

Universidade Federal de Juiz de Fora
Programa de Pós-Graduação em Ecologia

Victor Claudio Zarantonello Arantes

***Raphidiopsis raciborskii* (CYRF-01): HABILIDADE DE SECREÇÃO DE
VESÍCULAS EXTRACELULARES EM CONDIÇÕES DE CRESCIMENTO
NORMAL E EM RESPOSTA A ESTÍMULOS AMBIENTAIS EM CULTURA**

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Tese apresentada ao Programa de Pós-Graduação em Ecologia da Universidade Federal de Juiz de Fora, como parte dos requisitos necessários à obtenção do grau de Doutor Ecologia Aplicada a Conservação e Manejo de Recursos Naturais.

Orientadora: Prof^a. Dr^a Rossana Correa Netto de Melo

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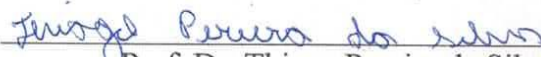
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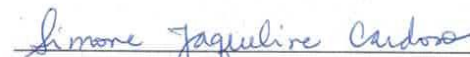
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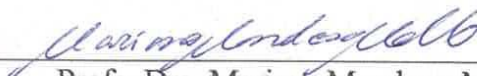
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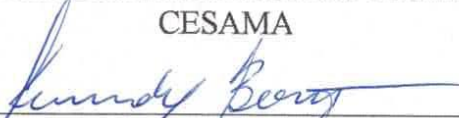
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CESAMA



Prof. Dr. Kennedy Bonjour de Oliveira Ferreira
NOVA Faculdade

Para a flor mais linda que desabrocha hoje longe de mim.

Cuidei, reguei, cultivei e sei que hoje cada pétala sua
servirá para perfumar todos os caminhos da minha vida.

A você, de novo, e sempre, Nana.

Do seu Dito...

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RESUMO

A secreção de vesículas na superfície celular, denominadas coletivamente de vesículas extracelulares (VEs), é um processo biológico importante das células eucarióticas e procarióticas associado com atividades funcionais e transferência de DNA. Esse processo tem sido demonstrado em bactérias de ecossistemas aquáticos, mas ainda é pouco estudado em cianobactérias. No presente trabalho, investigamos se *Raphidiopsis raciborskii* (CYRF-01), espécie de cianobactéria de distribuição global e crescente em ambientes de água doce, é capaz de liberar VEs quando cultivada em condições normais ou exposta a estressores ambientais (radiação ultravioleta ou co-cultura com outra espécie de cianobactéria - *Microcystis aeruginosa* - MIRF-01). Culturas não axênicas de CYRF-01 foram submetidas à radiação ultravioleta (UVA + UVB) por 6 h, tratamento que induz danos estruturais a esta espécie ou co-cultivadas com MIRF-01 por 24 h. Após incubação, a densidade e a viabilidade celular foram analisadas e as amostras processadas para microscopia eletrônica de transmissão (MET). As análises ultraestruturais revelaram que *R.raciborskii* libera constitutivamente VEs a partir da membrana externa (VMEs) durante crescimento normal e amplifica essa capacidade em resposta a estressores ambientais. Ambas as situações induziram formação significativa VMEs por CYRF-01, comparado aos controles. Análises quantitativas por MET mostraram aumento de 48% (UV) e 60% (interação) nos números de VMEs. Considerando todos os grupos, as VMEs variaram de 20 a 300 nm de diâmetro, com a maioria mostrando diâmetros entre 20 e 140 nm. Análise molecular do envoltório celular revelou que a liberação de VMEs envolve exposição à fosfatidilserina, evento também observado nas células eucarióticas secretoras de VEs. Em conjunto, identificamos pela primeira vez que *R.raciborskii* tem competência para secretar VMEs e que, sob situações de estresse, a gênese dessas vesículas é aumentada. A capacidade ampliada das cianobactérias para liberar VMEs pode estar associada com respostas adaptativas às mudanças nas condições ambientais e pode contribuir na comunicação celular interespecie.

Palavras-chave: cianobactérias, cultura, vesículas extracelulares, microscopia eletrônica de transmissão, microscopia de luz, ultraestrutura, interação interespecífica, vesículas de membrana externa.

ABSTRACT

Secretion of vesicles at the cell surface, collectively termed extracellular vesicles (EVs), is an important biological process of both eukaryotic and prokaryotic cells associated with functional activities and DNA transfer. This process has been demonstrated in bacteria from aquatic ecosystems, but it is still poorly characterized in cyanobacteria. In the present work, we investigated if *Raphidiopsis raciborskii* (CYRF-01), a globally distributed cyanobacterium species with increasing presence in freshwater environments, is able to release EVs when exposed to environmental stressors (ultraviolet radiation or co-culture with another cyanobacterium species - *Microcystis aeruginosa*). Non-axenic cultures of *R.raciborskii* (CYRF-01) were exposed to ultraviolet radiation (UVA + UVB) for 6 h period, treatment that induces structural damage to this species while co-cultures with MIRF-01 were kept for 24 h. After the incubation times, cell density and viability were analyzed, and samples were processed for transmission electron microscopy (TEM). Our ultrastructural analyses revealed that *R.raciborskii* constitutively releases EVs from the outer membrane during its normal growth and amplifies such ability in response to environmental stressors. Both situations induced significant formation of outer membrane vesicles (OMVs) by *R.raciborskii* compared to control cells. Quantitative TEM revealed an increase of 48% (UV) and 60% (interaction) in the OMV numbers. Considering all groups, the OMVs ranged in size from 20 to 300 nm in diameter, with most OMVs showing diameters between 20 and 140 nm. Additionally, molecular analysis of the cellular envelope revealed that formation of OMVs is accompanied by phosphatidylserine exposure, an event also observed in EV-secreting eukaryotic cells. Altogether, we identified for the first time that *R.raciborskii* has the competence to secrete OMVs and that under different stress situations the genesis of these vesicles is increased. The amplified ability of cyanobacteria to release OMVs may be associated with adaptive responses to changes in environmental conditions and interspecies cell communication.

Key words: cyanobacteria, culture, extracellular vesicles, transmission electron microscopy, light microscopy, ultrastructure, interspecific interaction, outer membrane vesicles.

INDICE DE ABREVIATURAS

CYRF-01	Cepa de <i>Raphidiopsis raciborskii</i> utilizada no trabalho
MIRF-01	Cepa de <i>Microcystis aeruginosa</i> utilizada no trabalho
MET	Microscopia eletrônica de transmissão
ML	Microscopia de luz
VEs	Vesículas Extracelulares
VG	Vesículas de gás
VMEs	Vesículas de membrana externa
UV	Ultravioleta

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ANEXOS: “The Cyanobacterium *Raphidiopsis raciborskii* (CYRF-01) Responds to Environmental Stresses with Increased Vesiculation Detected at Single-Cell Resolution”

“Increased production of outer membrane vesicles by cultured freshwater bacteria in response to ultraviolet radiation”

1 INTRODUÇÃO

1.1 ASPECTOS GERAIS DAS CIANOBACTÉRIAS

O grupo das cianobactérias é considerado um dos mais antigos do Planeta. São consideradas os primeiros organismos fotossintetizantes que apresentam clorofila a e realizam fotossíntese através dos fotossistemas I e II. Estima-se que seu surgimento tenha sido há 3,5 bilhões de anos, sendo os primeiros produtores primários a liberarem oxigênio na atmosfera (MUR; SKULBERG; UTKILEN, 1999). A produção de compostos orgânicos e a alta eficiência no uso do oxigênio para obtenção de energia, permitiram a esses organismos o aumento da capacidade de carga biológica e, ao mesmo tempo, o surgimento de uma elevada diversidade de espécies capazes de se adaptar às diferentes condições ambientais (TSUKAMOTO et al., 1993). As cianobactérias apresentam ampla distribuição ocupando os mais diversos habitats, aquáticos e terrestres.

Há uma imensa diversidade morfológica no grupo e pode se apresentar em formas unicelulares ou coloniais (Fig. 1). O arranjo das colônias pode ser irregular, radial ou em planos ordenados. O diâmetro celular varia de 0,5 μm a 100 μm (DUFRESNE et al., 2003). Os indivíduos unicelulares podem ter formas arredondadas, oblongas, elípticas, cilíndrico-arredondadas, fusiformes e piriformes. As células apresentam ou não envoltório mucilaginoso, dependendo da espécie (SANT'ANNA, 2006).

Em algumas espécies de cianobactéria, os indivíduos unicelulares (Fig. 1A) podem se reunir formando colônias (Fig. 1B-V). As cianobactérias coloniais podem ser formadas por poucas células (2 a 16) ou centenas delas; apresentando diferentes morfologias (Fig. 1B-V). As cianobactérias filamentosas, podem formar um tricoma, quando as células se dispõem linearmente (Fig. 1G-V). Assim, o filamento é formado pelo conjunto de bainha mucilaginosa e o tricoma (SANT'ANNA, 2006).

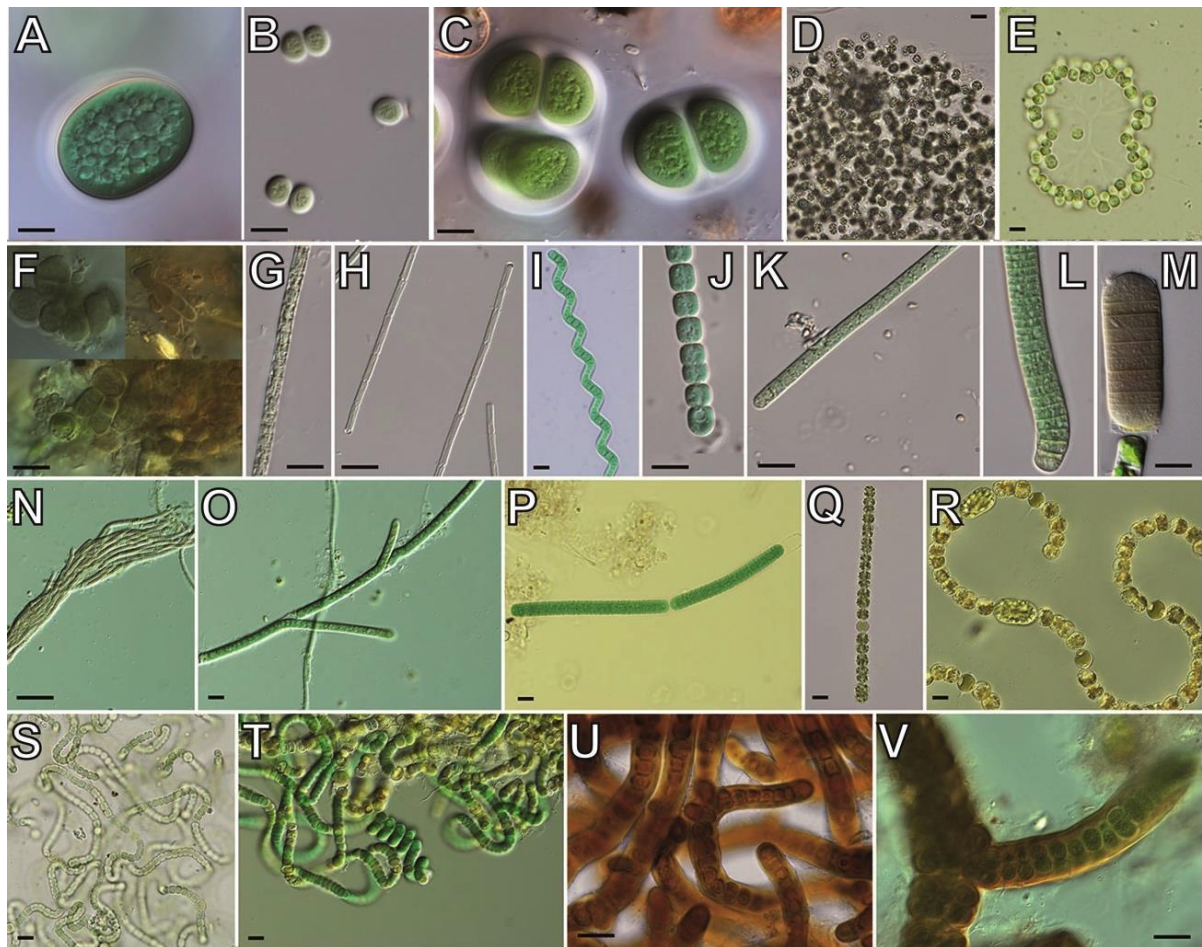


Figura 1: Diferentes tipos de morfologia encontrados no grupo das cianobactérias. Em (A), observe cianobactéria unicelular, e em (B-V), cianobactérias coloniais. As colônias podem conter duas (B) ou mais células (C-V), que se arranjam em forma de grumos (D-F) ou filamentos (G-V). Barra= 10 μm (A-U), 20 μm (V). Imagem adaptada de DVOŘÁK et al. (2015).

