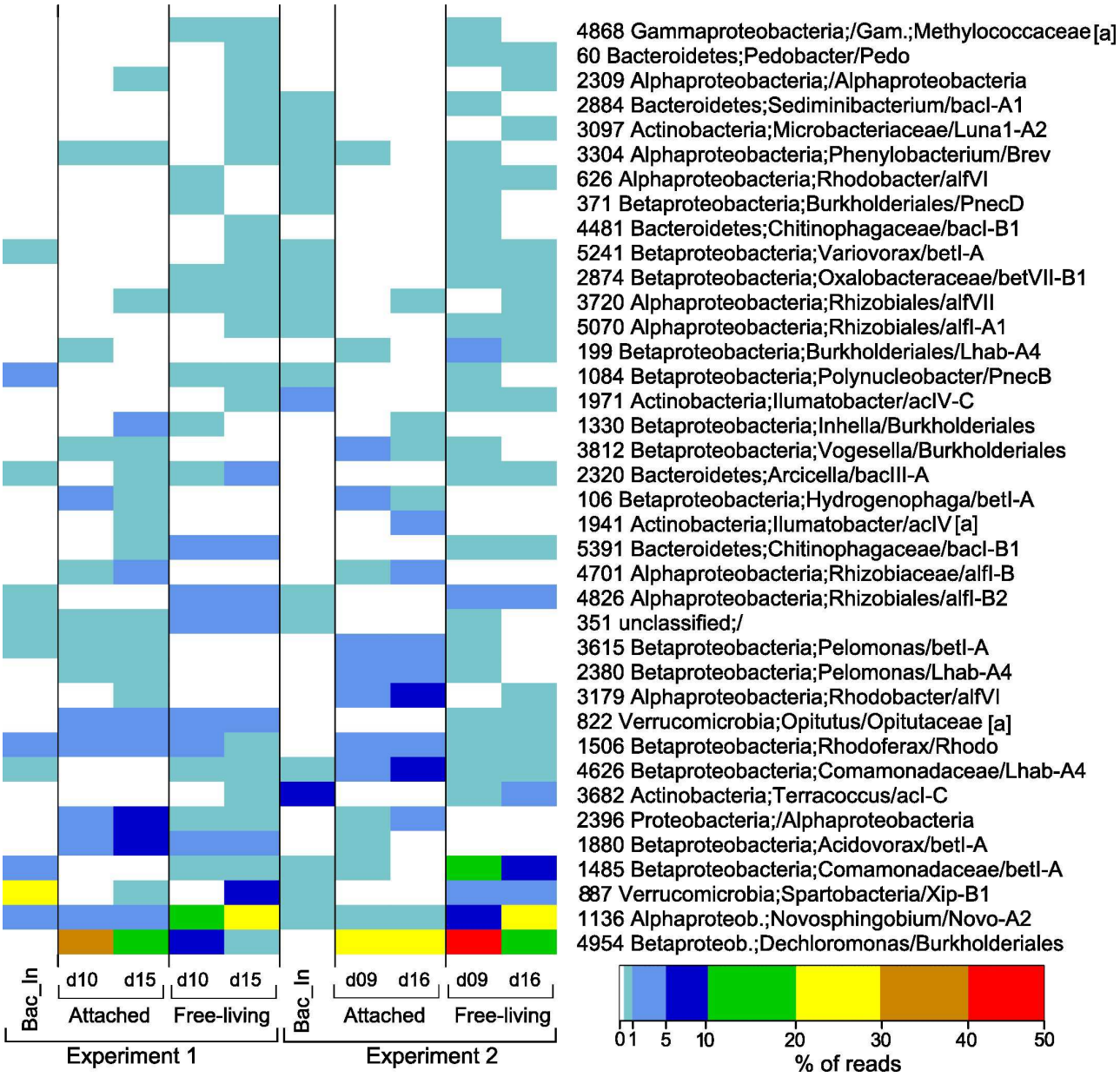

Figure 2.5 – Heatmap displaying the OTUs that occurred in both experiments of *A. granulata*. Taxonomic affiliation of two classification databases is shown after identification number: rdp/FW. Day of sampling is indicated by dxx; Bac_In is the bacterial inoculum. Frequencies are given by relativizing OTUs against the total number of reads of the sample. [a] indicates OTUs that did not occur associated with *M. aeruginosa.*

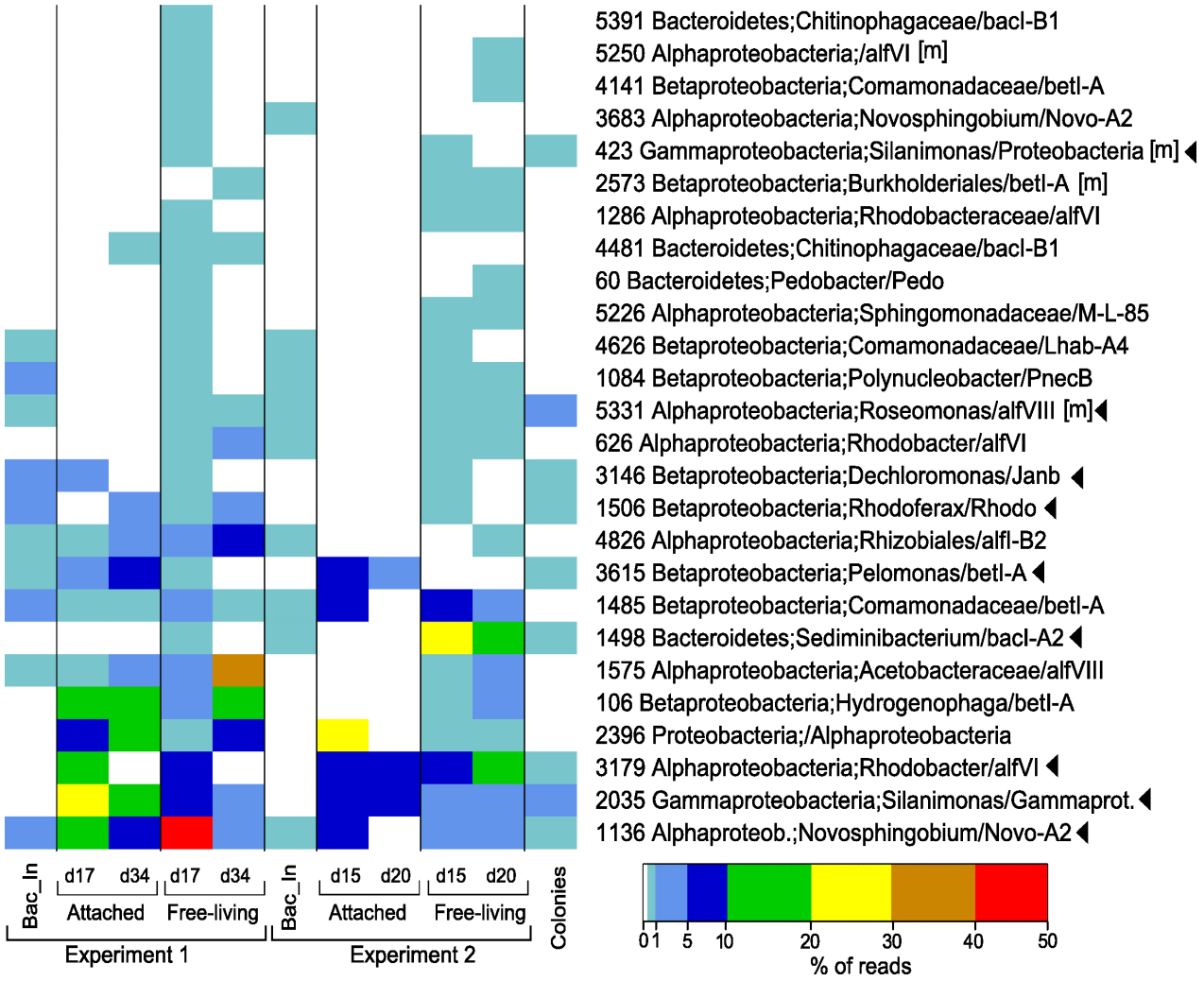

Figure 2.6 – Heatmap displaying the OTUs that occurred in both experiments of *M. aeruginosa*. Taxonomic affiliation of two classification databases is shown after identification number: rdp/FW. Day of sampling is indicated by dxx; Bac_In is the bacterial inoculum. Frequencies are given by relativizing OTUs against the total number of reads of the sample. [m] indicates OTUs that did not occur associated with *A. granulata*

Among the recurrent *M. aeruginosa*-associated OTUs the most abundant were the #1136 (*Novosphingobium*, Novo-A2), # 2035 (*Silanimonas lenta*), #3179 (*Rhodobacter*, alfVI), #2396 (*Alphaproteobacteria*) and the #106 (*Hydrogenophaga*, betI-A). We could also see differences in relative abundance of the OTUs between fractions, noteworthy among the attached in both experiments, the OTUs #3615 (*Pelomonas*, betI-A), the #2396 (*Alphaproteobacteria*) and the #2035 (*Silanimonas*). The OTU #5331 (*Roseomonas*-related) was found in both experiments and in the colonies, but was not detected in *A. granulata* cultures (even singleton).

Nine of the OTUs associated with *Microcystis* also occurred in the colonies isolated from the Reservoir (Figure 2.6). Among them, five have closely related OTUs previously found associated with *Microcystis* or cyanobacterial blooms around the world (Table 2.2). Among the recurrent OTUs in *A. granulata*, only one has been reported in association with diatoms, one with algae breakdown, and others with cyanobacterial blooms, however some of them were recurrent only in the diatom cultures in our study (Table 2.2).

Bacteria were not detected by inoculation of control cultures in bacterial growth media, but a few reads of non-cyano/chloroplast origin were detected in the controls: 108 non-cyano reads (from 6988 in total) in the first experiment and 3 in the second experiment (from 6947 reads in total) for *M. aeruginosa*; and in *A. granulata* cultures 13 non-chloroplast reads (from 6393 reads) were detected in the first experiment and 23 (form 10844 reads) in the second experiment. These contaminants likely represent contamination during DNA extraction and/or PCR reactions, except, perhaps, for the *M. aeruginosa* control in the first experiment, which could have presented a slight contamination during cultivation. The main contaminant OTU (98 reads) in this culture was from the genus *Cupriavidus*, all the other contaminant OTUs had less than 3 reads each.

Table 2.2 – Recurrent OTUs (occurred in triplicates in both experiments of each phytoplankton species) that presented closely related OTUs (similarity score in RDP database > 0.97) previously reported in association with phytoplankton blooms. A = *Aulacoseira granulata* cultures; M=*M. aeruginosa* cultures and Mcol = *M. aeruginosa* colonies from Barra Bonita Reservoir.

'Recurrent' OTUs in the present study			**Related OTUs in other studies**			
Occurrence	OTU #	Related taxon	Occurrence	location	accession number	citation
M/Mcol	2035	*Silanimonas lenta*	*Microcystis* bloom cyanobacterial mats	China Netherlands	GU559788 GQ441207	(Xing et al. 2010) (Severin et al. 2010).
M/Mcol	1498	*Sediminibacterium* (bacI-A2)	Cyanobacterial bloom	China	KC253319	(Xia and Xie Unpublished)
M/Mcol	5331	*Roseomonas* (alfVIII)	Cyano bloom	Sweden	AY509418	(Eiler and Bertilsson 2004)
			Microcystis bloom	China	GU559787	(Xing et al. 2010)
			Cyano bloom	China	KC253364	(Xia and Xie Unpublished)
M/Mcol/A	3179	*Rhodobacter* (alfVI)	*Microcystis* bloom	China	GU559802	(Xing et al. 2010)
			Microcystis bloom	China	KC836040	(Niu Unpublished)
			Cyano bloom	China	FJ572035	(Li et al. Unpublished)
M/A	626	*Rhodobacter* (alfVI)	Cyano bloom	Sweden	AY509406	(Eiler and Bertilsson 2004)
M/A	4481	*Chitinophagaceae* (bacI-B1)	Bentic diatom		EU580485	(Bruckner et al. 2008)
A	3304	*Phenilobacterium* (Brev)	Cyano bloom	Greece	EF203203	(Kormas et al. 2010)
			Microcystis	China	GU559783	(Xing et al. 2010)
A	551	unclassified	*Microcystis*		HQ856351	(Dziallas and Grossart 2011)
A	2309	*Alphaproteobacteria*	Cyano bloom	China	HQ827945	(Cai et al. Unpublished)
A	3682	*Terracoccus* (acI-C)	Cyano bloom	China	KC253354	(Xia and Xie Unpublished)
			Cyano bloom	China	HQ827947	(Cai et al. Unpublished)
A	3720	*Rhizobiales* (alfVII)	Algae outbreak	China	GU305792	(Chen and Yang)
			Microcystis bloom	China	KC836024	(Niu Unpublished)
A	1971	*Ilumatobacter* (acIV-C)	Cyano bloom	China	KC253346	(Xia and Xie Unpublished)

Bacterial community structure and differences between phytoplankton using higher taxonomic levels

We could observe some patterns analyzing the BCC. Some differences among classes (Figure 2.7) between *A. granulata* and *M. aeruginosa* were consistent in both experiments, as higher proportion of *Gammaproteobacteria* associated with cyanobacterial cultures and higher relative abundance of *Verrucomicrobia* associated with diatom cultures. Comparison of classes also showed that one of the major groups associated with *M. aeruginosa* in the second experiment ("Capítulo 1"), the *Gemmatimonadetes,* was not important in the first experiment.

The statistical analysis of the community dissimilarities considering higher taxonomic levels also revealed the same pattern obtained using OTUs analysis: the differences between phytoplankton species were greater than between experiments, and most of the PERMANOVA tests were significant (Table 2.3). However, using groups instead of OTUs increased the differences between the variation explained (R^2) by phytoplankton (*Aulacoseira* x *Microcystis*) and the variation explained by experiment (experiment 1 x experiment 2). For example, using families, the phytoplankton species explain 40.4% of the variation in the BCC, whereas differences between experiments (mainly bacterial inocula) explain 7.7% (difference of 32.7%). Comparing OTUs, we obtained that 27.7% was explained for differences in phytoplankton species, whereas 13.5% was explained by differences between experiments (difference of 13.6%) (Tables 2.1 and 2.3).

The variance in BCC explained by phytoplankton species was higher when comparing family than when comparing the other taxonomic levels. The main differences of families between phytoplankton species were higher proportion of *Rhodocyclaceae* and one family of *Spartobacteria* associated with *A. granulata,* and higher proportion of *Xanthomonadaceae, Rhodobacteraceae* and *Acetobacteraceae* associated with *M. aeruginosa* (Figure 2.8).

Table 2.3 - Results of Permutational MANOVA comparing bacterial community composition between phytoplankton species and experiment, using higher taxonomic levels. M is *M. aeruginosa*; A is *A. granulata;* and exp1 and exp2, experiment 1 and experiment 2, respectively.

PERMANOVA		Class (36 classes)			Order (75 orders)		
		F-stats	**R^2**	**p**	**F-stats**	**R^2**	**p**
Both experiments	phytoplankton	30.30	0.367	0.001	30.93	0.368	0.001
	experiment	5.63	0.068	0.022	7.24	0.086	0.005
	phyto*exp	2.73	0.033	ns	1.94	0.023	ns
M x A exp1	phytoplankton	22.56	0.506	0.003	20.24	0.479	0.001
M x A exp2	phytoplankton	11.10	0.335	0.003	12.72	0.366	0.002
Mexp1 x Mexp2	experiment	3.47	0.136	0.045	3.90	0.151	0.01
A exp1 x A exp2	experiment	5.53	0.201	ns	5.591	0.203	0.01
		Family (148 families)			**Genus (311 genera)**		
		F-stats	**R^2**	**p**	**F-stats**	**R^2**	**p**
Both experiments	phytoplankton	36.21	0.404	0.001	35.966	0.387	0.001
	experiment	6.91	0.077	0.005	9.401	0.101	0.002
	phyto*exp	2.52	0.028	ns	3.506	0.038	0.025
M x A exp1	phytoplankton	22.32	0.504	0.001	24.931	0.531	0.002
M x A exp2	phytoplankton	16.80	0.433	0.001	15.652	0.416	0.001
M exp1 x M exp2	experiment	4.96	0.184	0.004	8.196	0.271	0.002
A exp1 x A exp2	experiment	4.40	0.167	0.017	4.192	0.16	0.017