Os tricomas podem apresentar apenas células vegetativas ou células modificadas (heterocisto e acineto), que possuem extrema importância fisiológica e ecológica. Os heterocistos são células de parede fina e protoplasma hialino, especializadas na fixação de nitrogênio. Os acinetos são células grandes, igualmente de parede fina, com capacidade de reserva que permite a sobrevivência da espécie sob condições desfavoráveis (SANT'ANNA, 2006).

1.2 ULTRAESTRUTURA DE CIANOBACTÉRIAS

As cianobactérias apresentam envoltório celular típico de bactérias Gram-negativas, apresentado três camadas distintas: membrana plasmática, parede celular e membrana externa. Seu envoltório, porém, é consideravelmente mais espesso que o das demais bactérias gram-negativas. Exibe grande variação de acordo com a espécie e pode atingir até 700 nm de espessura (HOICZYK; HANSEL, 2000).

Algumas cianobactérias secretam polissacarídeos extracelulares que podem formar uma mucilagem ou uma bainha fibrilar externamente à parede celular (HOICZYK; HANSEL, 2000). Tais camadas podem em conjunto com a parede celular conectar fortemente as células em um filamento.

Em relação às estruturas citoplasmáticas, as membranas de tilacoides são as estruturas mais abundantes no citoplasma na maioria das cianobactérias. Os tilacoides são projeções intracitoplasmáticas da membrana plasmática, que formam variados arranjos no interior das células. Nos tilacoides localizam-se o aparato fotossintético (fotossistemas I e II) e aderidos a eles podem-se encontrar estruturas concêntricas, chamadas ficobilissomos. Os ficobilissomos (Fig. 3, cabeça de seta) são formados por pigmentos acessórios (ficocianina, ficoeritrina e aloficocianina) que funcionam como complexos de antenas na captação da luz e transferência de energia para a reação de fotossíntese. Os pigmentos fotossintéticos, como a clorofila a e os demais componentes celulares estão dispersos no citoplasma. Os tilacoides podem se arranjar concentricamente seguindo o formato celular ou apresentarem-se dispersos em formas variadas de acordo com a espécie (FLORES; HERRERO, 2008).

Diversas estruturas internas podem ser encontradas nas cianobactérias, dentre elas as principais são os ribossomos 70S típico de células procarióticas, os corpos poliédricos, ou carboxissomos (Fig.2, CP) que participam da fixação de carbono atmosférico, vesículas de gás (Fig.2, VG) com função a flutuação no meio aquático, e uma variedade de grânulos com diversas funções, como

por exemplo, os grânulos de fosfato (Fig. 2, GP) onde ocorre o armazenamento de fósforo na forma de grãos de polifosfatos no citoplasma.

Trabalhos com a descrição da ultraestrutura de diferentes gêneros de cianobactérias, por exemplo, *Anabaena*, *Mycrocistis*, *Oscillatoria* e *Synechococcus*, mostram uma grande variedade na distribuição das estruturas na célula e a influência do ambiente e da condição de crescimento sobre a presença e dispersão das mesmas (ALLEN, 1984; CASAMATTA et al., 2005; FALCÓN et al., 2004; FREDRIKSSON; BERGMAN, 1997; GROMOV; GAVRILOVA; KONOVALOV, 1986; JENSEN, 1993; PALÍNSKA; KRUMBEIN; SCHLEMMINGER, 1998). Por exemplo, NOYMA, N. (2009) investigou, pela primeira vez, a ultraestrutura da cianobactéria *Raphidiopsis raciborkii* e mostrou um envoltório celular trilaminar típico e um citoplasma contendo um sistema de membranas intrincado (tilacoides) com ficobilissomos associados e outras estruturas, tais como grânulos de polifosfato e carboxissomos (Fig. 2). Além disso, este estudo revelou que a ultraestrutura, principalmente as estruturas relacionadas ao aparato fotossintético, podem ser alteradas pela exposição a radiação ultravioleta (UV).

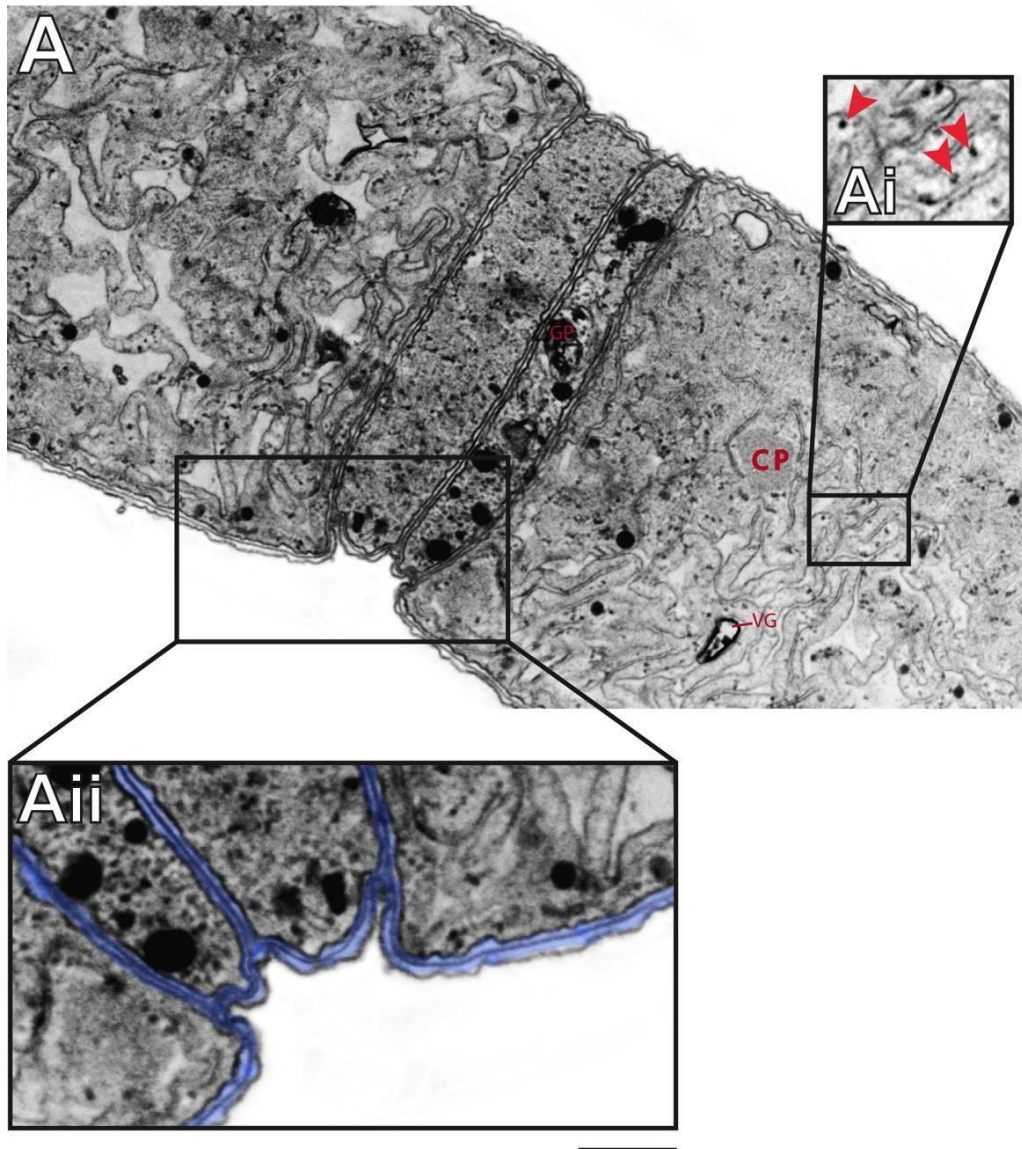


Figura 2: Fotomicrografia eletrônica mostrando as principais estruturas encontradas em *Raphidiopsis raciborskii* (CYRF-01). (A), ultraestrutura de duas células mostrando grânulos de polifosfato (GP), corpos poliédricos (CP) e vesículas de gás (VG). Em (Ai), os ficobilissomos são evidenciados (cabeças de setas vermelhas) associados às membranas dos tilacoides. Em (Aii), o envoltório da cianobactéria foi colorido em azul, evidenciando a parede celular. Barra = 1 μ m. Retirado de NOYMA, N. (2009).

1.2.1 *Raphidiopsis raciborskii*

Raphidiopsis raciborskii Seenayya et SubbaRaju (1972) é uma cianobactéria filamentosa pertencente à ordem Nostocales. Tem como característica a formação de tricomas solitários retos ou ligeiramente curvados, com vesículas de gás e heterocisto terminal em uma ou nas duas extremidades do filamento, células vegetativas cilíndricas e acinetos oblongos-ovais (EVERSON et al., 2011; SHAFIK et al., 2003). Embora tenha sido descrita como uma espécie tropical/subtropical, sua ocorrência em ambientes de regiões temperadas tem crescido na última década (EVERSON et al., 2011; HAMILTON et al., 2005; PADISÁK, 1997; SINHA et al., 2012).

A expansão geográfica de ocorrência de *R.raciborskii* é parcialmente atribuída a combinação de adaptações fisiológicas com a habilidade de sobrevivência em condições desfavoráveis. Tais fatores incluem a formação de acinetos e a tolerância a baixa disponibilidade de fósforo e nitrogênio. *R.raciborskii* é caracterizada como uma espécie com alta tolerância ao sombreamento, com baixo requerimento de luz, apresentando alta taxa de assimilação de amônio e capacidade de fixar nitrogênio atmosférico. Além disso, essa espécie possui uma alta afinidade por fósforo, sendo capaz de estocar este nutriente, além de ser resistente a predação pelo zooplâncton (PADISÁK, 1997). Possui ainda a capacidade de regular flutuação através de vesículas de gás denominadas aerótopos, possibilitando ao organismo certo controle sob a sua posição na coluna d'água, permitindo assim que se mova, verticalmente, em direção a uma intensidade luminosa favorável e disponibilidade de nutrientes. Entretanto, o aumento da temperatura global e eutrofização também têm sido considerados para explicar sua ampla distribuição (SINHA et al., 2012). *R.raciborskii* ocupou rapidamente uma extensa área geográfica, ocorrendo num amplo número de lagos, reservatórios e rios tropicais (PADISÁK, 1997), produzindo florações, algumas tóxicas, em muitos corpos d'água ao redor do mundo (BRANCO; SENNA, 1994; CHAPMAN; SCHELSKE, 1997; DOKULIL; MAYER, 1996)

R.raciborskii é uma espécie com alta tolerância ao sombreamento, baixo requerimento de luz, alta taxa de assimilação de amônio e capacidade de fixar nitrogênio atmosférico. Possui alta afinidade por fósforo, estocando este nutriente, sendo resistente à predação pelo zooplâncton (PADISÁK, 1997).

R.raciborskii, atraiu considerável atenção devido à sua ampla distribuição e potencial capacidade de produzir diversas toxinas, como hepatotoxinas e neurotoxinas, que causam impactos negativos à saúde ambiental (CARNEIRO; PACHECO; DE OLIVEIRA E AZEVEDO, 2013; MOLICA et al., 2002). Algumas cianotoxinas estão associadas a peixes, gado doméstico e mortalidades humanas (CODD; MORRISON; METCALF, 2005; DITTMANN; WIEGAND, 2006; SVIRČEV et al., 2017). Além disso, a produção de aleloquímicos por *R.raciborskii* foi proposta como uma vantagem adicional à dominância dessa espécie por inibição de outros concorrentes de cianobactérias, incluindo *Microcystis aeruginosa* (FIGUEREDO, CLEBER CUNHA; GIANI, 2009; MELLO et al., 2012).