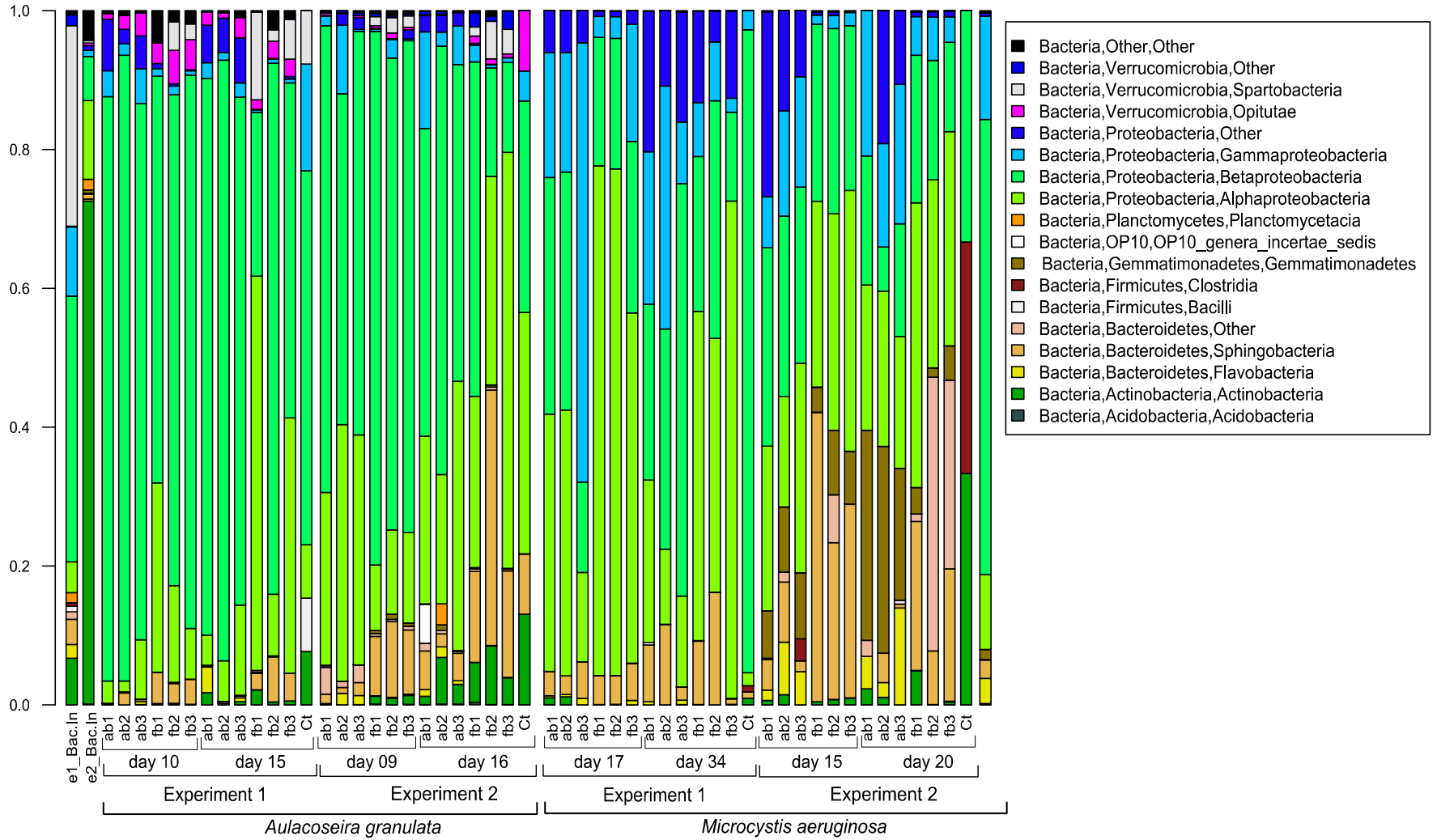

Figure 2.7 - Proportion of bacterial Classes in the replicates of each treatment of *A. granulata* and *M. aeruginosa*. Naïve bayesian classification was used. Numbers *1-3* represent the replicate, *ab* and *fb* indicate attached and free-living communities respectively, *Ct* indicates the controls and *e1_Bac.In* and *e2_Bac.In* represents inocula from experiment 1 and 2, respectively.

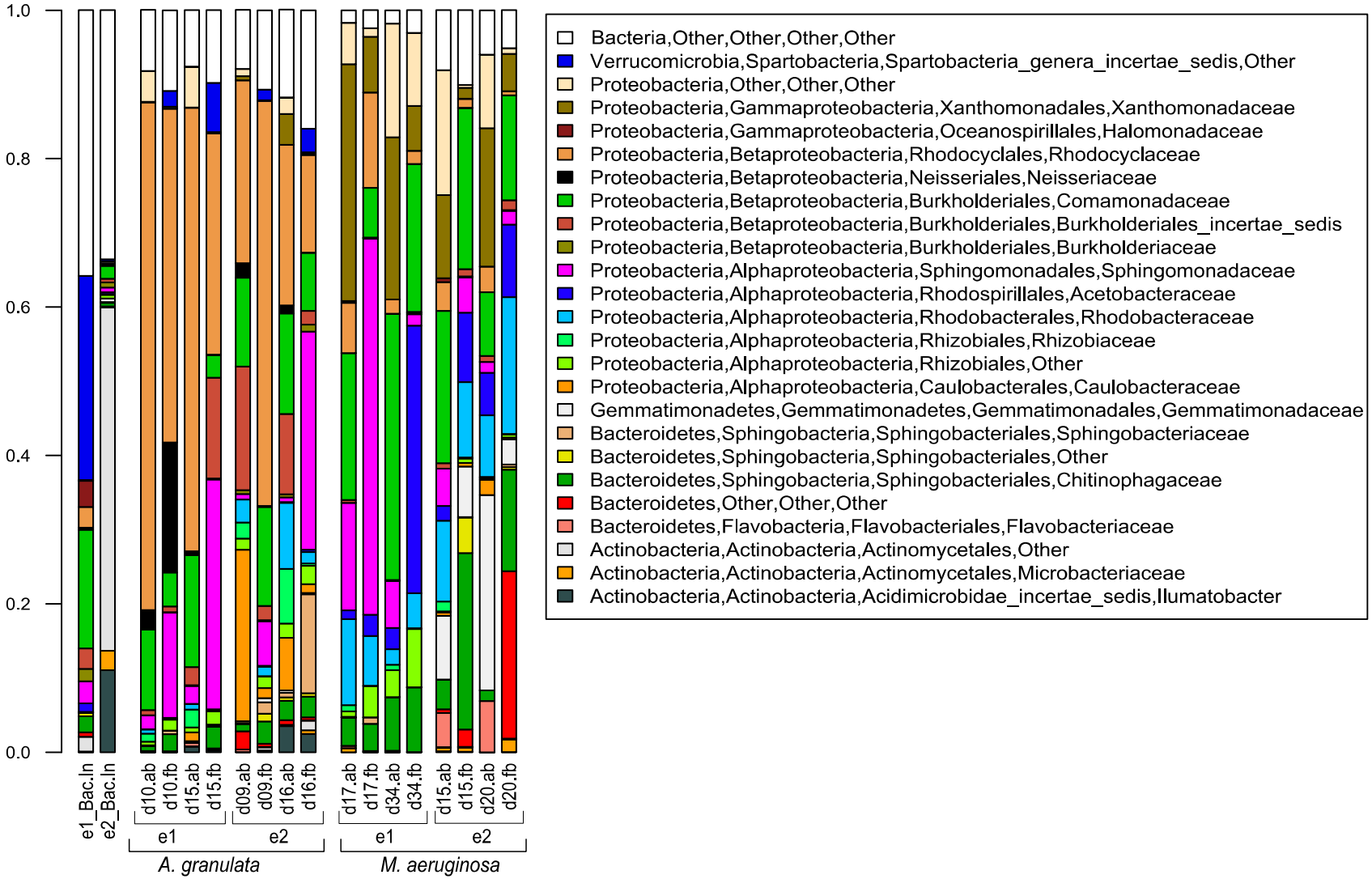

Figure 2.8 - Proportion of bacterial families in the treatments of *A. granulata* and *M. aeruginosa*. Naïve bayesian classification was used. Attached and free-living portions are represented by *ab* and *fb*, respectively; *d(xx)* represents sampling day; *e1* and *e2*, experiment 1 and experiment 2. *Bac.In* represents bacterial inocula.

2.5 . Discussion

Influence of bacterial community on phytoplankton growth

In both experiments bacterial community had no effect on the final biomass of *M. aeruginosa* cultures in comparison with the controls. However, the bacterial community accelerated *A. granulata* senescent phase in the first experiment and also reduced its growth yield in the second experiment. Casamatta & Wickstrom (2000) have shown that bacteria co-occurring with *M. aeruginosa* are more likely to be attracted and resistant to cyanobacterial exudates than bacterial communities from environments where *M. aeruginosa* blooms are not common. Since bacterial communities can be adapted to co-occurring phytoplankton, we could expect the same response for the phytoplankton species, in a co-adaptation. Although bacterial communities have not enhanced *M. aeruginosa* growth, they could bring some advantage by inhibiting the growth of other phytoplankton species, as observed for *A. granulata.*

However, more studies with isolated bacterial strains and/or cross-effects of exudates of one phytoplankton species altered by the bacterial community in the growth of other phytoplankton species could help understanding bacterial effects on phytoplankton ecology.

Effect of bacterial inocula and growth condition in the specificity of phytoplankton-bacteria association

We have found that the bacterial source had significant influence on the bacterial community that will develop with *A. granulata*. For free-living bacteria associated with *A. granulata,* only the bacterial inoculum was different between experiments and still BCC were significantly different. We cannot ensure that the main difference in bacterial communities associated with *M. aeruginosa* is due only to different inocula, since we also used different growth medium for the cyanobacteria. However, we can suggest a less tight phytoplankton-bacteria association for both phytoplankton species studied, since BCC associated with each phytoplankton species were different between experiments.

Previous work suggested a minor importance of the bacterial inocula on the development of bacterial communities associated with marine diatoms (Schäfer et al. 2002), suggesting more consistent association, but phytoplankton cultures were not previously axenic. However, the influence of the bacterial source has been demonstrated in a study with *M. aeruginosa* by Dziallas and Grossart (2011). These authors showed that the bacterial inoculum was more important to determine BCC associated with *M. aeruginosa* than environmental factors. Since the development of a specific bacterial taxon depends on its occurrence in the inocula, it could be expected some differences between experiments. Although the bacterial inocula have been sampled from the same reservoir, they presented different dominant taxa (Figures 2.5, 2.6 and 2.8), providing different proportions and/or different of bacterial OTUs.

Despite the significant differences among the bacterial communities associated with each phytoplankton species, we found that between-phytoplankton differences in bacterial community were greater than within-phytoplankton differences, even considering the change in growth medium for *M. aeruginosa* and differences in fractionation. This suggests that differences in the organic compounds released by the phytoplankton play a more important role in the selection of associated bacterial communities than differences in bacterial inocula or/and phytoplankton growth medium. In 'Capítulo 1' we have also demonstrated that different species may select different bacterial communities, as shown by previous studies (Riemann et al. 2000, Grossart et al. 2005). However, now we have shown that it may overcome the differences in bacterial inoculum and experimental design, at least for such different groups as Bacillariophyceae and *Cyanobacteria*. Paver et al. (2013) found that the bacterial community composition explained by phytoplankton treatment increased from 17 to 28% while the variation explained by bacterial source decreased from 84 to 30% in an experiment comparing phytoplankton communities from 3 environments incubated with bacterial community source from the same 3 environments. Although these authors have shown higher explained variation by bacterial source at the end of the experiment (30% compared to 28% for phytoplankton treatment), they have analyzed BCC only in the initial and 7^{th} day of incubation, and in our study we analyzed BCC during longer incubation time.

Recurrent phytoplankton-associated OTUs and main associated families distinguishing phytoplankton species

We have found 38 recurrent OTUs (i.e OTUs that occurred associated with the same phytoplankton species in both experiments) in *A. granulata* cultures and 26 recurrent OTUs in *M. aeruginosa* cultures.

The *Dechloromonas*-related OTU (#4954) represented a dominant group for *A. granulata,* but did not appear as an important OTU in the inoculum (Figure 2.5) or recurrent in *M. aeruginosa* cultures (due to non-occurrence in triplicates in the first experiment – not shown), suggesting a strong selectivity by the diatom. Eigemann et al. (2012) found a high selectivity in bacterial communities associated with algae in comparison to the inocula, and argued that it was an indication of a high host-specificity. The *Dechloromonas,* is a genus of perchlorate-reducing and benzene-degrading bacteria firstly described in 2001 (Achenbach et al. 2001, Coates et al. 2001), but we did not find previous reports about its occurrence during microalgal blooms.

Only one of the recurrent OTUs associated with *A. granulata* was found in previous studies with diatoms (#4481, *Chitophagaceae*), what could, however, be explained by the few available reports on freshwater diatom-associated bacteria. Despite the low sampling effort for *M. aeruginosa* colonies isolated from Barra Bonita reservoir, among the recurrent OTUs associated with the *Cyanobacteria*, 9 were also found in the colonies isolated from the field sample. Interestingly, 5 of them have already been reported occurring with *Microcystis* or other cyanobacterial blooms in other studies (Table 2.2), whereas only 2 of the other 17 *M. aeruginosa*-recurrent OTUs during this experiment also matched this criterion.

Among the OTUs with higher proportion in *M. aeruginosa* cultures, the *Rhodobacter* (# 3179) and the *Novosphingobium* (#1136) were also recurrent in *A. granulata,* however, the *Silanimonas* (#2035) was found among the recurrent only in the cyanobacterial cultures (with higher relative abundance in the attached fraction) and in the isolated colonies. This genus was also reported associated with cyanobacterial blooms in other studies, what can indicate a tight association. Groups often found in association with some phytoplankton species may be adapted and be selected for this association (Jasti et al. 2005). The information about the OTUs obtained in this experiment may help future works aiming to study the function of

isolated strains of some of the most common genus in the associations with these phytoplankton species, focusing on specific growth media to each bacterial taxon.

However, given the higher number of studies with *Microcystis* cultures and natural blooms (e.g. Eiler and Bertilsson 2004, Shi et al. 2009b, Shi et al. 2010, Dziallas and Grossart 2011), only six widespread OTUs (Table 2.2) seem to be a low number of specific OTUs associated with *M. aeruginosa*. Among the possible reasons, are the differences in experimental design and methods to detect biodiversity, primers used in the PCR (Dziallas and Grossart 2011), different phytoplankton strains (Sapp et al. 2007), environmental conditions and bacterial inocula (Langenheder et al. 2006, Dziallas and Grossart 2011, Eigemann et al. 2012). Thus, usually higher taxonomic levels rather than OTUs have been used to make comparisons among studies, still, with great variability.

Dziallas et al. (2011) reported a considerable variability of bacterial community during *M. aeruginosa* blooms around the world, but some groups were recurrent regardless the continent of sampling. Among the recurrent taxa, was the genus *Sphingomonas,* which, however, was not detected associated with *M. aeruginosa* in the present study. *Sphingomonadales* is usually associated with degradation of microcystin during cyanobacterial blooms, but Mou et al. (2013) suggested less importance of this group in the bacterial community in response to microcystin amendment, with other possible groups presenting the same function with an alternative pathway than the usually reported for microcystin degradation. The main OTU of *Sphingomonadaceae* in our study was related to *Novosphingobium,* which was also important in *A. granulata* cultures. The genus *Novosphingobium* has been associated with environments with high concentration of recalcitrant compounds as phenol (Tiirola et al. 2002, Hutalle-Schmelzer et al. 2010)

Using families, the differences between bacterial communities associated with distinct phytoplankton species were statistically more evident. In *M. aeruginosa* we found higher proportions of *Xanthomonadaceae* in the attached fraction (including the OTU #2035, a *Silanimonas*). This group was found to increase in relative abundance (analyzed by protein-encoding sequences), although not significant, with microcystin amendment in bacterial cultures (Mou et al. 2013). Furthermore, it has also been found as plant pathogens, especially due to its ability of attachment (Mhedbi-Hajri et al. 2011).

Other studies have found *Xanthomonadaceae, Rhodobacterales* (Shi et al. 2012) and *Comamonadaceae* (Li et al. 2012) among the most abundant taxa associated with *M. aeruginosa.* Also *Rhodobacteraceae* and *Acetobacteraceae* were found among most representatives *Alphaproteobacteria* during cyanobacterial blooms in Swedish lakes (Eiler and Bertilsson 2004).

Rhodocyclaceae was the main family associated to *A. granulata,* however, to the best of our knowledge, few works reported *Rhodocyclaceae* associated with diatoms. Zakharova (2013) reported that only 0.1% of the bacterial community associated with diatom degradation in Lake Baikal was assigned to *Rhodocyclales* order.

Higher taxonomic levels for the analysis of specificity in phytoplankton-bacteria association

As already discussed, even with similar methods, one of the main factors for the variability in bacterial communities is the bacterial source (e.g. our study, Langenheder et al. 2006, Paver et al. 2013). Since the initial bacterial community can show variation with different environments/experiments, it is expected that one missing OTU will be replaced by some other capable of play similar function and occupy the same niche (Teeling et al. 2012). The functional redundancy of bacterial OTUs is known, and is part of the discussion about species definition in Bacteria (Acinas et al. 2004). Teeling et al. (2012) found that differences in nutritional strategies (for example, in the expression of proteins related to transport systems for nutrient uptake) of the bacterioplankton accompanying a phytoplankton bloom were apparent even between classes.

We have found that phytoplankton-associated bacterial communities using compositional distances presented better between-phytoplankton separation when using higher taxonomic levels (specially genus and family), whereas the differences between experiments (different inocula/experimental design) decreased. We hypothesize that it could be due to ecological redundancy of OTUs that belonged to closer groups, which may play similar functions. Since the genetic differentiation may be predictive of bacterial functional properties (Martin 2002), the comparison of higher taxonomic levels could reduce the influence of the differences between inocula and could be more informative to understand phytoplankton-bacteria interaction in some cases.

Considering these results, the evaluation of families or genera could provide a good comparison to distinguish bacterial communities associated with the studied phytoplankton species, despite the bacterial inocula. This is in accordance with the recent published work of Steffen et al. (2012) that used pyrosequencing to characterize cyanobacterial blooms and found that the function of microbes within the environment is often more highly conserved than their phylogenetic/taxonomic identity.

Despite the differences in bacterial inocula (and growth medium for *M. aeruginosa*) we found some recurrent OTUs in each *A. granulata* and *M. aeruginosa* cultures, some of them being also reported associated with phytoplankton bloom around the world, suggesting strong interaction with some few OTUs. However, BCC was significantly different between phytoplankton species and when comparing experiments with the same phytoplankton species, what could suggest less tight association phytoplankton-bacterial community. On the other hand, when comparing higher taxonomic levels rather than OTUs the differences in BCC between-phytoplankton were enhanced, whereas differences within-phytoplankton decreased (although still significant), what may suggest that the analysis of phytoplankton-associated bacterial community could be better linked with ecological function and redundancy of bacterial OTUs than with the OTUs *per se*. Thus, future studies should consider the ecological guilds of bacterial communities in the specificity of phytoplankton-associated community (Sapp et al. 2007).