Um recente estudo observou cepas de *Raphidiopsis mediterranea* em lagos argentinos, utilizando abordagem polifásica, na qual não foi observada diferenciação dos heteocistos quando submetidas às condições de privação de nitrogênio. Isso suporta a ocorrência de populações nativas estáveis. Concluiu-se que esses dois gêneros (*Cylindrospermopsis* e *Raphidiopsis*) não devem ser considerados unidades genéricas separadas e sendo unificado sob o nome de *Raphidiopsis* (e não mais *Cylindrospermopsis*), respeitando o princípio da prioridade. (AGUILERA et al., 2018)

1.3 SECREÇÃO DE VESÍCULAS EXTRACELULARES

1.3.1 Aspectos gerais

A secreção de vesículas limitadas à membrana, denominadas coletivamente vesículas extracelulares (VEs), é um importante evento celular de células eucarióticas e procarióticas. As vesículas de membrana externa (VMEs) são estruturas formadas a partir da membrana externa de procariotos com envoltório celular tipicamente gram-negativos (KULP; KUEHN, 2010) (Fig. 3).

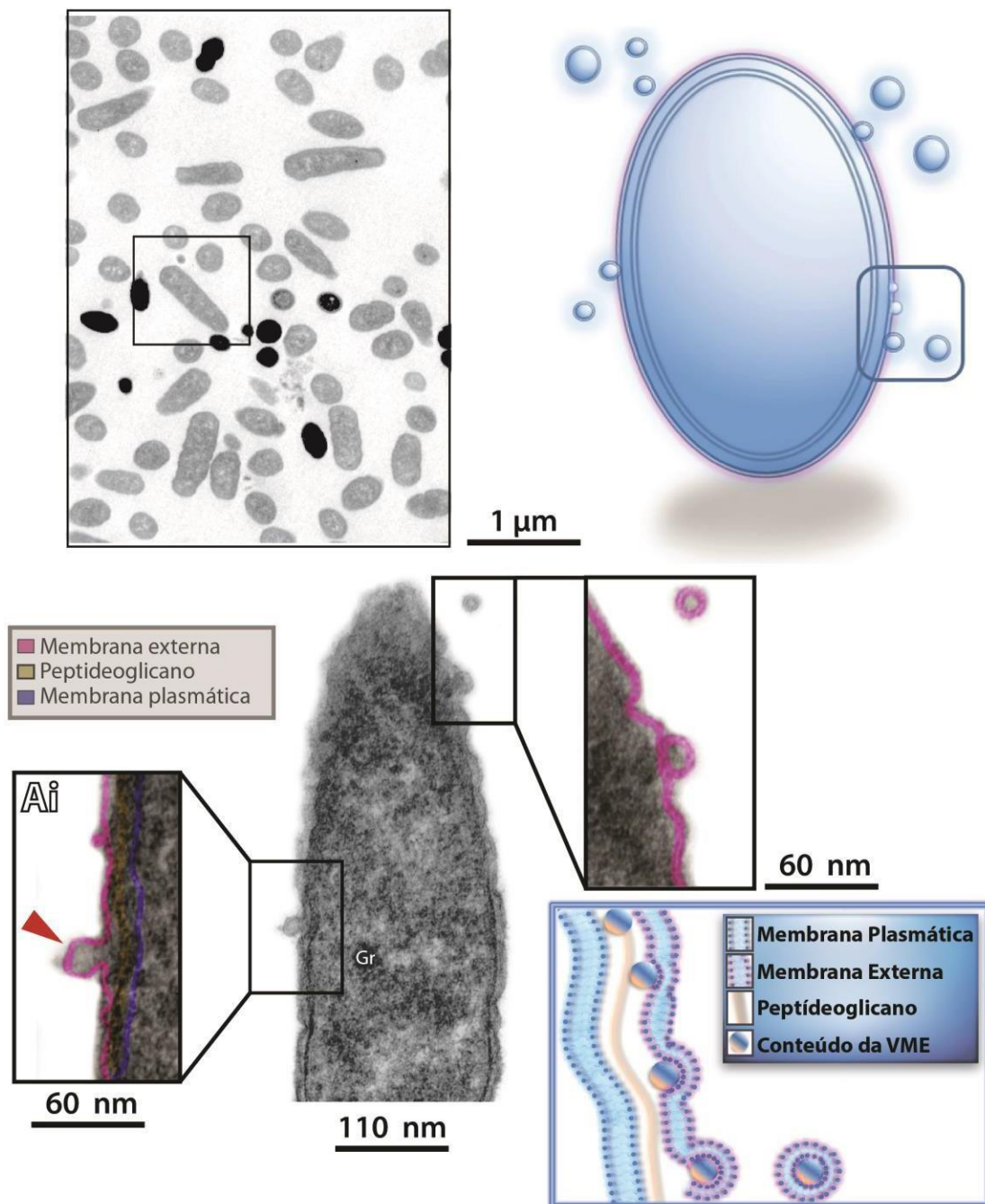


Figura 3: Processo de formação de vesículas extracelulares em bactérias Gram-negativas: Observe no painel inferior à esquerda uma comunidade de bactérias vistas ao MET em pequeno aumento. Em maior aumento, a superfície bacteriana mostra a formação de vesículas extracelulares. Note a estrutura trilaminar da membrana envoltora da vesícula. Retirado de GAMALIER et al. (2017).

A capacidade das bactérias de liberar VEs vem sendo demonstrada exponencialmente (KULP; KUEHN, 2010). Essas estruturas tem sido reconhecidas e estudadas principalmente em bactérias heterotróficas e suas funções sugeridas incluem: tráfego de toxinas e proteínas, transferência de DNA e comunicação celular (KULP; KUEHN, 2010; MASHBURN; WHITELEY, 2005). Essas vesículas, em nanoescala, têm sido associadas a processos biológicos fundamentais como a patogênese (KOLLING; MATTHEWS, 1999; RIVERA et al., 2010); defesa celular (BAUMGARTEN et al., 2012; MANNING; KUEHN, 2011), comunicação celular (MASHBURN-WARREN et al., 2008) e transferência de DNA (RUMBO et al., 2011).

Acredita-se que em microrganismos, a formação de VMEs pode estar relacionada com respostas adaptativas à sobrevivência devido a variações de fatores ambientais, como na competição por nichos (KULP; KUEHN, 2010; MCBROOM; KUEHN, 2007). Assim, é reconhecido que o conteúdo vesicular, o qual pode conter compostos tóxicos, fagos virais ou proteínas ativas, estão diretamente relacionados com mecanismo de defesa após a exposição a condições estressantes (ELLIS; KUEHN, 2010; MCBROOM; KUEHN, 2007). Além disso, já foi encontrado que microrganismos competidores no mesmo nicho ecológico são capazes de secretar moléculas antimicrobianas dentro de VMEs, que irão atuar seletivamente na morte de outras espécies (KADURUGAMUWA et al., 1998; LI; CLARKE; BEVERIDGE, 1998).

Foi relatado que as bactérias produzem VEs em ecossistemas de água doce (SILVA, T. P. et al., 2014) e que a radiação UV, um estressor ambiental, provoca aumento da vesiculação em bactérias heterotróficas da água doce (GAMALIER et al., 2017). A liberação amplificada de VEs pode ser uma resposta regulada, oferecendo um mecanismo adaptativo eficaz sob condições de diversos estresses naturais. As VEs podem empacotar uma variedade de cargos relacionadas à sobrevivência e persistência da população, remoção de proteínas indesejáveis do envelope (MCBROOM; KUEHN, 2007) ou moléculas de autodefesa (MANNING; KUEHN, 2011; RIVERA et al., 2010).

1.3.2 Vesículas extracelulares em cianobactérias

A formação de VMEs por microrganismos fotoautotróficos foi descrita pela primeira vez por BILLER et al. (2014) em cianobactéria do gênero *Prochlorococcus* e *Synechococcus*. Nestas cianobactérias, os autores documentaram a formação de VMEs contendo proteínas, DNA e RNA, além de demonstrarem sua abundância em amostras de ecossistemas marinhos costeiros (BILLER et al., 2014). Posteriormente, foi indicado que a cianobactéria *Synechocystis* PCC6803 produz VEs de maneira semelhante às bactérias gram-negativas, ou seja, a partir da membrana externa (PARDO et al., 2015). Diversas podem ser as funções das VEs secretadas por organismos procariotos em sistemas aquáticos, porém, devido à imensa dinâmica, que forma uma teia complexa de comunicação entre VEs, o estudo de cada uma delas se torna difícil de ser realizado, como revisado em SCHATZ; VARDI (2018), que propõem funções e implicações ecológicas da comunicação via VEs em ecossistemas microbianos aquáticos. Interessantemente, YIN et al. (2019) notaram que a cianobactéria *Synechococcus elongatus* PCC 7942 tem promissor potencial como uma estratégia para angiogênese terapêutica e cicatrização de feridas principalmente através da liberação de VEs.

No entanto, pouco se sabe ainda sobre a capacidade das cianobactérias de secretar vesículas de membrana externa (VMEs), sua biogênese e possíveis funções na biologia aquática.

2 JUSTIFICATIVA

A liberação de VEs é um processo celular considerado universalmente conservado nos três domínios da vida (revisado em GILL; CATCHPOLE; FORTERRE, 2019). A produção de VEs tem sido sistematicamente observada nos últimos anos em diferentes condições, sugerindo que todas as células são potencialmente capazes de produzir VEs. No entanto, a liberação de VEs por cianobactérias e seu papel em ambientes aquáticos são ainda muito pouco estudados na literatura. Em *R.raciborskii*, em específico, não há estudos que demonstrem a capacidade dessa cianobactéria de secretar VEs nos ecossistemas aquáticos. Por causa do pequeno tamanho das VEs, da ordem de nanômetros, VEs são observadas por microscopia eletrônica e, por esta razão aplicamos esta metodologia para investigar a formação nativa de VEs diretamente na superfície celular.

No presente trabalho, testou-se a hipótese de que a cianobactéria *R.raciborskii* da cepa (CYRF-01) é capaz de produzir VEs e aumenta essa capacidade como mecanismo adaptativo para sustentar as respostas a diferentes tipos de estressores ambientais, como radiação UV e interação com *M. aeruginosa*. A radiação UV afeta as células de *R.raciborskii*, levando a alterações estruturais e moleculares (NOYMA, N. P. et al., 2015), enquanto a interação interespecífica entre *R.raciborskii* e *M. aeruginosa* promove efeitos antagônicos mútuos através da alelopatia (FIGUEREDO, CLEBER CUNHA; GIANI, 2009; MELLO et al., 2012; RZYMSKI et al., 2014).

3 OBJETIVOS

3.1 OBJETIVO GERAL

Investigar a habilidade da cianobactéria *R.raciborskii* em secretar VEs durante seu crescimento e em resposta a estresses ambientais.

3.1.1 OBJETIVOS ESPECÍFICOS

- Investigar se *R.raciborskii* secreta VEs em condições de crescimento padrão em culturas.
- Avaliar a produção de VEs em resposta à radiação UV.
- Investigar se a interação interespecífica entre *R.raciborskii* e *M. aeruginosa* induz a formação de VEs por *R.raciborskii*.
- Investigar se a possível secreção de VEs, por *R.raciborskii*, encontra-se associada com alterações estruturais e moleculares do envoltório celular.
- Avaliar a viabilidade celular de *R.raciborskii* quando exposta a estresses ambientais em culturas.

4 MATERIAIS E MÉTODOS

4.1 CEPAS DE CIANOBACTÉRIAS E CULTURAS ESTOQUE

Cepas de *R.raciborskii* (CYRF-01) e *M. aeruginosa* (MIRF-01) foram obtidas da coleção de culturas de cianobactérias do Laboratório de Ecofisiologia e Toxicologia de Cianobactérias da Universidade Federal do Rio de Janeiro (LETC) (Brasil). Ambas as linhagens foram isoladas do Reservatório do Funil (Brasil) em 2005, onde essas espécies atuam como competidores naturais e apresentam dominância sazonal (SOARES et al., 2009). Em culturas, *R.raciborskii* (CYRF-01) cresce como colônias filamentosas, enquanto *M. aeruginosa* (MIRF-01) cresce predominantemente como células únicas (Bolch e Blackburn, 1996). Geralmente, CYRF-01 produz saxitoxinas, enquanto MIRF-01 produz microcistinas (BLÁHA; BABICA; MARŠÁLEK, 2009; FERRÃO-FILHO et al., 2009; MELLO et al., 2012). Ambas as cepas foram mantidas em meio de crescimento estéril ASM-1 (GORHAM et al., 1964) em frascos Erlenmeyer de 300 mL colocados em uma sala climatizada a 25 ° C, 35 μ mol de fótons m⁻²s⁻¹, com um fotoperíodo de 12 : 12 h (MELLO et al., 2012).

4.2 EXPOSIÇÃO À RADIAÇÃO UV

Para avaliar a produção de VEs por *R.raciborskii* em resposta à radiação UV, as culturas foram expostas à radiação UV artificial (UVA + UVB, 280-400 nm), conforme descrito em um trabalho anterior (NOYMA, N. P. et al., 2015). Resumidamente, amostras de *R.raciborskii* de culturas em fase de crescimento exponencial foram ressuspensas em 40 mL de meio ASM-1 fresco (Gorham et al., 1964) a uma concentração inicial de 10⁶ células / mL e expostas à radiação UV artificial fornecido pelas lâmpadas UVA (TL 40/05; Philips; pico de emissão a 365 nm) e UVB (TL 20/01; Philips; pico de emissão a 312 nm) (Noyma et al., 2015). As doses de UV utilizadas no experimento foram

de 254,88 KJ m² (UVA) e 11,66 KJ m² (UVB), enquanto as intensidades foram de 11,8 W m⁻² (UVA) e 0,54 W m⁻² (UVB). O tratamento controle foi realizado em frascos de quartzo (40 mL) sob radiação fotossinteticamente ativa (PAR; 400-700 nm) a 90 μ mol de fótons m⁻² s⁻¹. Os tratamentos foram realizados durante 6 horas à temperatura ambiente (TR; 20 \pm 1 ° C) e todas as amostras foram cuidadosamente homogeneizadas antes das análises subsequentes. Todas as experiências foram realizadas em tréplica.

4.3 INTERAÇÃO INTERESPECÍFICA DE CIANOBACTÉRIAS

Para investigar o potencial processo de vesiculação em *R.raciborskii* em resposta a uma interação interespecífica, utilizou-se como modelo a co-cultura das linhagens CYRF-01 e MIRF-01. Monoculturas de CYRF-01 e culturas mistas com 50% de CYRF-01/50% de MIRF-01 a uma concentração de 10⁶ células / mL foram estabelecidas a partir de culturas de estoque como antes (Mello et al., 2012). As culturas mono e mista foram mantidas em frascos Erlenmeyer de 125 mL contendo 40 mL de meio de crescimento ASM-1 nas mesmas condições controladas (25 ° C e 55 μ mol de fóton m⁻²s⁻¹, fotoperíodo 12:12 h). Não houve limitação de recursos durante o período do experimento. Após 24 h, alíquotas de monoculturas de *R.raciborskii* (controles e culturas mistas) foram coletadas para análises subsequentes. Todas as experiências foram realizadas em tréplica.

4.4 DENSIDADE CELULAR

As amostras foram coletadas de cada grupo experimental, como anteriormente, para determinação da densidade celular (SIPAÚBA-TAVARES; ROCHA, 2003). As amostras foram fixadas com solução de lugol para preservação e as células foram contadas em microscópio de luz (B \times 41, Olympus) com aumento de 400 \times , utilizando um hemocitômetro Neubauer

aprimorado (SIPAÚBA-TAVARES; ROCHA, 2003). As análises foram realizadas no início (0 h) e no final dos experimentos de UV (6 h) e interação (24 h).

4.5 VIABILIDADE CELULAR

A formação de VEs pode estar associada à morte / viabilidade celular (GAMALIER et al., 2017). A integridade da membrana celular foi investigada usando uma sonda fluorescente (Backlight) como um indicador de viabilidade celular (BOULOS et al., 1999). As amostras foram coletadas de cada grupo e a proporção de células vivas / viáveis e mortas / não viáveis foi determinada usando o kit de viabilidade LIVE / DEAD BacLight (Molecular Probes, Inc, Thermo Fisher Scientific, Eugene, OR, Estados Unidos). Este kit contém uma mistura de corantes fluorescentes, SYTO® 9 e iodeto de propídio, que diferem tanto em suas características espectrais quanto em sua capacidade de penetrar nas membranas celulares bacterianas saudáveis. Células com membranas intactas (células vivas) coram de verde e aquelas com membranas danificadas (células mortas) coram de vermelho (BARBESTI et al., 2000; BERNEY et al., 2007; HOEFEL et al., 2003; JOUX; LEBARON, 2000). Uma mistura de volumes iguais das manchas (volume total de 0,9 µL) foi adicionada a 300 µL de cada amostra e incubada por 20 min no escuro. As lâminas (n = 3) para cada ponto do tempo foram preparadas em uma citocentrífuga (ShandonCytospin 4, ThermoElectron) como antes (NOYMA, N. P. et al., 2015; SILVA, T. P. et al., 2014) a 28 × g durante 5 minutos em aceleração média e avaliados sob um microscópio de fluorescência (BX-60, Olympus, Melville, NY, Estados Unidos) com comprimentos de onda de excitação de 450 a 480 nm, que permitem a visualização simultânea de células vivas e mortas. Para cada grupo, foram contados 30 filamentos e determinada a porcentagem de células vivas / mortas. As imagens foram tiradas com a câmera digital Evolution VF (Media Cybernetics, Silver Spring, MD, Estados Unidos) e o software ImagePro-Plus 5.0 (Media Cybernetics, Silver Spring, MD, Estados Unidos).

4.6 MICROSCOPIA ELETRÔNICA DE TRANSMISSÃO (MET)

Amostras de *R.raciborskii* coletadas dos grupos tratados e respectivos controles foram imediatamente fixadas em uma mistura de aldeídos recém-preparados (paraformaldeído a 1% e glutaraldeído a 1%) em tampão fosfato 0,1 M, pH 7,3, por 1 h à temperatura ambiente antes de qualquer procedimento de centrifugação subsequente. Em seguida, as amostras foram lavadas duas vezes em tampão fosfato 0,1 M, pH 7,3 (3.000 × g por 10 min) e armazenadas a 4 ° C para uso subsequente. Após a fixação, a incorporação do ágar foi realizada como antes (NOYMA, N. P. et al., 2015; SILVA, T. P. et al., 2014), para que amostras distribuídas uniformemente pudessem ser processadas como blocos de células facilmente manipulados. As pastilhas de ágar contendo amostras de água foram pós-fixadas em uma mistura de 1% de tetróxido de ósmio tamponado com fosfato e 1,5% de ferrocianeto de potássio (concentração final) por 1 h. Após lavagem com tampão fosfato 0,1 M, pH 7,3, as pastilhas foram coradas em bloco em acetato de uranil a 2% em tampão fosfato 0,1 M, pH 7,3 à temperatura ambiente e lavadas no mesmo tampão antes da desidratação em etanóis graduados (70, 95 e 100 %) e infiltração e incorporação em uma sequência Epon de óxido de propileno (PolyBed 812, Polysciences, Warrington, PA, Estados Unidos) (SILVA, T. P. et al., 2014). Após polimerização a 60 ° C por 16 h, cortes finos foram cortados usando uma faca de diamante em um ultramicrotomo LKB (LKB Instruments, Gaithersburg, MD, Estados Unidos). As seções foram montadas em grades de cobre de malha 200 não revestidas (Ted Pella) antes da coloração com citrato de chumbo e cianobactérias serem examinadas usando um microscópio eletrônico de transmissão (TecnaiSpirit G12; FEI Company, Eindhoven, Holanda) a 80 kV.

4.7 ANÁLISES QUANTITATIVAS POR MET

Para as análises quantitativas das VEs, as micrografias eletrônicas foram capturadas aleatoriamente com ampliações de 30.000 a 75.000 ×. Um total de 122 micrografias eletrônicas (grupo UV: 40 secções celulares; grupo de interação interespecífico: 42 secções celulares; grupos controle: 40 secções celulares) e um total de 396 VMEs foram analisados. Em seguida, foram estabelecidos o número de VEs / secção celular e a proporção de vesículas liberadoras de *R.raciborskii*, bem como o número de VMEs em processo de brotamento externo ou intimamente associado à superfície celular. Além disso, os diâmetros das VEs foram medidos e agrupados em diferentes faixas (20–80, 81–140, 141–200, 201–260 e 261–320 nm). Essas análises foram feitas em secções claras de células exibindo envelopes intactos e cada célula de um filamento foi considerada separadamente. Todos os estudos quantitativos foram realizados com o software Image J (Institutos Nacionais de Saúde, Bethesda, MD, Estados Unidos).

4.8 ANÁLISES DE ANEXINA V

Em células eucarióticas, é reconhecido que a fosfatidilserina é realocada para o folheto da membrana plasmática em locais na superfície celular onde ocorre o brotamento de VEs (HUGEL et al., 2005; MURALIDHARAN-CHARI et al., 2010). Para detectar fosfatidilserina exposta, as células foram coradas com anexina V conjugada com FITC, um marcador para essa molécula (Invitrogen, Carlsbad, CA, Estados Unidos). As amostras (1×10^7 células / mL) foram coletadas, lavadas com solução salina tamponada com fosfato (PBS) (fosfato de sódio 0,1 M, cloreto de sódio 0,15 M, pH 7,4) e coradas com anexina -V - FITC (20 μ M) (DWYER et al., 2012). Após a incubação por 20 min, as amostras foram analisadas por contraste de interferência diferencial (DIC) e microscopia de fluorescência (BX-60, Olympus, Melville, NY, Estados Unidos) a comprimentos de onda de excitação de 450 a 480 nm (FITC). Para cada grupo,

foram contados 30 filamentos e determinada a porcentagem de células positivas para anexina. As experiências foram realizadas em tréplica.

4.9 ANÁLISES ESTATÍSTICAS

Os dados das análises de densidade e viabilidade celular de cianobactérias foram comparados usando ANOVA, seguido pelo teste de comparação de Turkey. O número de VMEs por análises de TEM e a proporção de células positivas para anexina-V por microscopia de fluorescência foram comparados usando o teste t de Student ($P < 0,05$). As análises estatísticas e gráficos foram realizados no software Prism 6.0.1 (software GraphPad, San Diego, CA, Estados Unidos).

5 RESULTADOS

5.1 ULTRAESTRUTURA DE *R.raciborskii*

Primeiramente foi analisada em detalhes a ultraestrutura de *R.raciborskii* em condições normais em cultura. De forma semelhante a outras espécies de cianobactérias filamentosas, *R.raciborskii* mostrou envoltório celular composto de três camadas: membrana plasmática (mais interna), espaço periplasmático preenchido por periplasma com uma fina camada de peptidoglicano e membrana externa (Fig. 4A). Secções longitudinais mostraram claramente a organização das células nos filamentos (Fig. 4B) com junções intercelulares, caracterizadas pelo espessamento do envoltório celular entre uma célula e outra. O citoplasma mostrou-se repleto de sistemas de membranas lamelares caracterizados como típicos tilacoides associados com pequenas estruturas arredondadas elétron-densas, os ficobilissomos. Estruturas não membranosas, como carboxissomos (Fig. 4Aiii) e grânulos de polifosfato (Fig. 4Ai) apareceram próximos ou associados aos tilacoides. Vesículas de gás também foram frequentemente observadas (Fig. 4 Aii).