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2.7 . Supporting Figures

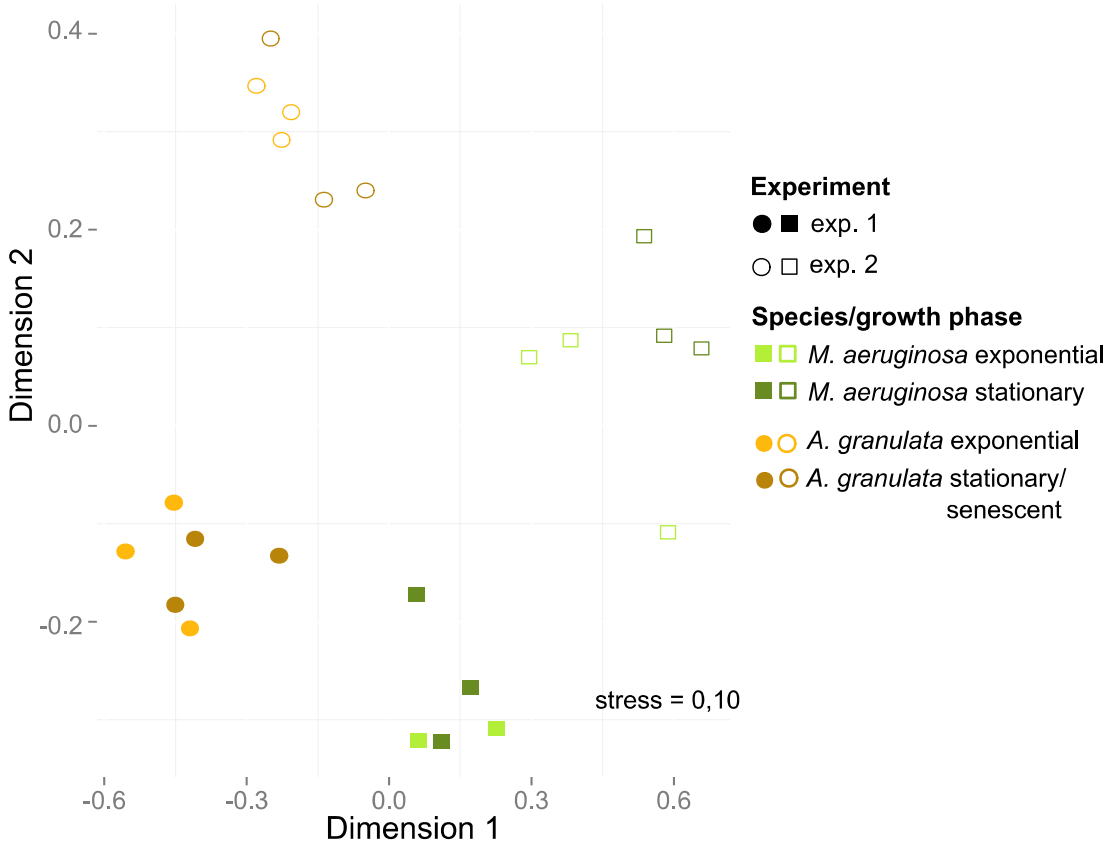

Figure 2.S1 - Non-metric multidimensional scaling plot showing differences between attached bacterial communities by phytoplankton species, experiment and growth phases.

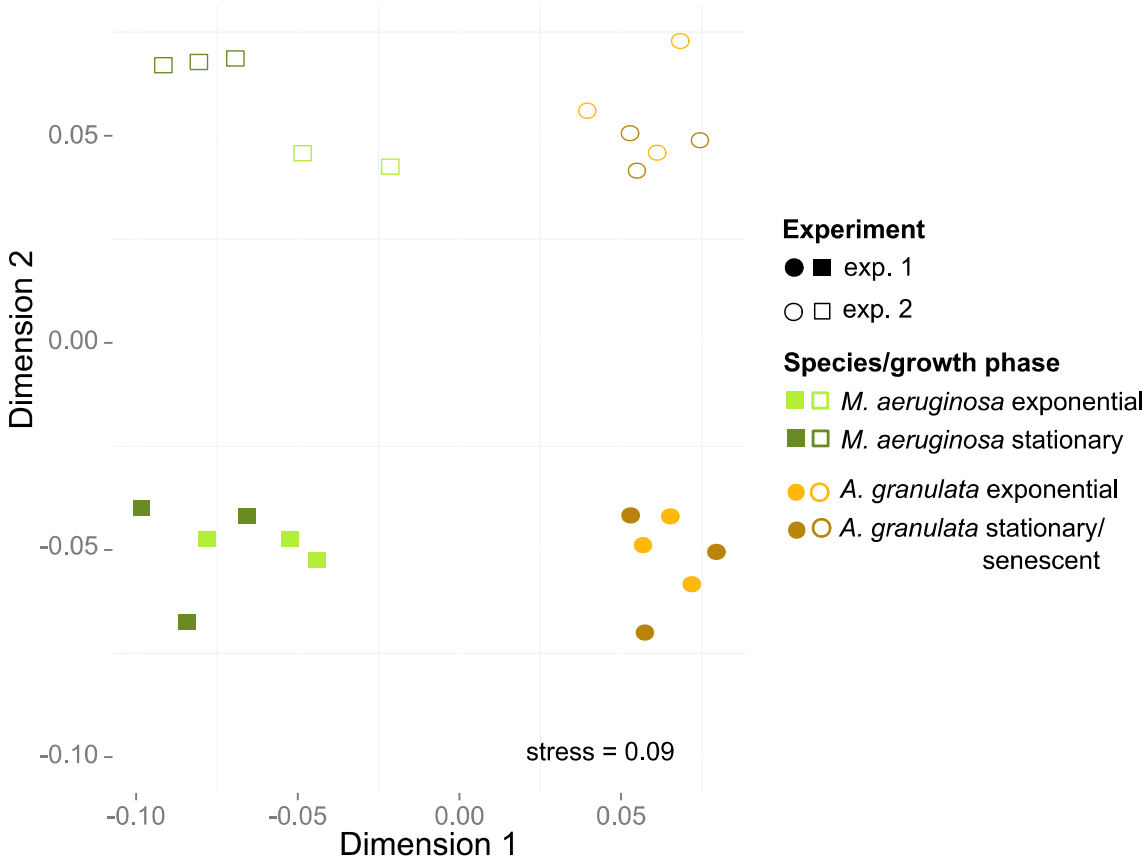

Figure 2.S2 - Non-metric multidimensional scaling plot showing differences between free-living bacterial communities by phytoplankton species, experiment and growth phases.

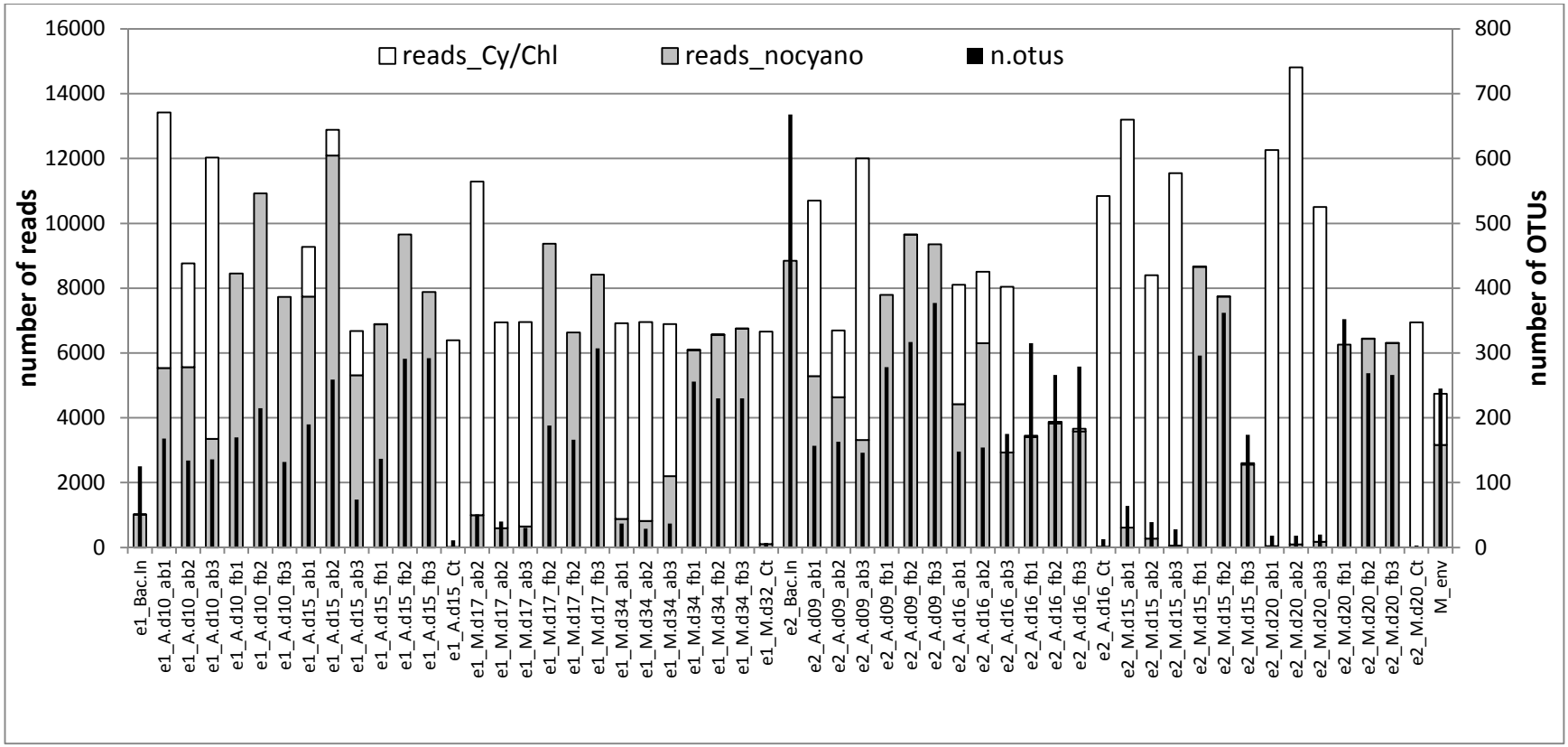

Figure 2.S3 – Numbers of non-cyano/chloroplast and of cyano/chloroplast reads, and number of OTUs per sample. Bac.In is the bacterial inoculum; e1 and e2 , experiment 1 and experiment 2, respectively. *A. granulata* (A) and *M. aeruginosa* (M) cultures, in different days (dxx) and fractions (ab, attached bacteria; fb, free-living bacteria). These data is partially presented in 'Capítulo 1'.

CAPÍTULO 3:

EFEITO DE LINHAGENS BACTERIANAS ADERIDAS A *A. GRANULATA* E *M. AERUGINOSA* SOBRE O CRESCIMENTO DESTAS ESPÉCIES E SUAS POSSÍVEIS IMPLICAÇÕES ECOLÓGICAS

Palavras-chave: diatomácea, cianobactéria, bactérias aderidas, efeitos inibitórios.

3.1. Resumo

A adesão de bactérias aos filamentos e colônias fitoplanctônicas pode indicar maior especificidade na associação fitoplâncton-bactéria. Embora as bactérias possam estar envolvidas com o estímulo ou declínio de florescimentos algais, pouco se sabe sobre os efeitos de linhagens bacterianas aderidas a uma espécie fitoplanctônica sobre a própria espécie e sobre outra coocorrente em ambiente natural. A partir de espécies fitoplanctônicas isoladas do Reservatório de Barra Bonita em cocultivo com a comunidade bacteriana proveniente do mesmo reservatório, foram isoladas 3 linhagens bacterianas de colônias de *M. aeruginosa* e 5 de filamentos de *A. granulata,* no entanto, duas destas últimas foram inicialmente detectadas também em *M. aeruginosa*. Os efeitos individuais das 8 linhagens, além do efeito conjunto das 5 linhagens isoladas de *A. granulata* e das 5 linhagens associadas à *M. aeruginosa* sobre o crescimento de ambas as espécies fitoplanctônicas foram avaliados em culturas estanques. Nenhum tratamento apresentou efeito significativo sobre a taxa de crescimento ou rendimento final da cianobactéria, porém alguns tratamentos acentuaram o declínio da cultura durante a fase estacionária. Em contrapartida, todos os tratamentos, exceto o cultivo em culturas binárias com as linhagens Bact 21 (uma *Rhizobiales*) e Bact 17.3 (uma *Acidovorax*), apresentaram efeitos negativos significativos na taxa de crescimento ou

rendimento máximo das culturas de *A. granulata*. Uma das linhagens, a Bact 6A (identificada como uma *Pseudomonadaceae*), isolada da diatomácea, apresentou forte efeito algicida sobre a espécie hospedeira. Entre as outras linhagens isoladas que apresentaram efeito sobre o crescimento de *A. granulata* estão bactérias associadas ao gênero *Rhizobium* e às famílias *Caulobacteraceae* (relacionada ao gênero *Asticcacaulis*) e *Comamonadaceae* (gêneros *Acidovorax* e possível *Pelomonas*). Os resultados sugerem que a comunidade bacteriana pode ser um fator importante para a dominância de *M. aeruginosa* no ambiente, por ser refratária à ação das bactérias que inibem o crescimento de outra possível espécie competidora. Futuros estudos com outras linhagens bacterianas e espécies fitoplanctônicas são necessários para a confirmação e extensão dessa hipótese.

3.2. Introdução

As bactérias e as espécies fitoplanctônicas (cianobactérias e microalgas) são os organismos numericamente dominantes em ambientes aquáticos e seu metabolismo controla o fluxo de energia e a ciclagem de nutrientes (Cole 1982, Azam & Malfatti 2007). A interação bactéria-fitoplâncton pode trazer benefícios para as bactérias, que utilizam os compostos orgânicos excretados pelo fitoplâncton (Bertilsson & Jones 2003, Giroldo et al. 2007) ou que se beneficiam de microambientes formados pelos agregados ou colônias fitoplanctônicas, que além de fornecerem uma matriz orgânica rica (Grossart & Simon 2007), oferecem proteção contra predadores (Jürgens & Güde 1994). O fitoplâncton pode também se beneficiar, seja pela remineralização de nutrientes (Paerl 1996), ou pela produção de vitaminas (Croft et al. 2005) e outros fatores de crescimento pelas bactérias (Fukami et al. 1997), que podem, portanto, ser fundamentais na formação de *blooms* e na dominância de certas espécies fitoplanctônicas (Liu et al. 2008).

Por outro lado, os efeitos inibitórios de bactérias sobre o fitoplâncton, e vice-versa, também são conhecidos. Algumas linhagens de cianobactérias, incluindo *Microcystis aeruginosa,* podem produzir substâncias que inibem o crescimento de outras bactérias (Ostensvik et al. 1998, Kreitlow et al. 1999). Contudo, as bactérias também podem prejudicar o crescimento fitoplanctônico, seja por competição por nutrientes (Danger et al. 2007) ou por produção de compostos que inibem o

crescimento de cianobactérias (Manage et al. 2000), diatomáceas (Mayali & Azam 2004) ou outras algas (Fukami et al. 1997), com possíveis efeitos sobre términos de *blooms* (Mayali & Azam 2004) e grande impacto no fluxo de carbono dos ambientes aquáticos (Azam 1998). Mesmo em interações alelopáticas entre espécies fitoplanctônicas, as bactérias podem agir sinergicamente à ação das microalgas ou podem degradar compostos bioativos (Hulot & Huisman 2004).

Sabe-se que a comunidade bacteriana varia ao longo de diferentes fases de *blooms* fitoplanctônicos, assim como entre espécies formadoras de *blooms* e, além disso, as bactérias livres podem diferir significativamente daquelas encontradas aderidas aos filamentos e às colônias fitoplanctônicas (Capítulos 1 e 2, Grossart et al. 2005, Dziallas & Grossart 2011, Eigemann et al. 2012, Teeling et al. 2012). Essa seletividade deve-se principalmente à variação na qualidade da matéria orgânica excretada (Bertilsson & Jones 2003, Pereira et al. 2009), à capacidade de degradação por parte das bactérias (Teeling et al. 2012) ou mesmo na resistência a antibióticos produzidos pelo fitoplâncton, como exposto anteriormente. Desta maneira, pode-se esperar alguma especificidade nas associações fitoplâncton-bactéria.

A força da interação e especificidade bactéria-fitoplâncton pode variar de acordo com a espécie fitoplanctônica (Eigemann et al. 2012), mas algumas UTOs (Unidades taxonômicas operacionais) bacterianas parecem ocorrer frequentemente associadas a determinadas espécies independentemente da localização geográfica (Capítulo 2). As bactérias aderidas, ou epifíticas, podem apresentar uma associação mais específica com o fitoplâncton do que as livres (Grossart et al. 2005, Sigee 2005), em alguns casos desenvolvendo uma associação com acoplamento metabólico e benefícios mútuos (Sigee 2005).

Apesar da importância da associação fitoplâncton-bactéria para ambos os organismos e para os ciclos biogeoquímicos em ambientes aquáticos (Malfatti & Azam 2009) e da possível especificidade nas associações, especialmente entre fitoplâncton-bactéria epifítica, o efeito das bactérias preferencialmente aderidas a determinadas espécies fitoplanctônicas sobre sua fisiologia e sobre possíveis espécies fitoplanctônicas competidoras ainda é pouco explorado. Grande parte dos relatos é sobre o efeito de bactérias isoladas de uma espécie (Shi et al. 2009) ou do ambiente onde a espécie fitoplanctônica é recorrente (Manage et al. 2000, Berg et al. 2009), sobre a própria espécie ou outras espécies formadoras de *blooms*, sendo

que para diatomáceas, grande parte dos trabalhos utiliza espécies marinhas (Mayali & Azam 2004, Paul & Pohnert 2011). Porém, os efeitos ecofisiológicos 'cruzados' de bactérias aderidas são ignorados. Além disso, as espécies para as quais há trabalhos descritos (algas e bactérias) são poucas, considerando-se o universo dos organismos envolvidos.