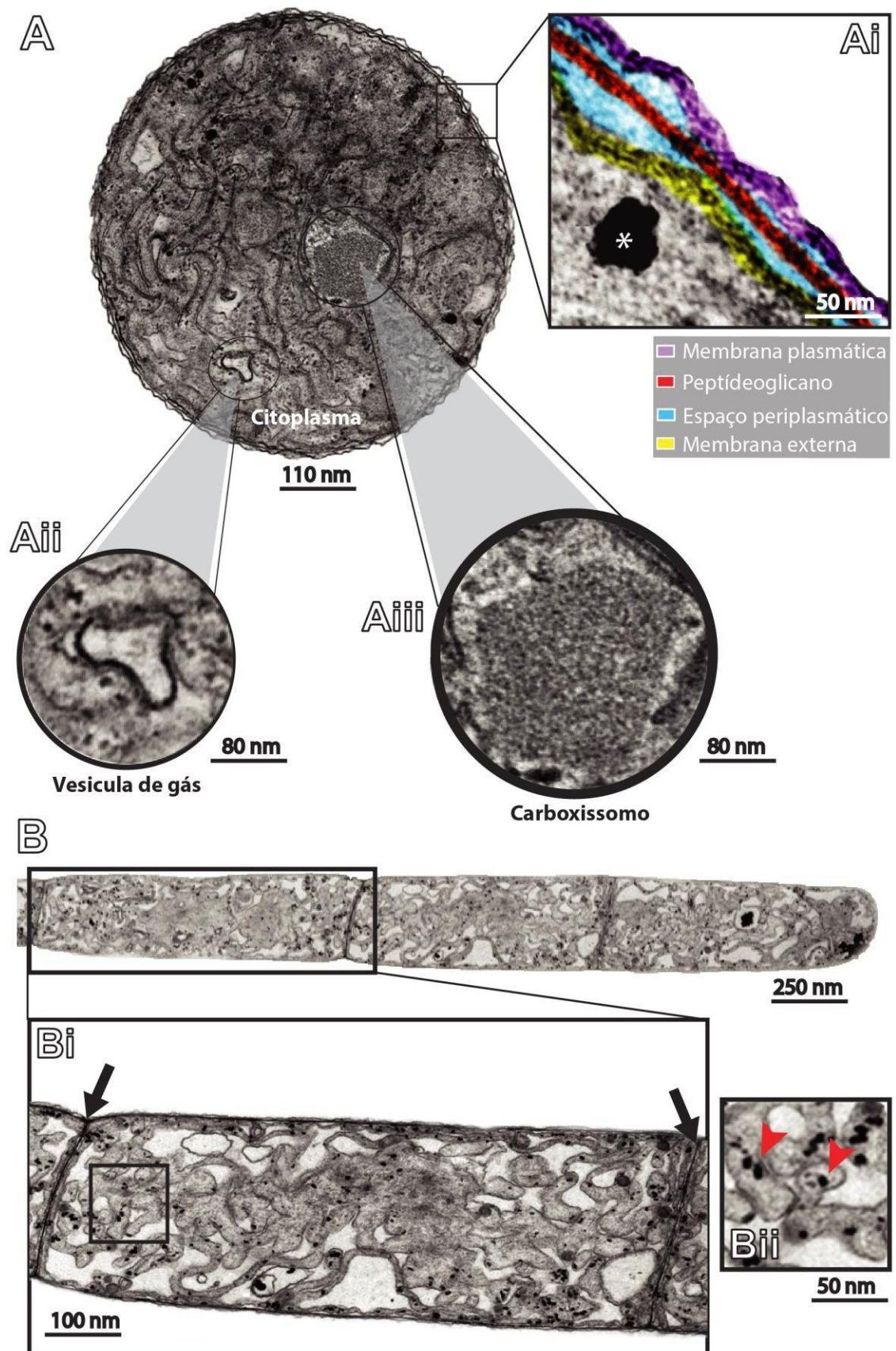


Figura 4: Ultraestrutura de *R.raciborskii*. (A) Secção transversal de uma célula de *R.raciborskii* mostrando diversas estruturas: em (Ai) o envoltório celular com seus diversos componentes, e um grânulo de polifosfato evidenciado pelo asterisco; em (Aii), destaque para uma vesícula de gás; em (Aiii), um carboxissomo é visto em maior aumento. (B) Secção longitudinal mostrando um filamento com três células de *R.raciborskii*; em (Bi) aparece em destaque as junções que formam os limites celulares; em (Bii), destaque para os ficobilissimos associados às membranas dos tilacoides.

5.2 *R.raciborskii* LIBERA VMEs EM CODIÇÕES NORMAIS DE CULTURA

Ao examinar as micrografias eletrônicas de *R.raciborskii* em culturas controle, uma observação marcante foi a presença de pequenas vesículas próximas ou intimamente associadas às células destas cianobactérias (Fig. 4). Análises ultraestruturais mais detalhadas revelaram que estas vesículas estavam brotando da membrana externa do envoltório celular das células de *R.raciborskii* (Figs. 6 Ai, 6 Aii). Essas VMEs foram identificadas como vesículas arredondadas limitadas por uma estrutura trilaminar típica das membranas fosfolipídicas (Figs. 6 Bi, Bii). Além disso, estas vesículas freqüentemente exibiam um revestimento amorfo externo, com a mesma estrutura observada na superfície do envelope celular e composto por substâncias poliméricas extracelulares (SPE) (Figs. 6 Ai, 6Aii, 6Aiii e 6Bii). Verificamos que $89,8 \pm 5,9\%$ (média \pm SEM, n = 396 vesículas) das VMEs exibiram essa camada de SPE.

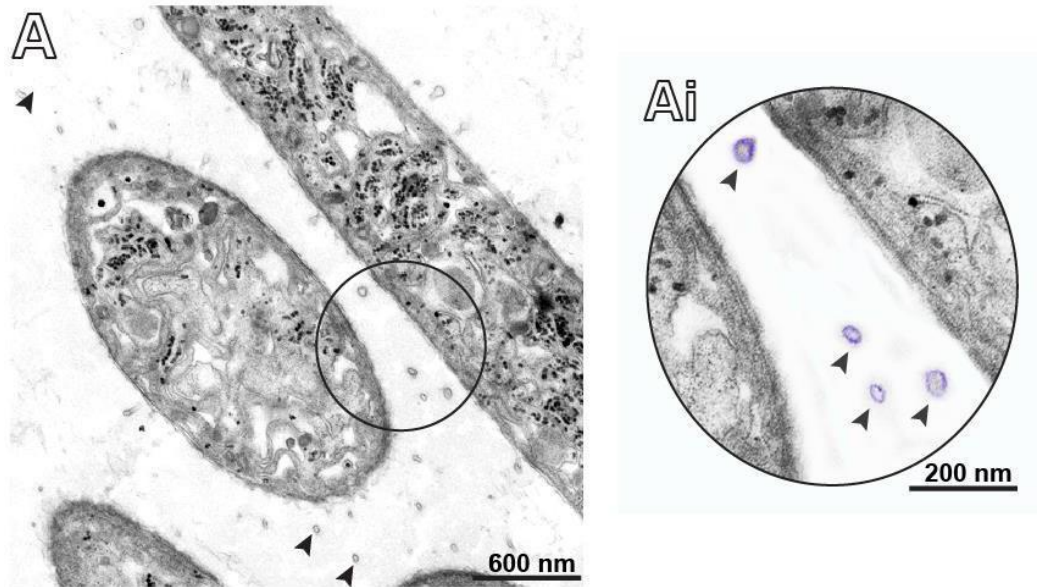


Figura 5: Secções longitudinais e transversais de *Raphidiopsis raciborskii* crescendo em condições de controle observadas por microscopia eletrônica de transmissão (TEM). Várias vesículas de membrana extracelular (indicadas pelas cabeças de setas em (A) e destacadas em roxo em (Ai) são observadas em torno das células cianobacterianas cultivadas.

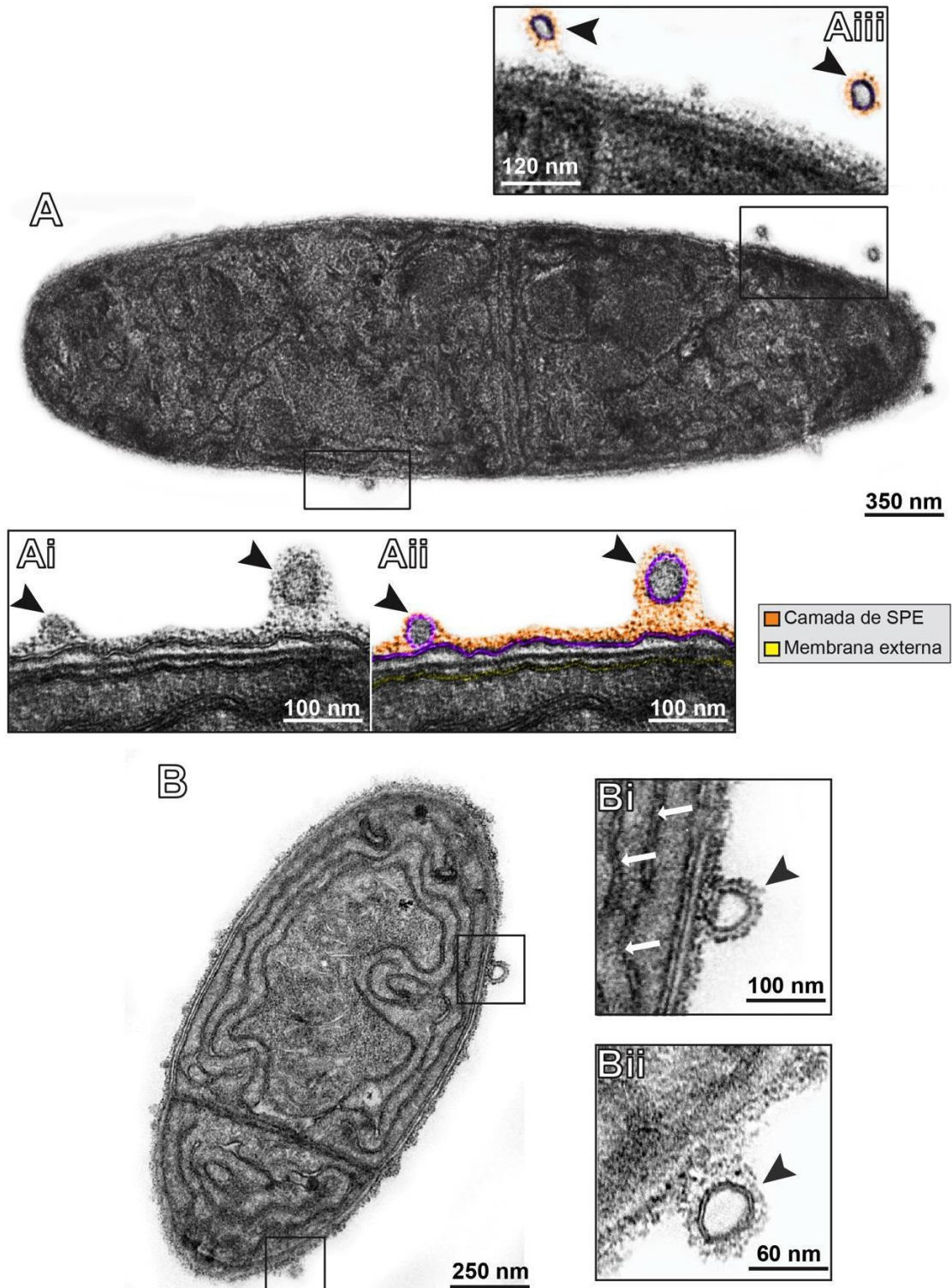


Figura 6: Micrografias eletrônicas representativas de células de *R. raciborskii* sob condições de cultura de controle (A, B). Em (Ai, Aii), o envelope celular é visto em alta ampliação. Essa estrutura é composta por duas membranas de duas camadas: a membrana interna ou plasmática (destacada em verde) e a membrana externa (destacada em roxo) que encerra o espaço periplásmico (azul claro) com uma fina camada de peptidoglicano (azul escuro). Observe a presença de VMEs (pontas de setas) com estrutura

trilaminar típica que claramente brotam da membrana externa. As vesículas segregadas freqüentemente exibiam um material amorfo externo (substâncias poliméricas extracelulares - EPS), como observado na superfície do envelope celular (destacado em laranja em Aii, Aiii). As membranas tilacoides são indicadas por setas brancas em (Bi).