Desta forma, uma grande questão permanece sem dados disponíveis na literatura: que efeitos a comunidade bacteriana aderida a uma espécie fitoplanctônica terá sobre a própria espécie e sobre outra espécie fitoplanctônica coocorrente no mesmo ambiente? Foi com o objetivo de obter dados que auxiliem a responder tal questão que foram estudados os efeitos de linhagens bacterianas aderidas a *Aulacoseira granulata* e *Microcystis aeruginosa*, isoladas a partir de coculturas com a comunidade bacteriana natural do Reservatório de Barra Bonita, sobre o crescimento das duas espécies fitoplanctônicas.

3.3. Material e Métodos

Espécies fitoplanctônicas

A cianobactéria *Microcystis aeruginosa* Kützing (BB005) e a diatomácea (Bacillariophyceae) *Aulacoseira granulata* var. *granulata* (Ehrenberg) Simonsen (BB001), foram isoladas do reservatório hipereutrófico de Barra Bonita, SP, Brasil. Os isolados foram axenizados mantidos na Coleção de Culturas do Departamento de Botânica da Universidade Federal de São Carlos (Centro de Dados Mundial de Microrganismos, WDCM No. 835).

Isolamento das linhagens bacterianas

Linhagens bacterianas aderidas aos filamentos de *Aulacoseira granulata* e às colônias de *Microcystis aeruginosa* foram isoladas a partir de culturas das respectivas espécies fitoplanctônicas em cocultivo com bactérias provenientes do Reservatório de Barra Bonita (São Paulo, Brasil).

A preparação do inóculo bacteriano, as condições de cultivo da diatomácea e da cianobactéria, assim como o fracionamento para a obtenção das bactérias aderidas, estão descritas no Capítulo 1. Em resumo, a separação entre frações em cada espécie fitoplanctônica foi realizada por filtração em membrana de acetato de

celulose com 8 µm de poro, seguida de centrifugação com gradiente de densidade da fração aderida (>8 µm) utilizando Percoll (GE-HealthCare).

Após a centrifugação com gradiente de densidade, as células da espécie fitoplanctônica foram recuperadas com pipetas de Pasteur estéreis e alíquotas dessa fração foram inoculadas em placas de Petri com ágar CPS (Casein, Peptone, Starch) (Regali-Seleghim 1992) para a obtenção das linhagens bacterianas aderidas à cianobactéria e à diatomácea. O ágar CPS isolou maior diversidade de bactérias, provenientes de água de um reservatório eutrófico raso, em comparação ao meio TGE 10% (Triptona, Glicose, Extrato de carne) (Regali-Seleghim 1992). As placas foram incubadas a 23±1 °C e após 5 dias algumas colônias foram reisoladas por estriamento ou espalhamento em placa. As linhagens isoladas foram mantidas em meio sólido por 2 meses e posteriormente armazenadas em glicerol 20% (em meio CPS) a -20 °C até a realização dos experimentos.

Neste trabalho foram utilizadas linhagens isoladas durante o crescimento exponencial de *A. granulata* (9º dia de cultivo) e *M. aeruginosa* (2º e 15º dias de cultivo) (Figura 1.1, Capítulo 1)

Microscopia Eletrônica de Varredura (MEV)

Alíquotas da fração contendo células fitoplanctônicas com bactérias aderidas, separadas como descrito acima, foram armazenadas em glutaraldeído 2% a 4 ºC por 2 a 3 meses até as análises de MEV. Além disso, alíquotas foram inoculadas em meio WC líquido e mantidas por 2 meses, permitindo o crescimento do fitoplâncton com a comunidade bacteriana aderida. Essas culturas também foram utilizadas para MEV. O método para preparação de amostras para MEV está descrito no Capítulo 1.

Seleção das linhagens bacterianas

Dentre as 23 linhagens isoladas, 8 foram selecionadas com base na morfologia da colônia em meio CPS e na posição da banda do produto de amplificação parcial (regiões V3-V5) do gene RNAr 16S após eletroforese em gel de poliacrilamida 6% com gradiente desnaturante (30-60%) (DGGE) (Muyzer et al. 1993) - dados não mostrados. Para a realização do DGGE o DNA foi extraído como descrito no Capítulo 1 e a amplificação do DNA realizada por PCR utilizando os *primers* 341*f* com grampo CG (Muyzer et al. 1993) e 805*r* (Herlemann et al. 2011), para o Domínio Bacteria.

Três linhagens foram isoladas de *M. aeruginosa* (denominadas Bact 14C, Bact 17.3 e Bact 21) e 5 de *A. granulata* (Bact 5A, Bact 6A, Bact 08, Bact 09 e Bact 22.2), no entanto, duas destas (Bact 08 e Bact 09) eram iguais a outras linhagens isoladas da cianobactéria em morfologia e posição da banda, e foram consideradas como sendo originalmente associadas às duas espécies fitoplanctônicas (Tabela 3.1). A presença dessas linhagens associadas às duas culturas foi confirmada pela análise de diversidade por pirossequenciamento apresentada nos capítulos 1 e 2, como será discutido posteriormente.

Efeitos das bactérias sobre o crescimento fitoplanctônico

Cada espécie fitoplanctônica em crescimento exponencial e axênico foi inoculada em 3,5 L de meio WC pH 7,0 suplementado com sílica (concentração final de $Na_2SiO_3.9\ H_2O$ = 56,84 mg L^{-1}) (Gorham et al. 1964). As culturas de cada espécie foram divididas assepticamente em alíquotas de 100 mL em frascos de cultura de 250 mL. Três culturas de cada espécie fitoplanctônica permaneceram como controle (axênicas), enquanto as outras foram inoculadas com as linhagens bacterianas isoladas e selecionadas como descrito anteriormente.

Cada uma das 8 linhagens bacterianas isoladas foi inoculada em culturas da diatomácea e da cianobactéria. Além das linhagens isoladas, também foi testado o efeito conjunto das linhagens originalmente associadas à *A. granulata* (Bact 5A, Bact 6A, Bact 08, Bact 09 e Bact 22.2) e das linhagens originalmente associadas à *M. aeruginosa* (Bact 08, Bact 09, Bact 14C, Bact 17.3 e Bact 21) (Tabela 3.1) sobre o crescimento de ambas as espécies fitoplanctônicas. Para cada tratamento, tréplicas independentes foram preparadas.

Tabela 3.1 – Linhagens bacterianas aderidas isoladas das culturas de *A. granulata* e *M. aeruginosa.* MLAulac e MLMicro indicam os tratamentos "Mistura de linhagens isoladas de *Aulacoseira*" e Mistura de linhagens isoladas de *Microcystis*" respectivamente.

Linhagens	Tratamento	Espécie de isolamento
Bact 5A	Bact 5A	*A. granulata*
Bact 6A	Bact 6A	*A. granulata*
Bact 22.2	Bact 22.2	*A. granulata*
Bact 08	Bact 08	*A. granulata* (M)[1]
Bact 09	Bact 09	*A. granulata* (M)[1]
Bact 14C	Bact 14C	*M. aeruginosa*
Bact 17.3	Bact 17.3	*M. aeruginosa*
Bact 21	Bact 21	*M. aeruginosa*
Bact 5A, Bact 6A, Bact 22.2, Bact 08, Bact 09	MLAulac	
Bact 08, Bact09, Bact 14C, Bact 17.3, Bact 21	MLMicro	

(M)[1] linhagens que também estavam presentes entre as isoladas de *M. aeruginosa* (baseando-se na morfologia das colônias e na posição das bandas no gel de DGGE) e foram, portanto, consideradas como originalmente presentes em ambas as culturas.

Aproximadamente 24 h antes do início do experimento, as linhagens bacterianas foram incubadas em meio CPS líquido a 30 °C. Essas culturas bacterianas foram adicionadas às culturas fitoplanctônicas experimentais em quantidades similares, determinadas por densidade ótica em 600nm (Bruckner et al. 2008) para uma concentração final estimada de $1x10^5$ células/mL. A densidade foi estimada por correlação linear (R^2 ajustado = 0,93) entre a densidade ótica e contagem das células bacterianas coradas com DAPI (Porter & Feig 1980) determinadas para duas das linhagens utilizadas no experimento: Bact 08 e Bact 6A (Figura 3.S1). A regressão obtida foi utilizada para todas as linhagens. No caso das misturas bacterianas, cada linhagem foi adicionada em densidade semelhante para obtenção final de concentração de aproximadamente $1x10^5$ células/mL de cultura fitoplanctônica. Os volumes de cultura bacteriana pipetados foram adicionados de meio CPS para um volume final de 220 µL, para que cada cultura fitoplanctônica recebesse quantidades semelhantes dos componentes do meio de cultura. O mesmo volume de meio CPS estéril foi também adicionado aos controles.

As culturas controle (axênicas) de *A. granulata* e *M. aeruginosa* e culturas adicionadas de bactérias foram incubadas a 23 ± 2 °C sob iluminação de 90±10 µmol fótons m^{-2} s^{-1} em ciclos de 12h:12h claro-escuro e agitação duas vezes ao dia.

Crescimento fitoplanctônico

Todas as culturas foram amostradas assepticamente nos seguintes dias de cultivo: 0, 2, 5, 8, 12 e 15. *M. aeruginosa* foi ainda amostrada no 17º dia, e *A. granulata* nos dias 18, 22, 25 e 29 de cultivo. O crescimento fitoplanctônico foi acompanhado por medidas de fluorescência *in vivo* (Grossart 1999, Sher et al. 2011). Para obtenção da fluorescência máxima (Fm), as amostras das culturas foram adaptadas ao escuro por 30 min., posteriormente adicionou-se DCMU [3-(3,4-diclorofenil)-1,1-dimetiluréia] para uma concentração final de 0,15 mM, as amostras foram homogeneizadas por 30 segundos (Parkhill et al. 2001) e a fluorescência quantificada em um fluorímetro Trilogy, Turner Designs (Sunnyvale, CA) com módulo de clorofila *in vivo*.

As taxas de crescimento foram calculadas para cada réplica por meio da equação (ln(Fdf)-ln(Fdi))/Δt, sendo ln o logaritmo neperiano, Fdf a fluorescência máxima em um ponto ao final do crescimento exponencial, Fdi a fluorescência máxima no dia 0 e Δt a variação do tempo entre os pontos de amostragem, em dias.

Para obtenção do rendimento máximo utilizou-se o ponto da curva de cada tratamento onde a média entre as tréplicas apresentou o maior valor de fluorescência (em unidade relativa de fluorescência – RFU). Para as culturas de *A. granulata* com a linhagem Bact 6A utilizou-se o 8° dia e com a mistura de bactérias de *A. granulata* (MLAulac) utilizou-se o 12° dia, embora tenha ocorrido um pequeno aumento da fluorescência no 2° dia de cultivo desta última.

A significância das diferenças entre os tratamentos e a respectiva cultura controle foi testada por meio do teste t não pareado ou teste t não pareado corrigido por Welch quando as variâncias eram significativamente diferentes. O nível de significância foi fixado em α=0,05.

Análises de nutrientes

Nitrato e fosfato dissolvidos foram quantificados em um sistema de cromatografia de íons Dionex ICS-1100 (Ion Chromatography System, Thermo Scientific) em amostras filtradas em filtros de 0,45 µm de poro.

Identificação das linhagens bacterianas

As linhagens bacterianas selecionadas para o experimento foram identificadas pelo sequenciamento de parte do gene RNAr 16S, utilizando os *primers*

341*f* e 805*r* (Herlemann et al. 2011). O DNA das linhagens isoladas foi extraído a partir de *pellets* obtidos de culturas puras, adicionados de 100 µL de solução de lise (50 ng μL^{-1} de proteinase K em tampão TE – Tris 10mM, EDTA pH 8,0) e 0,2g de pérolas de vidro (150-212 µm, Sigma-Aldrich) e submetidos a banhos térmicos de 55 e 80 °C durante 15 min cada. Posteriormente as amostras foram levadas ao vórtex (2500 rpm) por 10 s, centrifugadas a 16000 x*g* por 15 min e o sobrenadante de cada amostra foi imediatamente utilizado nas reações de PCR.

Cada linhagem foi amplificada em reações de 25 µL utilizando 2U de Taq DNA polimerase (Invitrogen) e 0.4 µM de cada *primer* e 0,2 mM de dNTPs. O programa de amplificação utilizado foi desnaturação inicial a 94 ºC por 4 minutos; 30 ciclos de 94 ºC por 1 minuto, 55 ºC por 45 segundos e 72 ºC por 2 minutos; extensão final a 72 ºC por 5 minutos e finalização com resfriamento a 4ºC. A amplificação foi verificada por eletroforese em gel de agarose 1% em tampão Tris-Borato-EDTA 1x (TBE).

Após a amplificação, o produto de PCR foi purificado com PEG 8000 (Polietilenoglicol) 20% e NaCl 1M (Lis & Schleif 1975, Rosenthal et al. 1993) e sequenciado na Macrogen (Korea) utilizando-se o primer *forward*.

As sequências obtidas (mínimo de 334 pb) foram classificadas utilizando Ribosomal Database Project – RDP (Wang et al. 2007). O RDP foi também utilizado para a obtenção de sequências com maior similaridade (>97%) às linhagens isoladas neste estudo. Além disso, essas sequências foram comparadas às sequências (complementares e reversas) obtidas por pirossequenciamento das culturas de onde as linhagens foram inicialmente isoladas (capítulos 1 e 2). A comparação com as sequências do pirossequenciamento foi realizada utilizando-se o comando pick_otus.py no software QIIME (Caporaso et al. 2010), com *cutoff* de 97%.

As sequências foram depositadas no GenBank sob o número de acesso (ainda não depositadas).

3.4. Resultados

Identificação das bactérias isoladas

Todas as linhagens isoladas pertencem ao Filo *Proteobacteria*, sendo 4 linhagens pertencentes à Classe *Alphaproteobacteria,* três à Classe *Betaproteobacteria* e 1 à *Gammaproteobacteria* (Tabela 3.2). As linhagens foram classificadas como pertencentes a 4 famílias e possivelmente a 5 gêneros (Tabela 3.2). As sequências de duas linhagens isoladas, a Bact 09 (isolada de *A. granulata*) e a Bact 14C (isolada de *M. aeruginosa*) apresentaram 99% de similaridade, desta maneira, tratam-se de uma mesma UTO.

Todas as bactérias identificadas apresentaram alguma UTO correspondente nos dados de pirossequenciamento (Tabela 3.3), no entanto, algumas destas UTOs não foram abundantes (Figura 1.5, Capítulo 1) ou recorrentes (Figuras 2.5 e 2.6, Capítulo 2) nas culturas de onde as linhagens foram isoladas. Apenas as linhagens Bact 6A e Bact 21 não ocorreram em ambas as espécies fitoplanctônicas de acordo com os resultados do pirossequenciamento (Tabela 3.3), e mesmo nas respectivas espécies hospedeiras, a proporção de leituras foi baixa (dados não mostrados).

A linhagem Bact 5A correspondeu à OTU #2396 (Figura 1.5, Capítulo 1) que ocorreu associada, com maiores proporções na fração aderida, a *A. granulata* e a *M. aeruginosa.* Esta linhagem foi classificada como pertencente ao gênero *Rhizobium*.

Ambas as linhagens Bact 17.3 e Bact 22.2, isoladas respectivamente de *M. aeruginosa* e *A. granulata*, foram classificadas como pertencentes ao gênero *Acidovorax.* A linhagem Bact 17.3 foi semelhante à UTO #1485 que ocorreu em ambas as culturas fitoplanctônicas nos 2 experimentos com a comunidade total de Barra Bonita (Capítulo 1, Figura 1.5 e Capítulo 2, Figuras 2.5 e 2.6). Já a linhagem Bact 22.2 está relacionada à UTO #1880, que ocorreu em maior proporção nas bactérias aderidas de *A. granulata* (Figura 2.5, Capítulo 2).

A linhagem Bact 08, relacionada ao gênero *Pelomonas*, também ocorreu associada a ambas as espécies fitoplanctônicas no experimento de onde foi isolada (experimento 2, Figuras 2.5 e 2.6, Capítulo 2).

As linhagens 09 e 14C foram classificadas como relacionadas à família *Caulobacteraceae* e também ocorreram em ambas as culturas das espécies

fitoplanctônicas de acordo com os dados de pirossequenciamento (Figura 1.5, Capítulo 1, UTO # 4085), em maiores proporções na fração aderida.

Efeito bacteriano sobre o crescimento de M. aeruginosa

Nenhuma das linhagens isoladas apresentou efeito significativo sobre a taxa de crescimento de *M. aeruginosa* (Figura 3.1) ou sobre o rendimento máximo de crescimento desta cianobactéria (Figura 3.2). No entanto, pode-se observar nas curvas de crescimento que em alguns tratamentos o rendimento máximo ocorreu em um dia de amostragem anterior ao de rendimento máximo do controle (Figura 3.3). Além disso, a fase de senescência foi mais acentuada para as culturas inoculadas com as linhagens bacterianas, com diferenças significativas ($p<0,05$) na fluorescência no 17° dia de cultivo em todos os tratamentos para *M. aeruginosa*, exceto com Bact 22.2 e MLAulac que se mostraram iguais ao controle.