5.3 PRODUÇÃO DE VMEs POR *R.raciborskii* AUMENTA COM O ESTRESSE INDUZIDO POR RADIAÇÃO UV

Em seguida, investigamos a produção de VMEs por *R.raciborskii* em resposta à radiação UV, como observado anteriormente para bactérias de ecossistemas aquáticos (GAMALIER et al., 2017). Depois de tratarmos as culturas de *R.raciborskii* com UV, como feito anteriormente (NOYMA, N. P. et al., 2015), as amostras foram imediatamente fixadas e processadas para MET. Micrografias de MET mostrando a membrana plasmática intacta foram adquiridas aleatoriamente e analisadas por processamento de imagem.

A exposição a essa radiação desencadeou a liberação de VMEs por *R.raciborskii* (Fig. 7A). Para quantificar o número de VMEs dos grupos experimental e controle, foram avaliadas seções de células de *R.raciborskii* ($n = 40$ células), e um total de 164 VMEs foram contadas. A radiação UV levou a um aumento significativo da produção de VMEs ($11,56 \pm 3,34$ VMEs / seção de células) em comparação com as células do grupo controle ($5,16 \pm 1,67$ VMEs / seção de células, $P < 0,0001$) (Fig. 7B). Além disso, ao pontuar o número de vesículas, descobrimos que em células não tratadas, 50% das células produtoras de VMEs liberaram 4-6 VMEs / seção de células, enquanto 70% das células tratadas com UV produziram 7-22 VMEs / seção de células (Fig. 7C). Esses dados demonstraram, portanto, uma hiper-vesiculação após o tratamento com radiação UV.

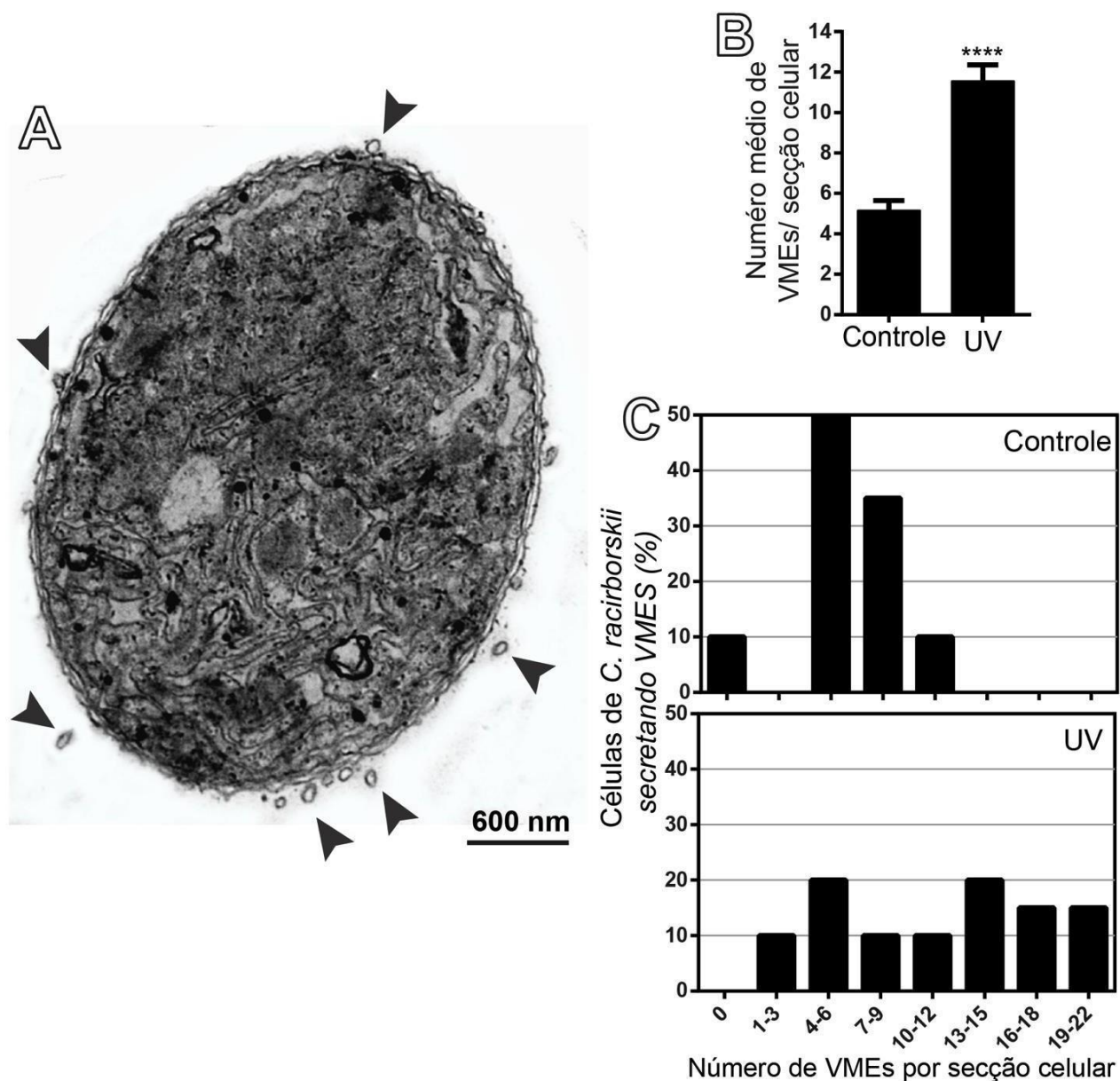


Figura 7: A liberação de vesículas de membrana externa (VMEs) por *R.raciborskii* aumenta em resposta à radiação UV. Em (A), micrografia eletrônica representativa de uma célula de *R.raciborskii* exposta à radiação UV mostra várias VMEs (pontas de setas) em diferentes graus de extrusão da superfície das cianobactérias. As VMEs por seção de célula e a proporção de células que liberam VMEs são mostrados em (B) e (C), respectivamente. Observe que a maioria das células (70%) produziu de 7 a 22 VMEs / seção de células no grupo tratado com UV, enquanto 50% das células produziu de 4 a 6 VMEs / seção de células no grupo controle. As contagens foram derivadas de três experiências com um total de 396 VMEs contadas em 122 micrografias eletrônicas. **** P <0,0001.

5.4 VESICULAÇÃO DE *R.raciborskii* AUMENTA EM RESPOSTA À INTERAÇÃO COM *M. aeruginosa*

Também detectamos um aumento da vesiculação por *R.raciborskii* quando cultivadas com uma cepa de *M. aeruginosa* (Figs. 8A, Ai). A análise quantitativa por MET mostrou aumento significativo no número total de VMEs secretados por *R.raciborskii* em co-culturas ($8,27 \pm 3,07$ VMEs / seção de células) em comparação com controles em monoculturas ($4,49 \pm 1,98$ VMEs / seção de células, $P < 0,001$) (Fig. 8B). Ao pontuar o número de VMEs, descobrimos que, enquanto 50% de *R.raciborskii* em monoculturas liberavam 4-6 VMEs / seção de células, 65% desta espécie em co-culturas produziam 7-22 vesículas / seção de células (Fig. 8C). Desta forma, a interação inter-específica também induziu hiper-vesiculação por *R.raciborskii*.

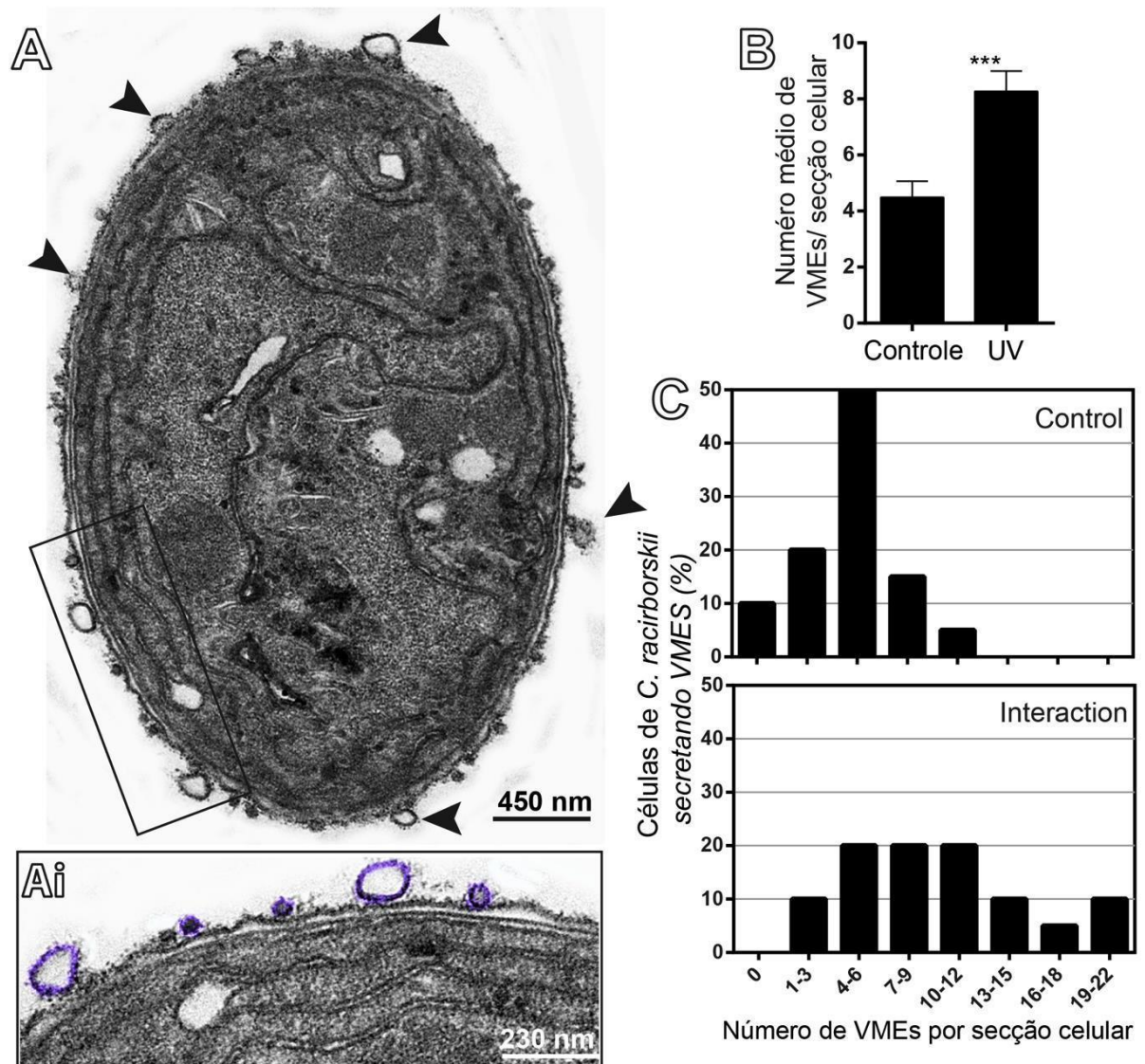


Figura 8: A co-cultura com uma cepa de *Microcystis aeruginosa* induz vesiculação por *R.raciborskii*. Em (A), micrografia eletrônica representativa de uma célula de *R.raciborskii*, coletada de uma cultura mista com *M. aeruginosa*, mostra várias VMEs (pontas de seta) na superfície da célula. Observe os diferentes tamanhos de VMEs nascentes (destacados em roxo em Ai). O número total de VMEs por seção de célula e a proporção de células que liberam VMEs são mostrados em (B) e (C), respectivamente. Enquanto a maioria das células de *R.raciborskii* (65%) produziu 7 a 22 VMEs / seção de células na cultura mista, a maioria das células (50%) no grupo controle liberou 4 a 6 VMEs / seção de células. As contagens foram derivadas de três experiências com um total de 396 VMEs contadas em 122 micrografias eletrônicas. *** P <0,0001.

5.5 A VESICULAÇÃO DE *R.raciborskii* É UM PROCESSO DINÂMICO

Nas células eucarióticas, a gênese das VEs é um evento rápido, com vesículas nascentes sendo observadas pelo MET em diferentes estágios de brotamento externo da membrana plasmática e/ou completamente liberados na superfície celular (AKUTHOTA et al., 2016). Em seguida, estudamos mais detalhadamente o processo de liberação de vesículas em *R.raciborskii* através da avaliação quantitativa de VMEs nascentes que estavam se desprendendo da membrana externa (VMEs em brotamento) ou livres na superfície celular (Fig. 9A-C). O número de VMEs emergentes nas células de *R.raciborskii* foi significativamente maior nos dois grupos tratados (Fig. 9D, E), em comparação aos controles.