O final da fase de crescimento exponencial em todas as culturas de *M. aeruginosa* foi acompanhado pela depleção ou baixas concentrações de nitrato e fosfato (Tabela 3.S1). As concentrações iniciais médias de nitrato e fosfato nas culturas controle foram de 47,02±2,38 e 2,20±0,25 $mg.L^{-1}$ respectivamente, enquanto no 8º dia, as concentrações foram de 3,06±3,79 mg de nitrato.L^{-1} de e 0,41±0,33 mg de fosfato.L^{-1}. Já nos tratamentos, os valores iniciais de nitrato variaram de 44,51±10,73 $mg.L^{-1}$ a 59,79±0,17 $mg.L^{-1}$ e de fosfato variaram entre 2,29±0,50 e 2,89±0,14 $mg.L^{-1}$. No 8º dia de cultivo, os valores de nitrato nas culturas inoculadas com bactérias foram de 0,01±0,02 a 6,83±5,19 $mg.L^{-1}$, enquanto os valores de fosfato variaram entre 0,00±0,00 e 0,54 ±0,41 $mg.L^{-1}$.

Tabela 3.2 – Classificação das sequências isoladas segundo RDP naive Bayesian rRNA Classifier. O limite de confiança ("confidence threshold", %) é mostrado para todos os gêneros. Para outros níveis taxonômicos apenas limites de confiança menores que 80% são mostrados*.

linhagem	Classe (*Proteobacteria*)	Ordem	Família	Gênero
Bact-09	Alphaproteobacteria	Caulobacterales (67%)	Caulobacteraceae (67%)	Asticcacaulis (56%)
Bact-14C		Caulobacterales (67%)	Caulobacteraceae (67%)	Asticcacaulis (56%)
Bact-5A		Rhizobiales	Rhizobiaceae	Rhizobium (98%)
Bact-21			Rhizobiaceae (49%)	Rhizobium (49%)
Bact-17.3	Betaproteobacteria	Burkholderiales	Comamonadaceae	Acidovorax (100%)
Bact-22.2				Acidovorax (92%)
Bact-08				Pelomonas (55%)
Bact-6A	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas (46%)

*O limite de confiança de 80% é usado pelo RDP para garantir uma classificação confiável até o nível de gênero (Wang et al. 2007, Claesson et al. 2009).

Tabela 3.3 – Linhagens isoladas das culturas de *A. granulata* (A) e *M. aeruginosa* (M) e as respectivas linhagens mais próximas no RDP e nos dados de pirossequenciamento das culturas de onde foram isoladas.

Linhagem	Espécie de isolamento	Linhagem mais próxima[2] no RDP (Núm. Acesso)		#UTO mais próxima[2] e ocorrência de acordo com dados de pirossequenciamento do experimento de onde foram isoladas		
Bact 5A	A	uncultured *Rhizobium* sp.	(JX523040)	#2396	Capítulo 1, Figura 1.5	A e M
Bact 6A	A	*Pseudomonas fluorescens*	(JN679857)	#4250	não mostrado	A
Bact 22.2	A	uncultured *Acidovorax* sp.	(DQ234163)	#1880	Capítulo 2, Figura 2.5	A
Bact 08	A (M)[1]	*Pelomonas* sp.	(KC464825)	#3615	Capítulo 2, Figs 2.5 e 2.6	A e M
Bact 09	A (M)[1]	*Asticcacaulis* sp	(HQ652555)	#4085	Capítulo 1, Figura 1.5	A e M
Bact 14C	M	*Asticcacaulis* sp	(HQ652555)	#4085	Capítulo 1, Figura 1.5	A e M
Bact 17.3	M	*Acidovorax* sp.	(HM214572)	#1485	Capítulos 1 e 2, Figs 1.5, 2.5, 2.6	A e M
Bact 21	M	*Rhizobium* sp.	(EF649779)	#2147	não mostrado	M

[1] considerada como sendo originalmente também isolada de *M. aeruginosa*; [2] similaridade >97%

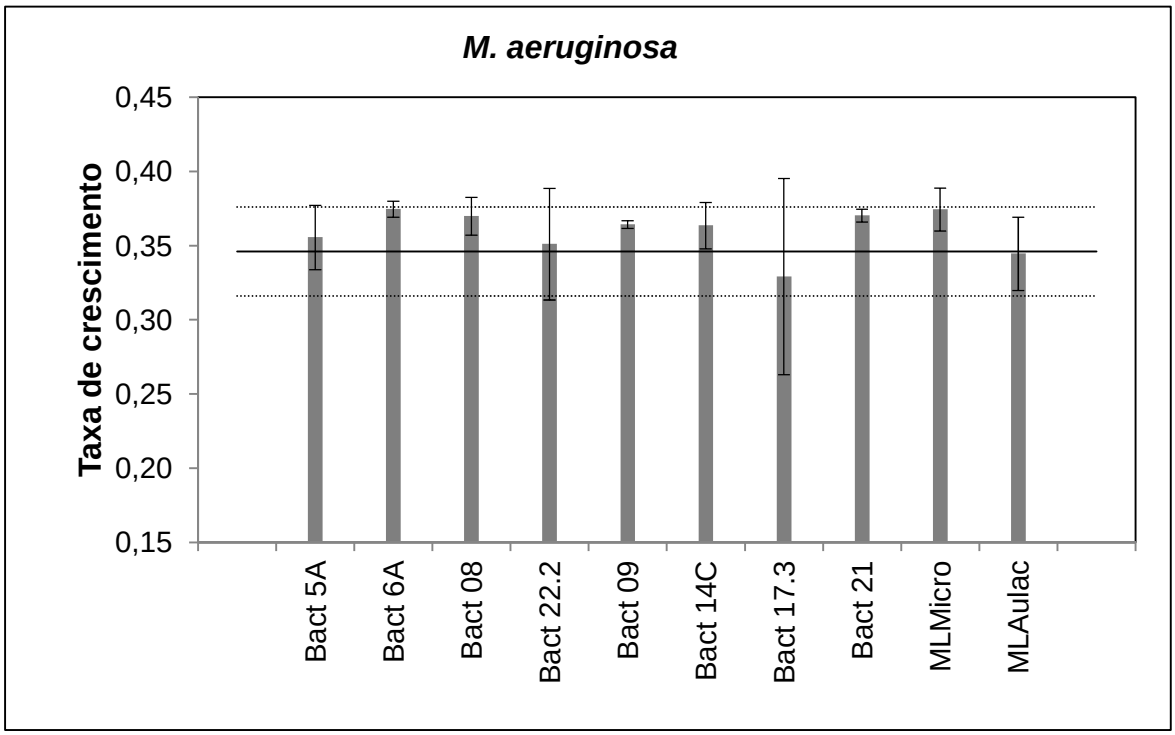

Figura 3.1 – Taxas de crescimento (RFU/dia) de *M. aeruginosa* em culturas binárias com as diferentes linhagens bacterianas e em culturas com mistura de linhagens originalmente associadas a *M. aeruginosa* (MLMicro) e originalmente associadas à *A. granulata* (MLAulac). A linha sólida representa a taxa de crescimento média e as linhas tracejadas representam o desvio padrão (n=3) das culturas controle (axênicas). As barras de erros representam os desvios padrões dos tratamentos (n=3).

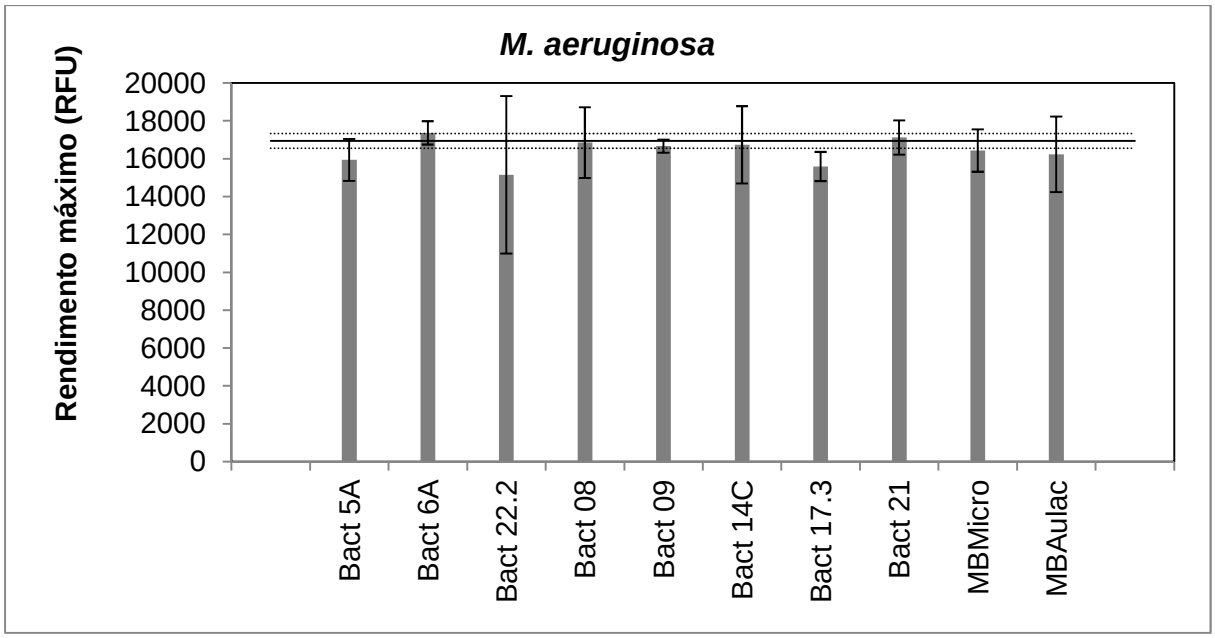

Figura 3.2 – Rendimento Máximo (em unidades relativas de fluorescência - RFU) de *M. aeruginosa* em culturas binárias com as diferentes linhagens bacterianas e em culturas com mistura de linhagens originalmente associadas a *M. aeruginosa* (MLMicro) e originalmente associadas à *A. granulata* (MLAulac). A linha sólida representa o rendimento máximo e as linhas tracejadas representam o desvio padrão (n=3) das culturas controle (axênicas). As barras de erros representam os desvios padrões de n=3.

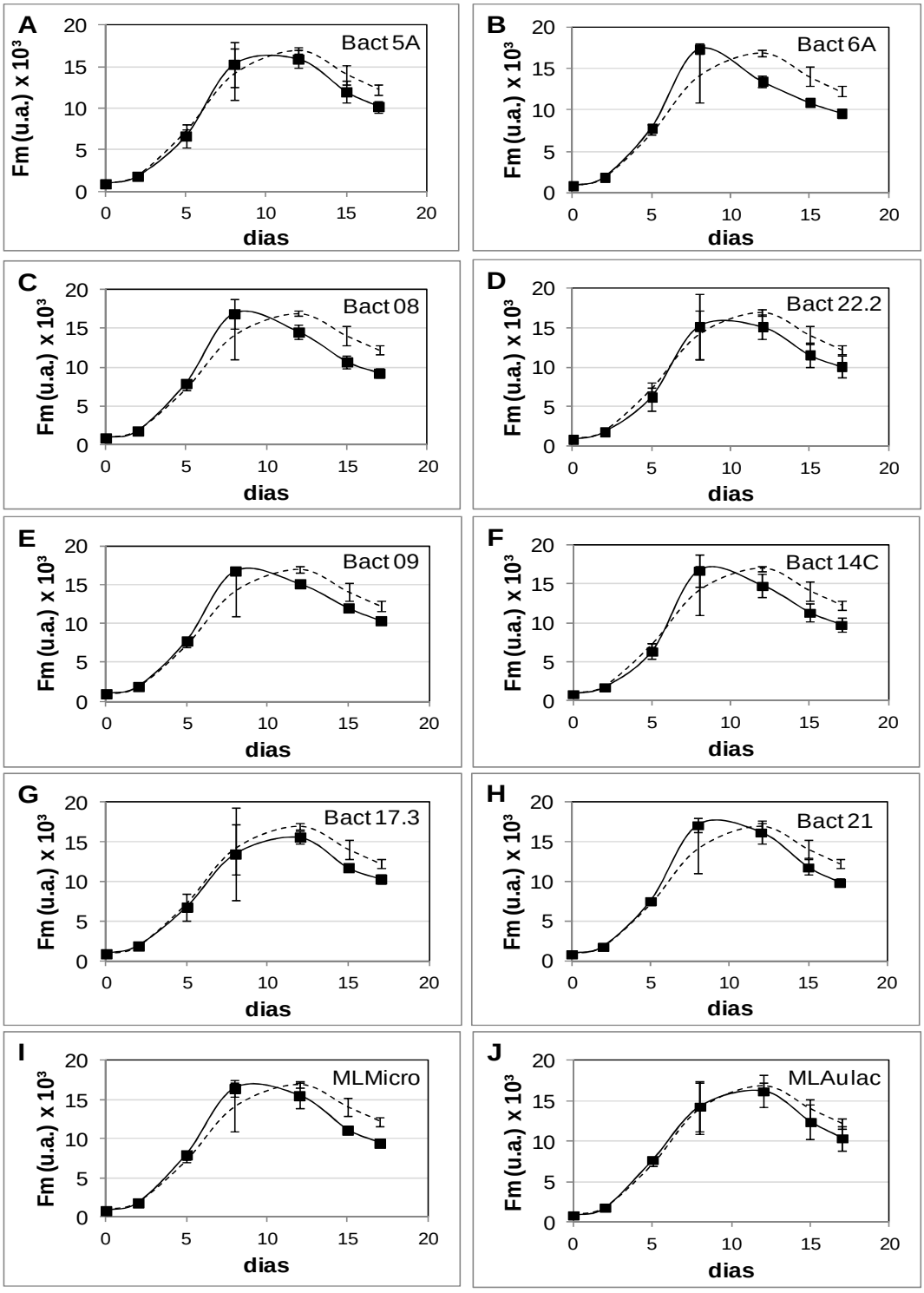

Figura 3.3 – Curvas de crescimento de *M. aeruginosa* em culturas binárias com diferentes linhagens bacterianas (A-H) e em culturas com mistura de linhagens originalmente associadas a *M. aeruginosa* (MLMicro) e originalmente associadas à *A. granulata* (MLAulac). As linhas tracejadas representam as culturas controle (axênicas) para *M. aeruginosa*. As barras de erros representam os desvios padrões de n=3.

***Efeito bacteriano sobre o crescimento de* A. granulata**

Diferente do resultado obtido para a *Cyanobacteria*, a crescimento de *A. granulata* foi significativamente menor na maior parte dos tratamentos. Apenas as linhagens Bact 17.3 e Bact 21, que foram isoladas de *M. aeruginosa*, não apresentaram efeito significativo sobre a taxa de crescimento da diatomácea (Figura 3.4). Estas linhagens também não afetaram o rendimento máximo de *A. granulata*, embora as culturas com a linhagem Bact 17.3 tenham apresentado uma média menor do que o controle (Figura 3.5).

O rendimento máximo de crescimento da diatomácea foi significativamente mais baixo em cocultura com as linhagens Bact 6A, Bact 09 e para os tratamentos com misturas das linhagens, tanto originalmente associadas à *A. granulata* como associadas à *M. aeruginosa* (Figura 3.5). Embora as linhagens Bact 09 e Bact 14C sejam a mesma UTO, elas apresentaram efeitos distintos sobre a diatomácea, com diferença significativa no rendimento máximo ($p<0,05$) entre os dois tratamentos.

É possível verificar pelas curvas de crescimento (Figura 3.6) que algumas linhagens e a mistura de bactérias isoladas originalmente de *M. aeruginosa* aumentaram consideravelmente a fase lag de crescimento da diatomácea. A linhagem Bact 6A (*Pseudomonadaceae*) apresentou o efeito mais drástico, com morte já detectada no 2° dia. A mistura de bactérias isoladas originalmente de *A. granulata*, que continha a Bact 6A, permitiu um leve crescimento da diatomácea até o segundo dia, mas posteriormente também apresentou efeito algicida (Figura 3.6).

Apesar das diferenças no crescimento entre controle e tratamentos, as concentrações de nitrato e fosfato não foram limitantes, nem mesmo para o controle (Tabela 3.S2). Os valores de nitrato para o controle nos dias 0 e 12 foram, respectivamente, 49,46±7,88 e 55,53±6,96 $mg.L^{-1}$, enquanto os valores de fosfato nos mesmos dias foram 2,21±0,57 e 2,45±0,33 $mg.L^{-1}$, respectivamente. Nos tratamentos, as concentrações de nitrato no dia 0 variaram entre 51,41±5,33 e 64,53±3,97 $mg.L^{-1}$. As concentrações de fosfato iniciais variaram de 2,37±0,24 a 2,96±0,18 $mg.L^{-1}$. Os dias finais do crescimento exponencial ou os dias com maior rendimento no crescimento variaram entre os tratamentos (Figura 3.5), mas considerando-se esses dias, os valores de nitrato variaram de 31,99±10,14 a 64,09±0,62 $mg.L^{-1}$ e os valores de fosfato, de 2,98±0,17 a 1,63±0,27 $mg.L^{-1}$.

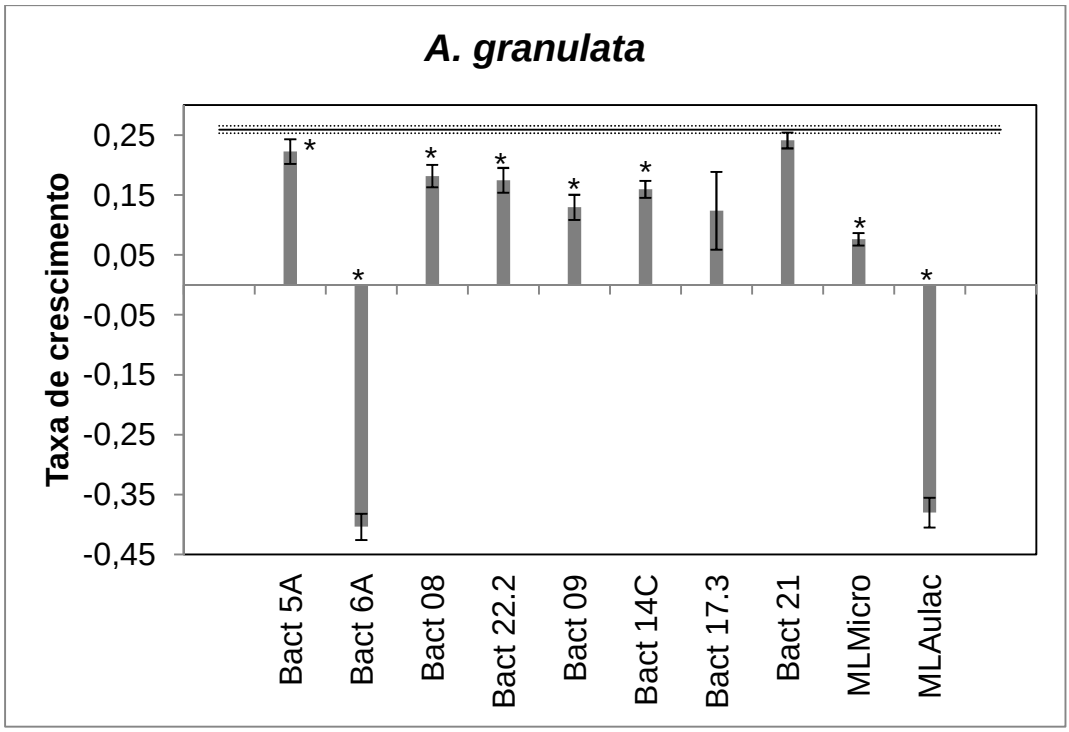

Figura 3.4 – Taxas de crescimento (RFU/dia) de *A. granulata* em culturas binárias com as diferentes linhagens bacterianas e em culturas com mistura de linhagens originalmente associadas a *M. aeruginosa* (MLMicro) e originalmente associadas a *A. granulata* (MLAulac). A linha sólida representa a taxa de crescimento média e as linhas tracejadas representam o desvio padrão (n=3) das culturas controle (axênicas). Os asteriscos representam diferenças significativas em relação ao controle ($p<0,05$) e as barras de erros representam os desvios padrões de n=3.

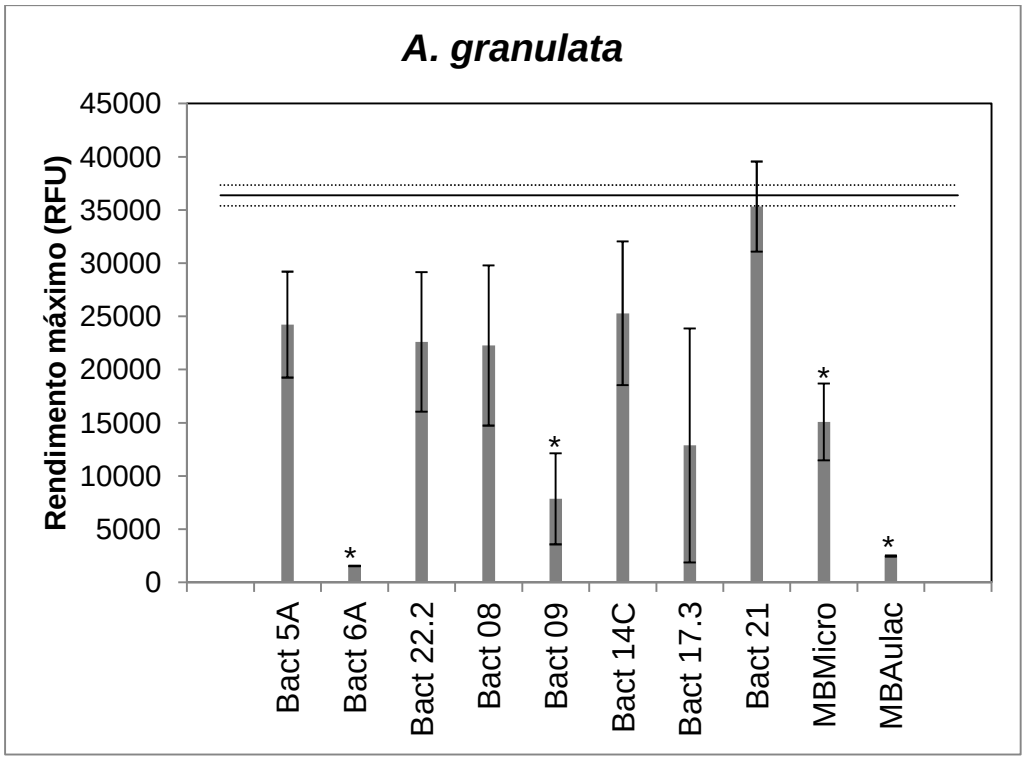

Figura 3.5 – Rendimento Máximo (em unidades relativas de fluorescência - RFU) de *A. granulata* em culturas binárias com as diferentes linhagens bacterianas e em culturas com mistura de linhagens originalmente associadas a *M. aeruginosa* (MLMicro) e originalmente associadas à *A. granulata* (MLAulac). A linha sólida representa o rendimento máximo das culturas controle (axênicas) e as linhas tracejadas representam o desvio padrão (n=3). Os asteriscos representam diferenças significativas em relação ao controle ($p<0,05$). As barras de erros representam os desvios padrões de n=3.

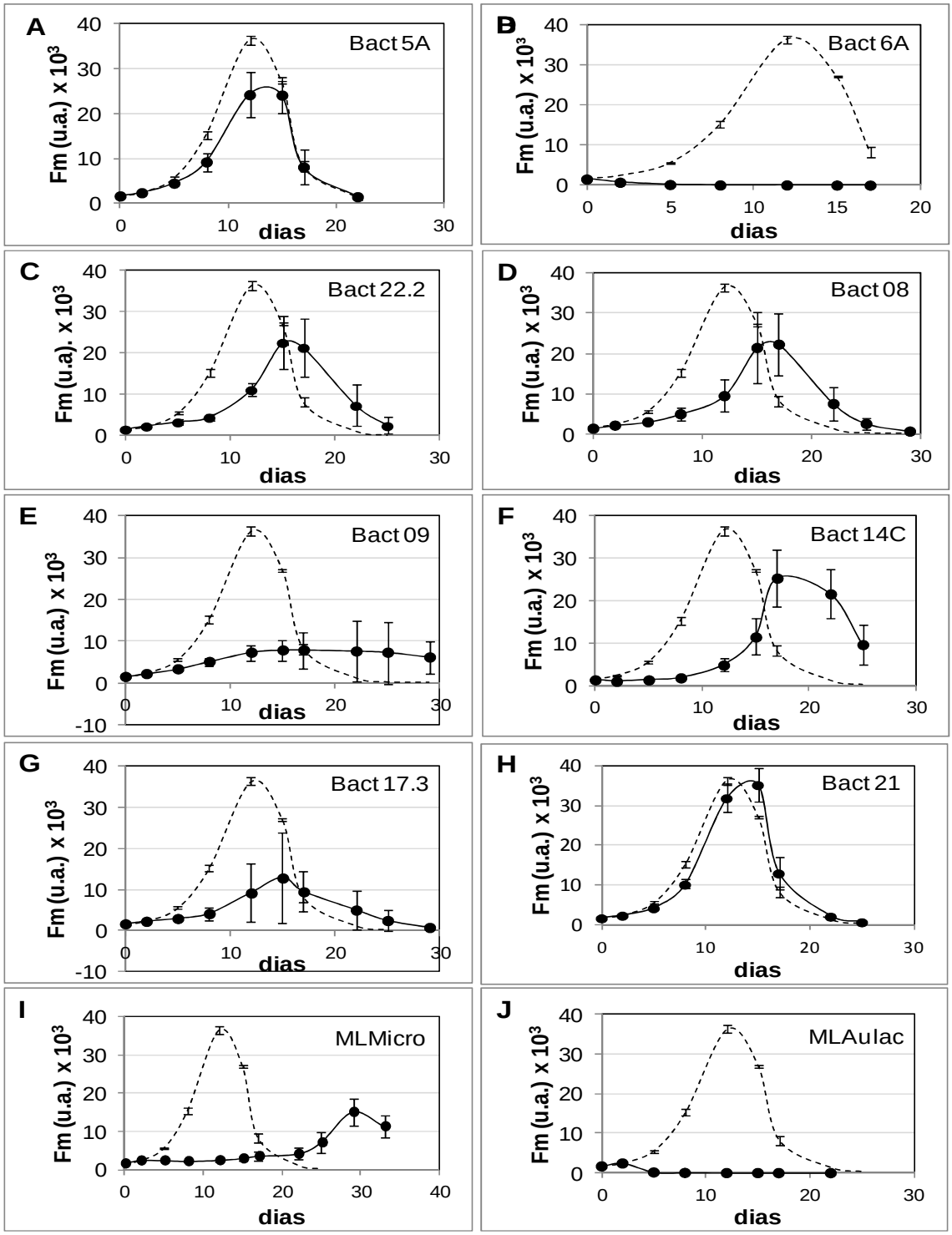

Figura 3.6 – Curvas de crescimento de *A. granulata* em culturas binárias com diferentes linhagens bacterianas (A-H) e em culturas com mistura de linhagens originalmente associadas a *M. aeruginosa* (MLMicro) e originalmente associadas à *A. granulata* (MLAulac). As linhas tracejadas representam as culturas controle (axênicas) para *A. granulata.* As barras de erros representam os desvios padrões de n=3.

3.5. Discussão

Efeito das bactérias isoladas sobre o crescimento de M. aeruginosa

Para alguns tratamentos de *M. aeruginosa*, o ponto de amostragem com maior rendimento foi anterior ao de maior rendimento do controle, desta maneira, existe a possibilidade de que essas linhagens tivessem um efeito positivo no crescimento da cianobactéria em caso de maior suprimento ou suprimento contínuo de nutrientes para a espécie fitoplanctônica. No entanto, em culturas estanques, com a concentração de nutrientes do meio WC, não foi possível verificar diferenças significativas nas taxas de crescimento e rendimento final nas culturas de *M. aeruginosa.* Shi et a.l (2009) estudaram o efeito de 14 linhagens bacterianas isoladas de colônias de *M. aeruginosa* sobre o crescimento da cianobactéria e encontraram 4 com efeitos inibitórios, em que a biomassa final com bactéria foi menor do que o controle, 4 linhagens aumentaram significativamente a biomassa final, enquanto 6 não apresentaram diferença significativa em relação ao controle. Entre as linhagens bacterianas utilizadas por Shi et a.l (2009) , três eram relacionadas ao gênero *Pseudomonas,* duas elas apresentaram efeitos positivos e uma apresentou negativo sobre o crescimento da cianobactéria. Esses autores não consideraram os efeitos inibidores como 'algicidas', pois não causaram uma queda abrupta na biomassa da cianobactéria (Salomon et al. 2003) e argumentaram que a adesão de bactérias 'algicidas' parece não ocorrer em colônias saudáveis de *M. aeruginosa.*

A única diferença significativa no crescimento da cianobactéria ocorreu ao final da fase estacionária/senescência para a maioria das linhagens, exceto para a Bact 22.2, isolada de *A. granulata* e que só ocorreu na diatomácea de acordo com os dados de pirossequenciamento (Tabela 3.3), e para mistura de linhagens isoladas de *A. granulata*. Desta maneira, pode haver maior susceptibilidade da cianobactéria à competição por fósforo (Danger et al. 2007) ou ao 'ataque' por outras bactérias durante a fase de senescência (Manage et al. 2000).

***Efeito das bactérias isoladas sobre o crescimento de* A. granulata**

A maior parte das linhagens alterou significativamente a taxa de crescimento ou o rendimento máximo de *A. granulata*. Sabe-se que sob limitação de fósforo, as bactérias podem ter feito negativo sobre o crescimento de diatomáceas (Brussaard & Riegman 1998), no entanto, nenhum dos tratamentos esteve sob limitação de nutrientes, indicando que outros mecanismos como produção de antibióticos ou enzimas possam estar envolvidos (Sigee 2005).

Apenas as linhagens Bact 17.3 e Bact 21, que foram isoladas de *M. aeruginosa*, não alteraram significativamente a taxa de crescimento da diatomácea. Ambas as linhagens também não afetaram o rendimento final, embora as culturas com a linhagem Bact 17.3 tenham apresentado uma media menor do que o controle. A linhagem Bact 17.3, classificada como pertencente ao gênero *Acidovorax*, ocorreu associada às duas espécies fitoplanctônicas nos experimentos de onde foi isolada, enquanto que a Bact 21, relacionada ai gênero *Rhizobium*, ocorreu apenas em *M. aeruginosa.* Apesar de a Bact 21 ter sido a única linhagem que ocorreu apenas em *M. aeruginosa*, foi a que apresentou menor efeito sobre *A. granulata*, no entanto, a proporção de sequências dessa linhagem nas culturas de *M. aeruginosa* de onde foi isolada, foi muito baixa (dados não mostrados), o que pode indicar pouca interação com a cianobactéria.

O rendimento máximo de crescimento foi significativamente mais baixo apenas em cocultura com as linhagens Bact 6A, Bact 09 e para os tratamentos com misturas das linhagens, tanto as originalmente associadas à *A. granulata* como associadas à *M. aeruginosa*. Bruckner et al. (2008) também encontrou efeitos distintos de bactérias isoladas e de misturas de bactérias sobre a diatomácea *Cymbella microcephala*, que apresentou maior crescimento quando em presença de mistura de bactérias epilítcas do que em culturas binárias com cada bactéria.

A linhagem Bact 6A, relacionada ao gênero *Pseudomonas* (e mais próxima de uma *P. fluorescens*) ocorreu apenas em *A. granulata.* Shi et al. (2009) encontraram bactérias do gênero *Pseudomonas* com efeitos deletérios e outras com efeitos benéficos à *M. aeruginosa*. A espécie *P. fluorescens* já foi descrita como promotora de crescimento em vegetais superiores (Andrews & Harris 2000). No entanto, essa linhagem causou um declínio acentuado na biomassa da diatomácea, podendo ser classificada como uma linhagem algicida (Salomon et al. 2003) para *A. granulata*.

Provavelmente a Bact 6A foi também responsável pelo mesmo efeito no tratamento MLAulac, que apresentou um leve crescimento até o segundo dia e depois morreu, o que poderia ser explicado pelo menor número de células iniciais da Bact 6A neste tratamento em relação ao tratamento em culturas binárias. Embora tenha apresentado um efeito bastante importante, essa linhagem foi detectada em baixas proporções nas culturas de onde foi isolada (dados não mostrados), podendo não representar uma linhagem importante no ambiente natural.

A linhagem Bact 09 foi isolada de *A. granulata*, mas ocorreu também em *M. aeruginosa* nas culturas originais, e está relacionada à ordem *Caulobacterales.* Embora a linhagem Bact 14C tenha sido identificada como sendo a mesma UTO da Bact 09, elas afetaram o rendimento máximo da diatomácea de maneiras diferentes, o que poderia ser explicado por densidade bacteriana diferente nos dois tratamentos, ou seja, por erro metodológico. No entanto, elas foram isoladas de espécies fitoplanctônicas diferentes, e mesmo com regiões do gene RNAr 16S indicando uma mesma UTO, podem apresentar modificações em regiões menos conservadas do DNA, resultando em funções diferentes (Palys et al. 1997, Acinas et al. 2004). Além disso, as linhagens foram identificadas utilizando um fragmento pequeno (~340 pb) do gene RNAr 16S. Portanto, contagens bacterianas e estudo sobre as funções dessas linhagens ou sequenciamento de outras regiões do DNA das bactérias podem ajudar a esclarecer as diferenças entre os tratamentos. Alguns membros dessa ordem, incluindo o gênero *Asticcacaulis,* relacionado a esta linhagem, compõem o grupo de “Bactérias Prostecadas Dimórficas” (DPB, do inglês Dimorphic Prosthecate Bacteria), que possuem como característica a variação na morfologia das colônias de linhagens isoladas a partir de uma única linhagem (Poindexter 2006), o que pode explicar a escolha da mesma linhagem apesar da triagem inicial por colônias com morfologias diferentes.

As DPB possuem uma reprodução que resulta em duas células morfologicamente diferentes, uma séssil com prosteca que permanece aderida e a outra móvel, com flagelo (Poindexter 2006). A UTO correspondente a essas linhagens no experimento de onde foram isoladas ocorreu em maior proporção na fração ‘aderidas’ (Capítulo 1, Figura 1.5) e imagens de microcopia eletrônicas das culturas originais, mostraram algumas bactérias com prosteca em culturas de *A. granulata* (Figura 3.S2). De acordo com Poindexter (2006) o gênero *Asticcacaulis* é

raramente isolado em culturas, mas membros do DPB, especialmente *Caulobacter* são frequentemente encontrados como epibiontes de algas, com preferência para diatomáceas, geralmente com benefício mútuo entre a alga e a bactéria (Kaczmarska et al. 2005, Poindexter 2006). No entanto, foram encontrados efeitos negativos sobre a taxa de crescimento de *A. granulata* e, para uma das linhagens, também sobre o rendimento máximo. Klaveness (1982) encontrou uma bactéria do gênero *Caulobacter* aderida a *Cryptomonas*, também sem efeito sobre seu crescimento.

Ambas as misturas de bactérias, MLMicro e MLAualc apresentaram efeitos significativos na taxa de crescimento e no rendimento de *A. granulata*. No caso da MLAulac, possivelmente o principal efeito tenha sido devido à presença da linhagem algicida Bact 6A.