Além disso, também estabelecemos o tamanho médio das VMEs liberadas pelas células de *R.raciborskii* em $86,23 \pm 4,86$ nm (média \pm SEM) de diâmetro nas condições de controle e $99,64 \pm 4,53$ e $101,50 \pm 4,62$ (média \pm SEM) nm após a exposição aos raios UV e interação com *M. aeruginosa*, respectivamente. Considerando todas as condições (grupos controle e tratado), os diâmetros de VMEs variaram de 20 a 320 nm, com a maioria das VMEs mostrando diâmetros entre 20 e 140 nm (Figs. 8F, G). As VMEs liberadas em resposta ao estímulo ao estresse (exposição aos raios UV e interação das espécies) foram maiores em comparação aos controles (Fig. 9F, G). Curiosamente, nossas análises de MET permitiram detectar a vesiculação por células individuais intimamente inter-relacionadas no mesmo filamento (Fig. 10).

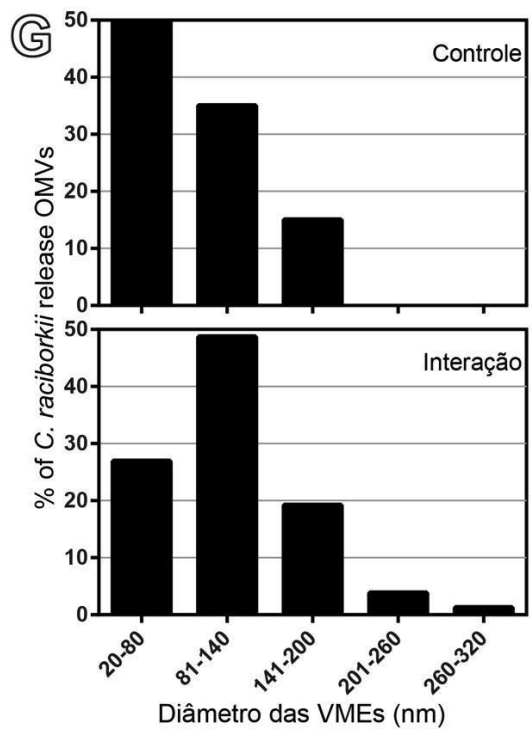
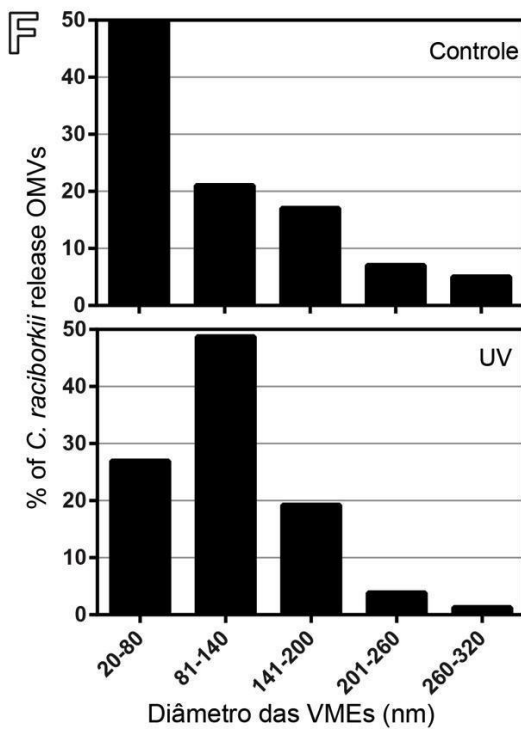
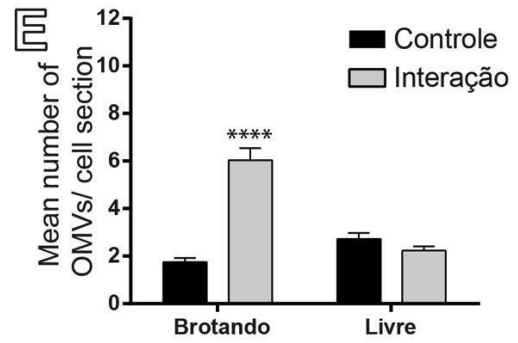
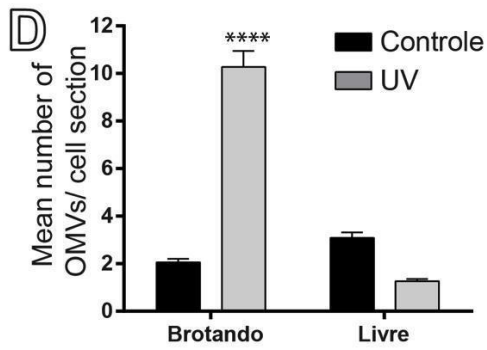
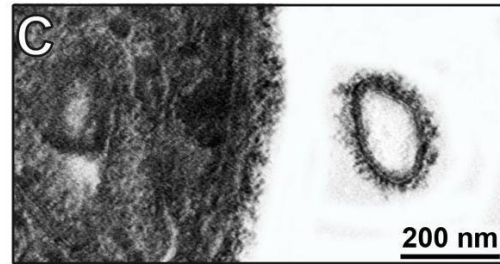
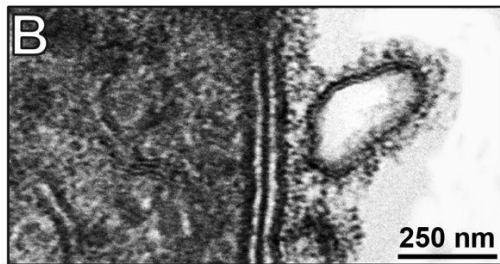


Figura 9: Caracterização de VMEs nascentes e taxa de brotamento de células de *R.raciborskii*. Em (A – C), VMEs com tamanhos diferentes e em brotamento externo progressivo da superfície celular são observados. (Ai, Aii) correspondem às áreas enquadradas em (A), vistas em maior ampliação. (D, E) O número de VMEs em processo de brotamento aumentou significativamente em resposta à radiação UV (D) e à interação com *M. aeruginosa* (E), em comparação com os respectivos controles. (F, G) Ambos os tratamentos provocaram secreção de VMEs com maior tamanho em comparação com os controles. As contagens foram derivadas de três experimentos com um total de 396 VMEs contados em 122 micrografias eletrônicas. **** P <0,0001.

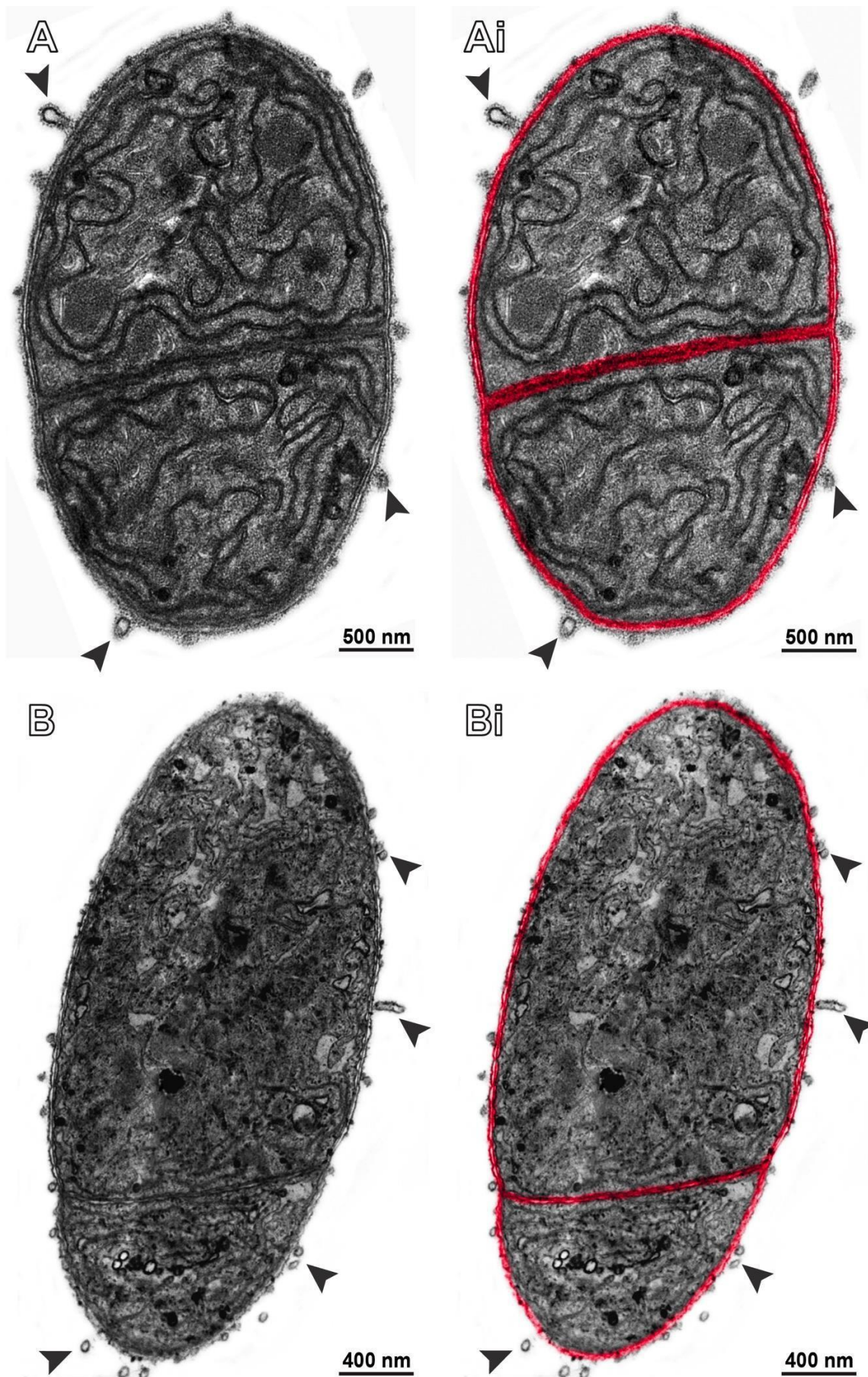


Figura 10: Vesiculação como evento dinâmico em células de *R.raciborskii* no mesmo filamento. (A e B) Micrografias eletrônicas de um filamento representativo de *R.raciborskii* após exposição a UV mostram duas células em processo de vesiculação. Observe o alto número de VMEs (indicadas por pontas de seta) sendo formadas na superfície da célula. Em (Ai e Bi), o envoltório da célula foi colorido em vermelho para destacar os contornos das células individuais.

5.6 A FOSFATIDILSERINA SE REALOCA NA MEMBRANA EXTERNA DE *R.raciborskii*

Como observado, a realocação de fosfatidilserina pode estar associada à secreção de VEs (HUGEL et al., 2005; MURALIDHARAN-CHARI et al., 2010). Assim, estudamos se o mesmo fenômeno ocorreria na membrana externa das cianobactérias. Células foram marcadas com anexina-V-FITC e as amostras foram analisadas por microscopia de fluorescência (Fig. 11A). Uma proporção maior de *R.raciborskii* mostrou coloração positiva para anexina V após tratamento com UV e interação com *M. aeruginosa* em comparação com células de controle (Fig. 11B).

5.7 VIABILIDADE CELULAR E DENSIDADE DE *R.raciborskii*

Em paralelo às análises com anexina, também investigamos a concentração e a viabilidade celular, usando avaliações clássicas da densidade celular e marcadores moleculares para viabilidade da membrana (baclight) (JOUX; LEBARON, 2000; SIPAÚBA-TAVARES; ROCHA, 2003). Como esperado, a densidade celular foi significativamente reduzida, enquanto a proporção de cianobactérias não viáveis aumentou significativamente após a exposição aos raios UV (Fig. 11C, Ci), como demonstrado anteriormente (NOYMA, N. P. et al., 2015). Por outro lado, nem a redução da densidade celular nem a perda da viabilidade celular foram observadas como resultado da interação com *M. aeruginosa* (Fig. 11D, Di).