As linhagens Bact 08 e Bact 09 estavam presentes em ambos os tratamentos. Uma vez que a linhagem Bact 09 apresentou efeito isoladamente sobre o rendimento da diatomácea, ela pode ter sido uma das principais responsáveis para a o menor rendimento no tratamento MLMicro. No entanto, a taxa de crescimento de *A. granulata* com a mistura MLMicro (Bact 08, 09, 14C, 17.3 e 21), foi menor do que com as linhagens isoladas, uma vez que a fase lag das culturas de *A. granulata* foi bastante estendida, o que pode indicar um efeito sinérgico das bactérias em mistura.

As linhagens Bact 5A, Bact 22.2, Bact 08, Bact 14C e Bact 17.3, apresentaram efeito negativo significativo apenas sobre a taxa de crescimento, no entanto, embora não significativo, o rendimento máximo foi menor no tratamento com essas linhagens isoladas do que o controle, o que em ambiente natural, pode significar uma desvantagem competitiva.

Sob o efeito das *Rhizobiaceae* (*Rhizobium* ou relacionada à *Rhizobium*, respectivamente Bact 5A e Bact 21) as culturas de *A. granulata* apresentaram as médias de taxa de crescimento mais próximas da média do controle, embora uma tenha apresentado efeito negativo significativo e a outra não. O efeito das *Commamonadaceae* (Bact 17.3, 22.2, ambas *Acidovorax,* e Bact 08, relacionada à *Pelomonas*) foi variável em significância, embora em todas as linhagens, a taxa de crescimento e rendimento máximo tenham sido inferiores ao controle. O gênero *Acidovorax* contém linhagens com atividades algicidas contra a diatomácea *Stephanodiscus hantzschii* (Kang et al.). De acordo com Sher et al. (2011), tanto

interações antagônicas como estimulantes podem revelar uma assinatura filogenética, com espécies mais relacionadas causando efeitos semelhantes em culturas de *Prochlorococcus*. No entanto, não foi possível observar esse padrão neste estudo.

Possíveis efeitos ecológicos

Os resultados mostraram que *Microcystis aeruginosa* é pouco susceptível a efeitos deletérios das linhagens abordadas neste estudo, isoladamente ou em misturas. Mesmo a linhagem Bact 6A, que foi letal para *Aulacoseira granulata*, teve pouca interferência no crescimento da cianobactéria. É interessante notar que única linhagem que não apresentou nenhum efeito sobre a diatomácea foi a Bact 21, isolada de *M. aeruginosa*, com poucas leituras nos dados de pirossequenciamento e que não ocorreu em *A. granulata.*

No entanto, a maior parte das bactérias isoladas de *M. aeruginosa*, que apresentaram maior abundância do que a Bact 21 nas culturas com a comunidade bacteriana total, afetou significativamente o crescimento de *A. granulata*, sendo que o maior efeito foi observado para a mistura das bactérias. Além disso, o pirossequenciamento das culturas de onde as linhagens foram isoladas (Figura 1.5, capítulo 1) revelou que a linhagem 5A, isolada inicialmente somente de *A. granulata,* foi mais abundante associada a *M. aeruginosa* do que em *A. granulata*. Assim, não somente um *bloom* de *A. granulata*, mas também um *bloom* de *M. aeruginosa,* pode estimular o crescimento das bactérias que afetam o crescimento da diatomácea.

Desta maneira, as bactérias associadas à cianobactéria durante um *bloom* natural, podem reduzir a capacidade competitiva da diatomácea, ou mesmo prevenir seu rápido crescimento e formação de *bloom* logo após o declínio de um *bloom* da cianobactéria. Isso permitiria que *M. aeruginosa* pudesse formar um novo florescimento quando as condições se tornassem favoráveis novamente, com menor competição de outra espécie fitoplanctônica (neste estudo, podemos afirmar isso apenas para *A. granulata*). Kang et al. (2008) também sugerem o papel inibitório de bactérias associadas ao fim de *blooms* de *Stephanodiscus* e *Perinidinium* na prevenção de *bloom* de novas espécies.

Apesar da coocorrência no ambiente natural e da possível baixa competição entre as espécies fitoplanctônicas estudadas, uma vez que a diatomácea ocorre em

profundidades maiores (Vieira et al. 2013), em casos de condições desfavoráveis para o crescimento da cianobactéria, o efeito inibitório das bactérias associadas à *M. aeruginosa* poderia evitar que *blooms* da diatomácea se desenvolvessem rapidamente. Além disso, esse efeito pode ocorrer também sobre outras espécies fitoplanctônicas competidoras, porém, mais estudos são necessários para comprovação.

Utilizando a comunidade bacteriana total de Barra Bonita, também se observaram efeitos negativos no crescimento da diatomácea e nenhum efeito sobre a cianobactéria (Capítulo 1). No entanto, os efeitos foram principalmente no rendimento total e não na taxa de crescimento, como observado no presente estudo. Uma possível explicação é a concentração inicial das bactérias possivelmente deletérias (Salomon et al. 2003), ou mesmo o efeito benéfico de outras linhagens associadas à *A. granulata,* seja por competição entre as bactérias ou por degradação de possíveis compostos inibitórios. No entanto, com as bactérias isoladas, pudemos demonstrar que as linhagens que cresceriam em *blooms* de *M. aeruginosa* e não somente as associadas a *blooms* de *A. granulata* podem inibir o crescimento da diatomácea.

Esse efeito favorável à cianobactéria em quase todos os tratamentos pode indicar menor susceptibilidade de *M. aeruginosa* aos efeitos deletérios dos compostos produzidos pelas bactérias associadas, devido à maior adaptação/seleção entre a cianobactéria e comunidade bacteriana do Reservatório de Barra Bonita, uma vez que esta espécie predomina neste ambiente durante quase todo o ano (Vieira et al. 2013). Linhagens isoladas de colônias que apresentaram efeitos negativos no crescimento de *M. aeruginosa* são conhecidas (Shi et al. 2009), no entanto, na maior parte dos casos as cianobactérias parecem coexistir em equilíbrio positivo com as bactérias epífitas e não como antagonistas (Sigee 2005). Casamatta e Wickstrom (2000) mostraram que bactérias isoladas de *blooms* de *M. aeruginosa* apresentavam maior quimiotaxia e maior resistência aos compostos produzidos pela cianobactéria do que as bactérias isoladas de locais onde os *blooms* de *M. aeruginosa* não eram frequentes, sugerindo alguma adaptação da comunidade bacteriana. Quimiotaxia de bactérias com efeitos benéficos sobre espécies fitoplanctônicas (Lupton & Marshall 1981) e deletérios (Lovejoy et al. 1998) já foram reportadas.

Os resultados sugerem que algumas interações de importância podem ocorrer quando comunidades bacterianas que se associam a uma espécie fitoplanctônicas entram em contato com outra espécie diferente, como ocorreu com a mistura de linhagens isoladas a partir de *M. aeruginosa* que inibiram o crescimento de *A. granulata*. Desta forma, ressaltamos a importância de estudos sobre as comunidades bacterianas associadas às espécies fitoplanctônicas na dominância dessas espécies no ambiente natural. Trabalhos futuros com mais linhagens bacterianas isoladas e sua ação sobre diferentes espécies formadoras de *blooms*, com estudos também sobre suas funções e modo de ação, como produção de antibióticos, proteases ou 'predação', podem ajudar a elucidar alguns mecanismos envolvidos na formação e término de *blooms*, além da dominância perene de algumas espécies fitoplanctônicas em determinados ambientes.

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3.7. Material Suplementar

Tabela 3.S1 – Concentrações de Nitrato e Fosfato dissolvidos (mg.L-1) iniciais e ao final do crescimento exponencial nas culturas de *M. aeruginosa* no controle e nos tratamentos.

M. aeruginosa

Controle	Nitrato		Fosfato	
dia	Média	DP	Média	DP
0	47,02	2,38	2,20	0,25
8	3,06	3,79	0,41	0,33
12	0,10	0,09	0,05	0,09

Bact 14C	Nitrato		Fosfato	
dia	Média	DP	Média	DP
0	56,55	3,16	2,70	0,21
8	0,68	1,18	0,06	0,10

Bact 5A	Nitrato		Fosfato	
dia	Média	DP	Média	DP
0	49,07	1,91	2,31	0,11
8	3,37	1,70	0,41	0,13
12	0,05	0,09	0,00	0,00

Bact 17.3	Nitrato		Fosfato	
dia	Média	DP	Média	DP
0	59,79	0,17	2,86	0,14
8	6,83	5,19	0,54	0,41

Bact 6A	Nitrato		Fosfato	
dia	Média	DP	Média	DP
0	44,51	10,73	2,29	0,50
8	0,01	0,01	0,00	0,00
12	0,23	0,03	0,00	0,00

Bact 21	Nitrato		Fosfato	
dia	Média	DP	Média	DP
0	55,72	9,68	2,68	0,51
8	0,13	0,12	0,00	0,00

Bact 08	Nitrato		Fosfato	
dia	Média	DP	Média	DP
0	49,48	7,76	2,36	0,37
8	0,64	0,37	0,00	0,00
12	0,14	0,06	0,24	0,42

Bact 22.2	Nitrato		Fosfato	
dia	Média	DP	Média	DP
0	55,00	6,93	2,72	0,24
8	2,80	4,85	0,25	0,32

Bact 09	Nitrato		Fosfato	
dia	Média	DP	Média	DP
0	55,13	1,59	2,71	0,13
8	0,01	0,02	0,04	0,04

MLMicro	Nitrato		Fosfato	
dia	Média	DP	Média	DP
0	48,25	9,36	2,46	0,59
8	1,90	2,36	0,048	0,068

MLAulac	Nitrato		Fosfato	
dia	Média	DP	Média	DP
0	45,05	4,94	2,38	0,47
8	1,95	2,20	0,16	0,22

Tabela 3.S2 – Concentrações de Nitrato e Fosfato dissolvidos (mg.L-1) iniciais e ao final do crescimento exponencial nas culturas de *M. aeruginosa* no controle e nos tratamentos.

A. granulata

Controle	Nitrato		Fosfato	
dia	Média	DP	Média	DP
0	49,46	7,88	2,21	0,57
12	55,53	6,96	2,45	0,33
15	47,15	9,90	2,24	0,23

Bact 17.3	Nitrato		Fosfato	
dia	Média	DP	Média	DP
0	60,53	5,16	2,60	0,26
8	53,57	8,43	2,33	0,36
12	41,32	6,77	1,56	0,37
15	31,99	10,14	1,95	0,29

Bact 5A	Nitrato		Fosfato	
dia	Média	DP	Média	DP
0	56,69	6,39	2,48	0,57
12	64,09	0,62	2,98	0,17

Bact 21	Nitrato		Fosfato	
dia	Média	DP	Média	DP
0	64,53	3,97	2,87	0,17
12	51,55	4,04	2,11	0,22
15	50,79	5,63	2,22	0,09

Bact 6A	Nitrato		Fosfato	
dia	Média	DP	Média	DP
0	51,41	5,33	2,37	0,24
5	56,61	7,78	2,35	0,26
12	60,60	4,19	2,54	0,16

Bact 22.2	Nitrato		Fosfato	
dia	Média	DP	Média	DP
0	63,31	4,49	2,96	0,18
12	40,79	4,28	1,79	0,41
15	37,46	10,02	2,14	0,18

Bact 08	Nitrato		Fosfato	
dia	Média	DP	Média	DP
0	56,73	9,14	2,53	0,50
12	55,48	-	2,59	0,23
18	41,04	6,27	1,63	0,27

MLMicro	Nitrato		Fosfato	
dia	Média	DP	Média	DP
0	62,14	4,53	2,64	0,29
12	56,57	3,51	1,81	0,37
29	49,96	8,06	2,23	0,23

Bact 09	**Nitrato**		**Fosfato**	
dia	Média	DP	Média	DP
0	62,99	2,60	2,65	0,10
12	42,57	-	1,89	0,71
25	40,53	2,55	1,80	0,04

MLAulac	Nitrato		Fosfato	
dia	Média	DP	Média	DP
0	60,33	4,96	2,49	0,10
12	58,46	5,94	2,52	0,25
15	56,72	5,15	2,16	0,17

Bact 14C	Nitrato		Fosfato	
dia	Média	DP	Média	DP
0	55,05	8,24	2,51	0,12
12	47,09	11,79	1,86	0,45
18	45,40	3,31	2,02	0,23

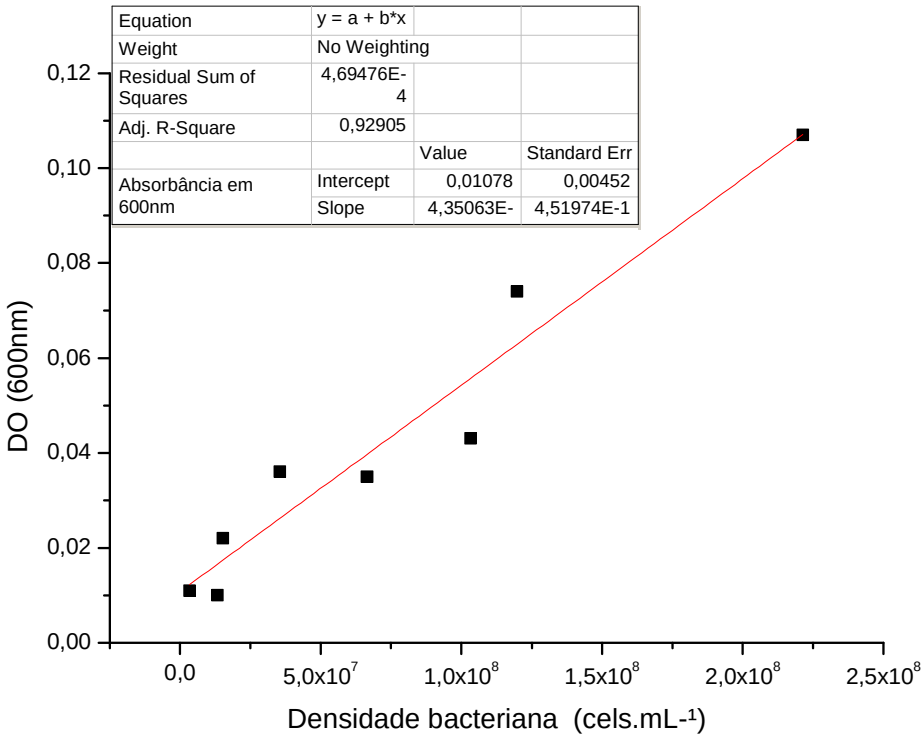

Figura 3.S1 – Correlação entre densidade ótica (DO) em 600 nm e densidade bacteriana (cels.mL-1) de culturas puras das linhagens Bact 6A e Bact 08 em meio CPS.

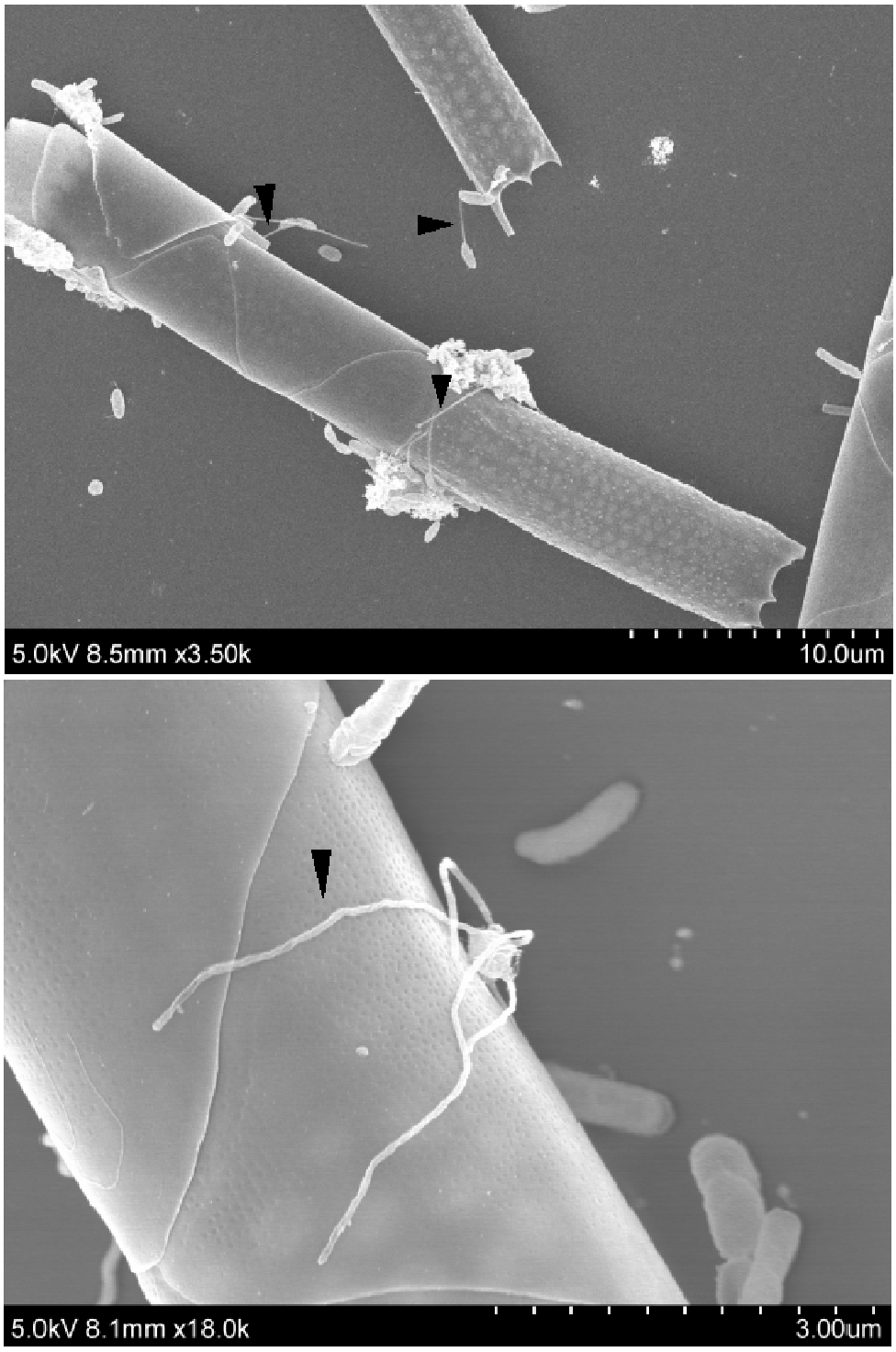

Figura 3.S3 – Fotomicrografia de Microscopia Eletrônica de Varredura de possíveis *Caulobacterales* aderidas à *A. granulata* após 9 dias de cultivo com a comunidade bacteriana total de Barra Bonita (A) e apenas com as bactérias aderidas dessa comunidade mantidas em co-cultura por aproximadamente 2 meses (B). As setas indicam a prosteca.

DISCUSSÃO GERAL

No presente estudo procurou-se analisar a especificidade das interações fitoplâncton-bactéria em diferentes escalas: 1) especificidade fitoplâncton-UTO (unidade taxonômica operacional), seja por ocorrência em associação a uma única espécie fitoplanctônica quando comparada a outras espécies, utilizando o mesmo inóculo bacteriano e condições de cultivo, seja por especificidade de ocorrência (recorrência) em culturas da mesma espécie fitoplanctônica com diferentes inóculos bacterianos e/ou diferenças experimentais. 2) especificidade fitoplâncton-comunidade bacteriana, também comparando as comunidades associadas a diferentes espécies fitoplanctônicas quando utilizado o mesmo inóculo bacteriano ou comparando comunidades associadas à mesma espécie, mas submetidas a diferentes inóculos bacterianos e/ou condições de cultivo.

Observou-se que a comunidade bacteriana se desenvolveu de maneira similar associada à mesma espécie quando foi utilizado o mesmo inóculo bacteriano, uma vez que houve reprodutibilidade entre as triplicatas experimentais. Para uma mesma espécie fitoplanctônica, foram observadas diferenças nas comunidades bacterianas nas diferentes fases de crescimento da cultura e nas diferentes frações (livres e aderidas). Além disso, com o mesmo inóculo bacteriano inicial, comunidades significativamente diferentes se desenvolveram associadas a diferentes espécies fitoplanctônicas (*Aulacoseira granulata, Cylindrospermopsis raciborskii* e *Microcystis aeruginosa*), sugerindo seleção pelos compostos excretados por cada espécie e demonstrando a importância do fitoplâncton em determinar a diversidade bacteriana. Esses resultados corroboraram a primeira hipótese do trabalho. No entanto, o sequenciamento de DNA em larga escala mostrou que as UTOs que ocorrem associadas apenas a uma das espécies fitoplanctônicas são poucas e apresentam baixa abundância relativa, desta maneira, a principal diferença entre as espécies, foi na proporção das UTOs mais abundantes, o que é mais difícil de ser observado com as técnicas usualmente utilizadas para comparar comunidades bacterianas, como o DGGE, como discutido no Capítulo 1.

Em relação à comparação de comunidades bacterianas com diferentes inóculos ou condições de crescimento do fitoplâncton, também foram encontradas diferenças significativas entre as comunidades que se desenvolvem com a mesma espécie fitoplanctônica. Esse resultado contestou parcialmente nossa segunda

hipótese inicial, entretanto, na escala fitoplâncton-UTO bacteriana, foram encontradas algumas UTOs relativamente abundantes que ocorrem comumente associadas às espécies mesmo em condições variáveis e que possivelmente apresentam maior especificidade à espécie hospedeira.

Observou-se que embora a variação no inóculo bacteriano e/ou condições de crescimento sejam importantes na especificidade de interação bactéria-fitoplâncton, as diferenças nas espécies fitoplanctônicas explicaram maior parte da variação entre as comunidades do que as diferenças entre inóculo/condições de crescimento, corroborando as evidências (Grossart et al. 2005, Kent et al. 2007, Teeling et al. 2012, Paver et al. 2013) de que o fitoplâncton é um importante fator na determinação da betadiversidade das comunidades bacterianas.

Um fato interessante foi que comparando grupos taxonômicos mais elevados, como família e gênero, a explicação da variação pelas espécies fitoplanctônicas aumentou, enquanto a explicação da variação por diferenças experimentais diminuiu. Esses resultados sugerem que embora as UTOs sejam diferentes entre os experimentos (inóculos), elas podem ser filogeneticamente próximas. Uma vez que diferenciação genética pode ser preditiva de propriedades funcionais (Martin 2002), uma UTO poderia ocupar o nicho de outra filogeneticamente próxima quando esta não estivesse presente ou ocorresse em menor proporção no inóculo. Assim, talvez a comparação de gêneros e famílias seja mais informativa para avaliar a especificidade de comunidades bacterianas associadas ao fitoplâncton do que a comparação entre UTOs, especialmente em casos de variação inicial devido à fonte bacteriana distinta. Futuros trabalhos sobre especificidade fitoplâncton-bactéria devem considerar também a redundância ecológica das OTUs e avaliar as funções dos táxons associados (Sapp et al. 2007) e não somente sua identificação.

Apesar das diferenças entre as comunidades bacterianas associadas à mesma espécie fitoplanctônica, a caracterização aprofundada dessas comunidades permitiu a identificação de algumas UTOs que possivelmente apresentam maior especificidade às espécies hospedeiras, o que possibilita a utilização de meios de cultura mais específicos para isolar os grupos bacterianos de interesse em futuros trabalhos. Além disso, os dados de pirossequenciamento foram importantes para verificar que algumas das bactérias isoladas que retardaram o crescimento de *A.*

granulata também ocorreram associadas à *M. aeruginosa* e em importantes proporções e/ou recorrência.

Tanto a população bacteriana total quanto a maioria das linhagens isoladas apresentaram efeitos negativos sobre o crescimento de *A. granulata* e pouco ou nenhum efeito sobre *M. aeruginosa*. Esses resultados contrariaram parcialmente a terceira hipótese inicial deste trabalho, pois mesmo as bactérias aderidas à *A. granulata* provocaram efeitos negativos no crescimento da diatomácea. As possíveis explicações para isso são: maior resistência de *M. aeruginosa* e das cianobactérias ao 'ataque' de outras bactérias quaisquer (Sigee 2005, Shi et al. 2009) e/ou maior resistência devido à seleção e adaptação da comunidade bacteriana à *M. aeruginosa* (Casamatta & Wickstrom 2000), uma vez que esta cianobactéria apresenta blooms frequentes em Barra Bonita.

Essas observações podem ter implicações ecológicas relevantes, pois a interação 'comunidade bacteriana-fitoplâncton' poderia ajudar a explicar a dominância de certas espécies fitoplanctônicas nos ambientes aquáticos em que são frequentes, não somente por remineralizarem nutrientes ou produzirem vitaminas e outras substâncias promotoras de crescimento (e.g. Sigee 2005), mas também por inibirem o crescimento de outras espécies fitoplanctônicas. Assim, os benefícios obtidos por uma espécie ao liberar grande quantidade de matéria orgânica dissolvida que possibilita o crescimento de bactérias potencialmente competidoras, visto como o paradoxo do fitoplâncton (Bratbak & Thingstad 1985) deve ser analisado não somente em relação aos nutrientes inorgânicos e sua remineralização, mas também a outras funções e aos diversos efeitos que diferentes linhagens podem apresentar sobre a própria espécie e sobre as possíveis espécies competidoras. Futuros trabalhos que visem avaliar o efeito de linhagens bacterianas associadas sobre a ecologia das espécies fitoplanctônicas devem incluir linhagens bacterianas isoladas de locais onde as espécies fitoplanctônicas formam *blooms* frequentes e de locais onde elas ocorrem, mas não produzem grande quantidade de biomassa.

Dessa forma, trabalhos futuros sobre interações fitoplâncton-bactéria devem analisar as funções das comunidades e das linhagens bacterianas, tanto para melhor compreensão da especificidade das associações como para compreensão de seus efeitos sobre as espécies formadoras de blooms.

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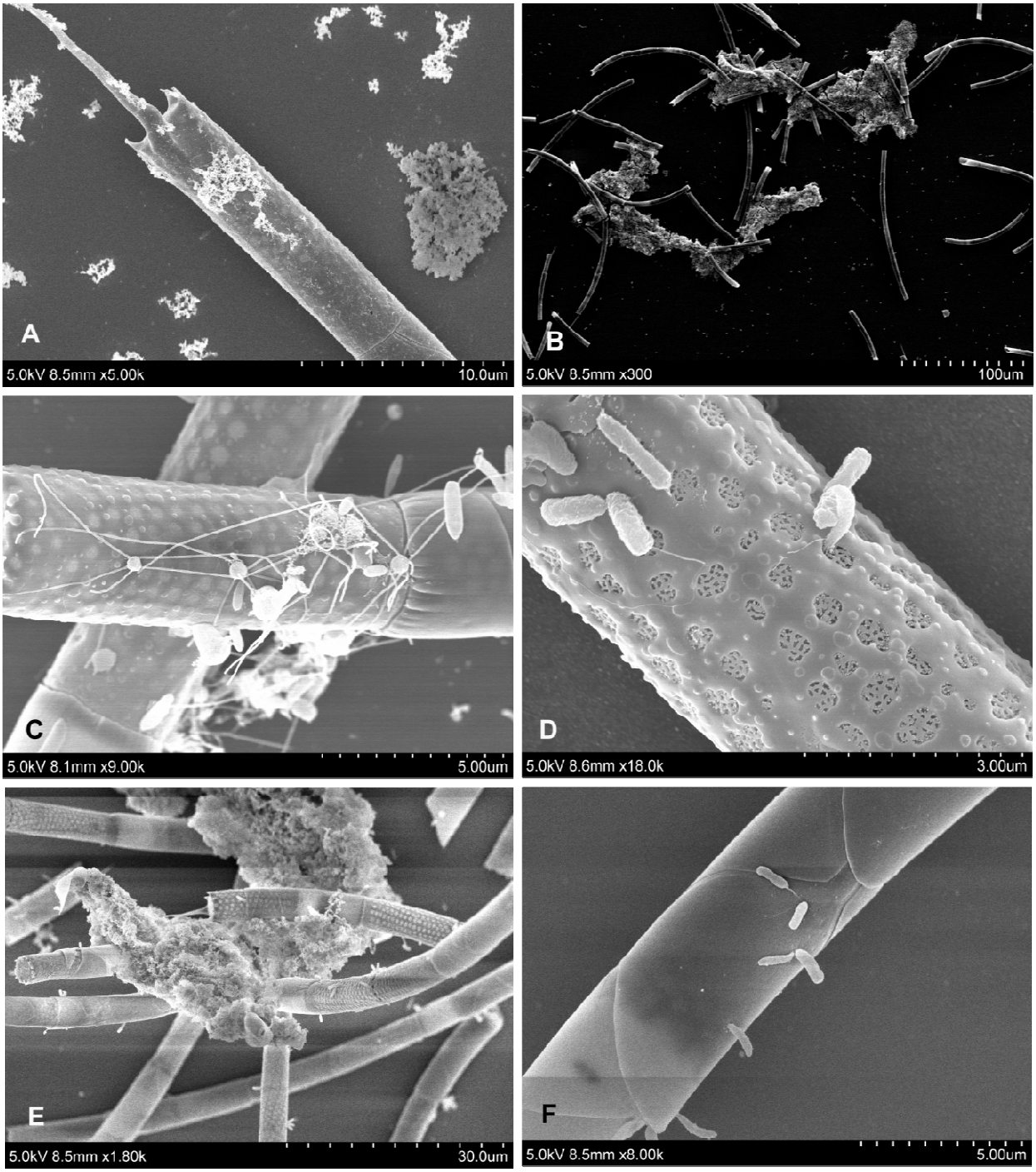
CONCLUSÕES

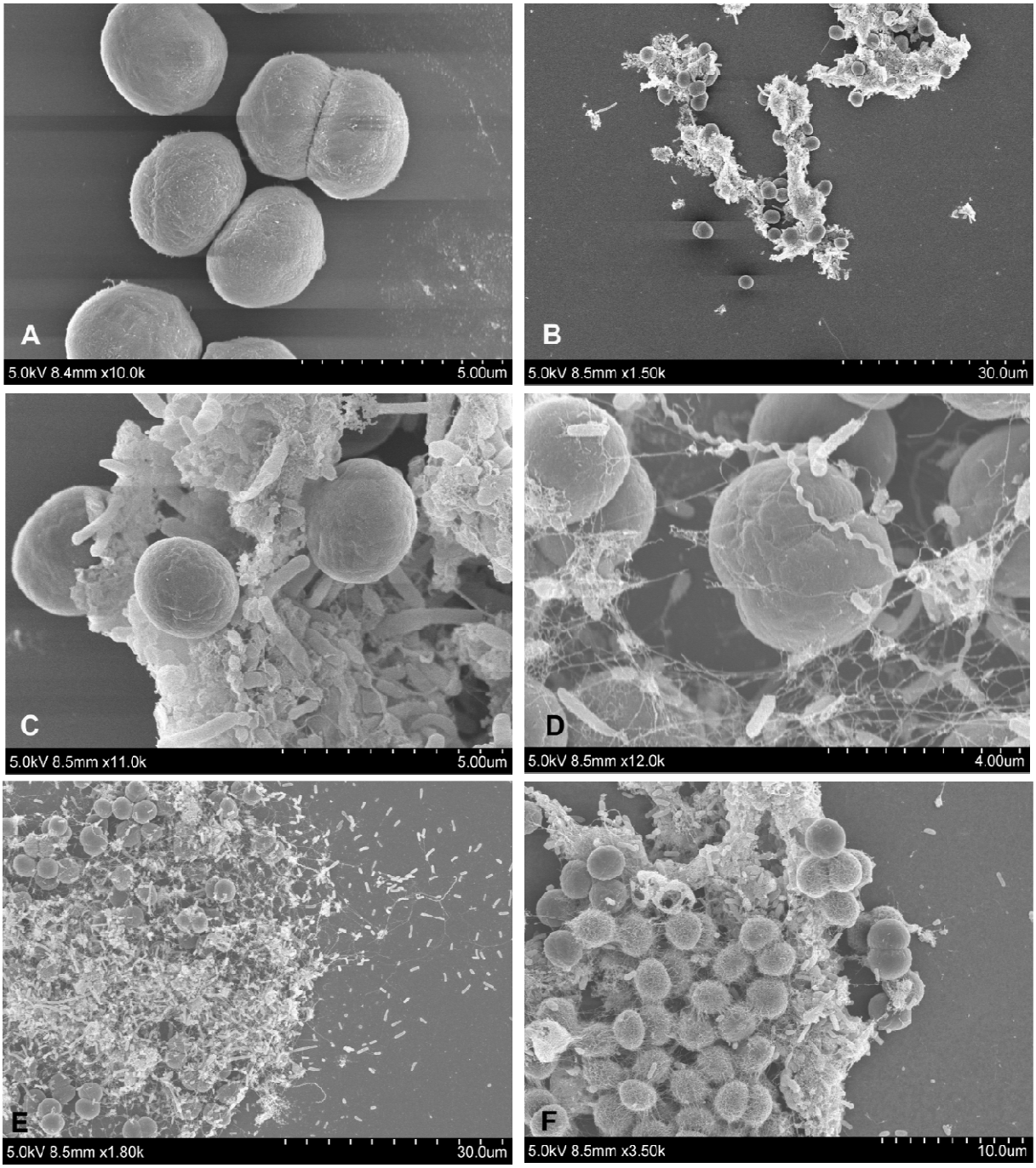
1) A comunidade bacteriana associada a uma mesma espécie fitoplanctônica varia com o estado fisiológico das células e é significativamente diferente entre as frações livre e aderida, demonstrando seletividade das bactérias que se aderem às células.
2) As espécies fitoplanctônicas quando expostas ao mesmo inóculo bacteriano selecionam diferentes comunidades bacterianas, indicando certa especificidade da comunidade bacteriana em relação à espécie fitoplanctônica hospedeira. No entanto, as principais diferenças são nas proporções das UTOs (unidades taxonômicas operacionais) e não em presença/ausência de UTOs específicas.
3) Fatores como inóculo bacteriano e/ou condições de cultivo são importantes na variabilidade da associação fitoplâncton-comunidade bacteriana, pois determinam comunidades significativamente diferentes associadas à mesma espécie fitoplanctônica.
4) As espécies fitoplanctônicas foram mais importantes do que as diferenças no inóculo bacteriano e entre experimentos na determinação das variações entre as comunidades bacterianas associadas.
5) Apesar das diferenças entre comunidades bacterianas associadas a uma mesma espécie fitoplanctônica em diferentes experimentos, ou seja, ausência de especificidade fitoplâncton-comunidade bacteriana, a recorrência de algumas UTOs abundantes indicou que especificidade fitoplâncton-UTO pode ocorrer.
6) Algumas linhagens bacterianas aderidas às espécies fitoplanctônicas têm efeitos distintos sobre a espécie hospedeira e sobre uma possível espécie competidora. Isso pode ser um dos fatores que explicam a dominância de *M. aeruginosa* em relação à *A. granulata* no reservatório de Barra Bonita.

ANEXOS

Anexo 1 – Fotomicrografia de microscopia eletrônica de varredura de *Aulacoseira granulata* em cultura axênica (A) e com bactérias associadas (B-F).



Anexo 2 – Fotomicrografia de microscopia eletrônica de varredura de *Microcystis aeruginosa* em cultura axênica (A) e com bactérias associadas (B-F).



Anexo 3 – Fotomicrografia de microscopia eletrônica de varredura de *Cylindrospermopsis raciborskii* em cultura sem bactérias associadas (A) e com bactérias associadas (B-D).