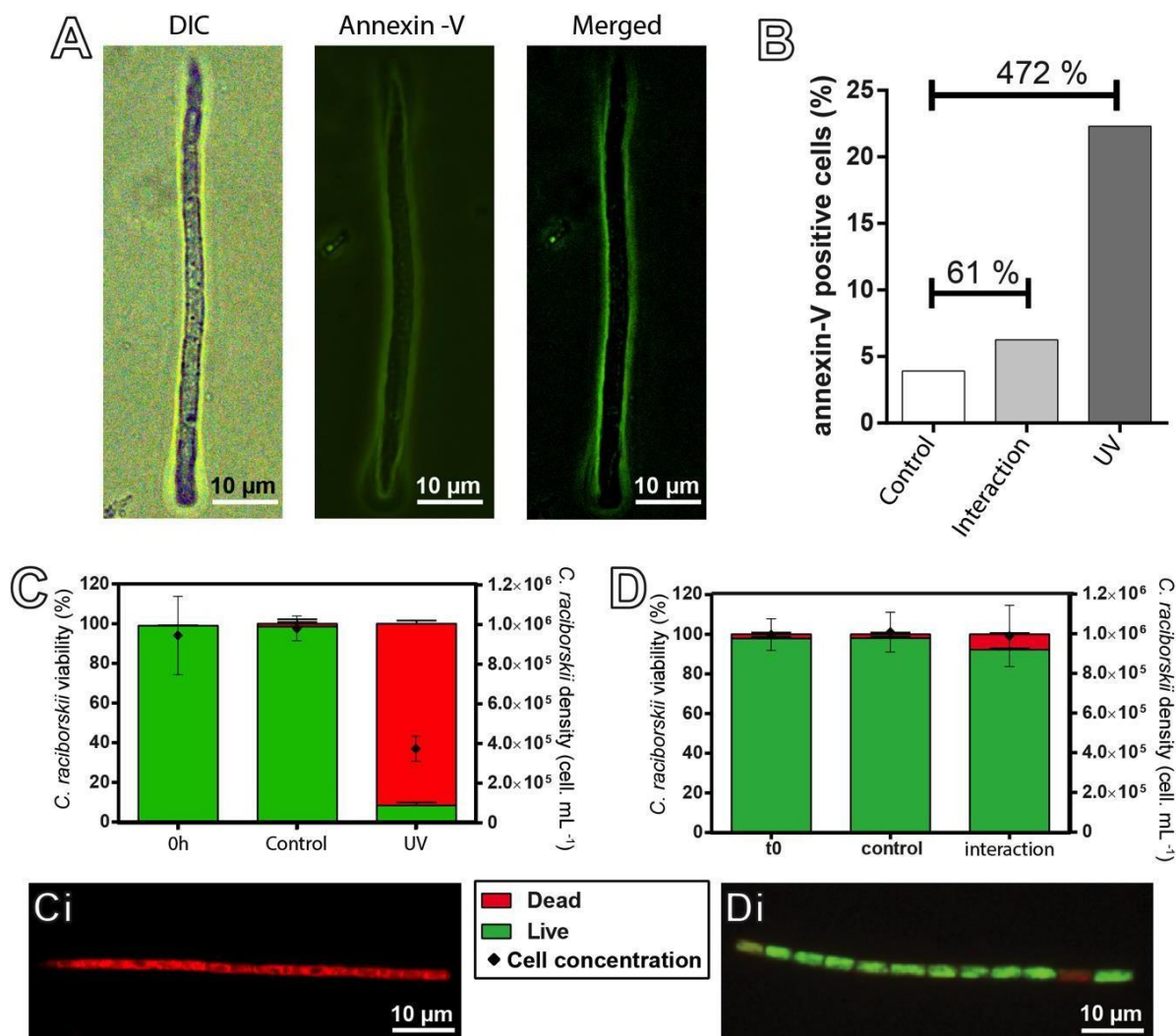


Figura 11: Relocação de fosfatidilserina, densidade celular e viabilidade celular de *R.raciborskii*. (A) Imagens representativas de células de *R.raciborskii* vistas por contraste de interferência diferencial (DIC) e microscopia de fluorescência (campos idênticos) após exposição ou não à radiação UV. A fluorescência verde indica células positivas para anexina V. (B) A proporção de células positivas para anexina-V aumentou após exposição à radiação UV e interação com *M. aeruginosa*. (C, D) A densidade e viabilidade de *R.raciborskii* diminuíram significativamente em resposta à radiação UV (C), mas não em culturas mistas com *M. aeruginosa* (D). (Ci, Di) Mostra imagens fluorescentes representativas de células coradas com Baclight. As células vivas são vistas em verde e as mortas em vermelho.

6 DISCUSSÃO

Os dados apresentados neste trabalho demonstram, pela primeira vez, que a vesiculação é um fenômeno comum para *R.raciborskii*. Ao estudar a cepa CYRF-01, mostramos que essa cianobactéria libera constitutivamente VMEs durante seu crescimento normal e amplia essa capacidade em resposta a estressores ambientais, como radiação UV e interação com um concorrente em potencial. Assim, reconhecemos que *R.raciborskii* tem competência para secretar VMEs e lidar com diferentes situações de estresse com o aumento da gênese dessas vesículas. Nossos dados são apoiados por trabalhos anteriores sobre outras espécies de cianobactérias e diferentes cepas que encontraram produção de VEs em resposta a estressores, como condições hiperosmóticas (hipersalinas) (XU et al., 2013), suplementação de antibióticos (OLIVEIRA, P. et al., 2016) ou temperatura reduzida (OLIVEIRA, P. et al., 2016). Além disso, o presente trabalho expande a compreensão acerca das cianobactérias como organismos capazes de liberar ativamente as VEs (BILLER et al., 2014; BRITO et al., 2017; OLIVEIRA, V. E. et al., 2015; PARDO et al., 2015; ZHENG et al., 2009).

Para identificar e caracterizar as VEs, usamos o MET, técnica crucial para distinguir as VEs de partículas não membranosas de tamanho semelhante (LÖTVALL et al., 2014). Isso ocorre porque as VEs são nanoestruturas delimitadas por uma membrana fosfolipídica, que pode ser visualizada de forma inequívoca pelo MET como uma estrutura "trilaminar" na qual as "cabeças" de fosfato hidrofílico são eletron-densas e as "caudas" de ácidos graxos hidrofóbicas são eletron-lúcidas (BOZZOLA; RUSSELL, 1999; LODISH et al., 2008). Por esse motivo, o uso do MET fornece a evidência mais direta para a produção de VEs. De fato, o MET é considerado uma técnica padrão para caracterizar as VEs individuais (AKUTHOTA et al., 2016; GAMALIER et al., 2017; LAWSON et al., 2016), conforme endossado pela Sociedade Internacional de Vesículas Extracelulares (ISEV) (LÖTVALL et al., 2014). Além disso, o ISEV recomenda que, para uma melhor caracterização do evento de vesiculação, as imagens de MET mostrem um campo amplo da superfície

celular e não apenas vesículas individualizadas de forma que o evento de vesiculação possa ser bem estabelecido e capturado (LÖTVALL et al., 2014). Nossas análises de MET incluíram uma abordagem quantitativa, que permitiu caracterizar claramente o tamanho e quantificar muitas VEs nascentes em toda a superfície celular (Fig. 4, 6), fornecendo evidências conclusivas para a produção de VEs por *R.raciborskii*. Também fornecemos, pela primeira vez, evidências diretas de que as VEs liberadas por esta cianobactéria são formadas por fissão da membrana externa, semelhante ao processo descrito para liberação de VMEs por espécies bacterianas gram-negativas (KULP; KUEHN, 2010). Essa semelhança de vesiculação da cianobactéria com a vesiculação de bactéria gram-negativa foi sugerida por estudos anteriores (BRITO et al., 2017; OLIVEIRA, V. E. et al., 2015; ZHENG et al., 2009). Em um estudo usando *Synechocystis* PCC6803, por exemplo, proteínas e lipídios derivados da membrana externa foram detectados em sobrenadantes derivados de *Synechocystis* (PARDO et al., 2015). Além disso, a abordagem de alta resolução permitiu a criação de imagens da camada SPE na maioria das VMEs nascentes. Isso significa que as VMEs apresentam uma camada de natureza polissacarídica, que pode fornecer integridade estrutural e proteção contra a radiação UV, importante para a persistência das VMEs no ambiente (PEREIRA et al., 2009). Outros papéis potenciais para a camada SPE nas VMEs incluem servir como armazenamento alternativo para compostos de carbono (PANNARD et al., 2016; PEREIRA et al., 2009). De fato, os açúcares são moléculas previamente sintetizadas para serem transportadas por VMEs em cianobactérias (XU et al., 2013).

Nosso estudo também demonstrou que o número de VMEs em processo de brotamento externo da membrana externa, ou seja, ainda fisicamente ligado a essa membrana, aumentou nas células sob estresse em comparação às células em crescimento normal (Fig. 6). A maior taxa de produção de VMEs após a exposição às condições de estresse possivelmente nos permitiu capturar com mais frequência os diferentes graus de vesícula que brotam da membrana externa. Além disso, nossos resultados revelaram que as VMEs nascentes mostram tamanhos variados (faixa de 20 a 320 nm, Fig. 6), indicando que existem populações morfologicamente diferentes de VMEs

secretadas por *R.raciborskii*. Também capturamos o evento de vesiculação por células individuais no mesmo filamento (Fig. 5), indicando que células diferentes são capazes de responder coletivamente aos agentes estressores.

Para explorar a ligação entre a liberação da vesícula e o estresse ambiental, expusemos *R.raciborskii* a diferentes condições, como a radiação UV, que é conhecida por promover claras alterações na densidade, viabilidade e estruturas fotossintéticas dessa cianobactéria (NOYMA, N. P. et al., 2015), bem como danos em outras espécies de cianobactérias (SINGH; HADER; SINHA, 2010). Os resultados mostraram um aumento na produção de VMEs em resposta à radiação UV. Notavelmente, as análises em alta resolução também mostraram que a vesiculação por cianobactérias tratadas com UV não era resultado de lise celular. Embora a maioria das células seja inviável, como detectado por um marcador de permeabilidade à membrana (Fig. 11), as VMEs nascentes foram liberadas a partir de cianobactérias estruturalmente intactas (Figs. 4 e 6). O mesmo fenômeno foi observado para bactérias gram-negativas expostas à radiação UV (GAMALIER et al., 2017), indicando que as células danificadas liberam VMEs antes da lise celular, provavelmente como um mecanismo de proteção contra a radiação UV para aumentar a sobrevivência das células, uma vez que compostos celulares como o DNA poderiam ser preservados dentro dessas nanovesículas.

Quando em co-culturas com *M. aeruginosa*, *R.raciborskii* pode inibir o crescimento de cepas de *M. aeruginosa* (FIGUEREDO, CLEBER C; GIANI; BIRD, 2007; MELLO et al., 2012; RZYMSKI et al., 2014). Aleloquímicos secretados por *R.raciborskii* provavelmente mediam essa ação (FIGUEREDO, CLEBER C et al., 2007; RZYMSKI et al., 2014). Como as VEs constituem uma maneira pela qual as células eucarióticas e bacterianas secretam produtos para o meio extracelular, hipotetizamos que *R.raciborskii* seria capaz de aumentar a vesiculação após a interação com *M. aeruginosa*. Nossos resultados demonstraram claramente essa capacidade. Embora a identidade química da carga dentro das vesículas ainda esteja por estabelecer, é claro que *R.raciborskii* responde à interação com a produção de vesículas secretoras, que

podem estar envolvidas no mecanismo de inibição do crescimento de *M. aeruginosa*.

A fosfatidilserina é um glicerofosfolípídeo aniônico presente nas membranas dos eucarióticos (KAY; GRINSTEIN, 2011) e de algumas células procarióticas (SOHLENKAMP; GEIGER, 2016). Em eucariotos, a fosfatidilserina está localizada no folheto interno da membrana plasmática e sua externalização, ou seja, sua exposição no folheto externo dessa membrana, tem sido relatada como um evento molecular distinto durante a formação de VEs (GONZÁLEZ-CANO et al., 2010; HUGEL et al., 2005; MURALIDHARAN-CHARI et al., 2010), sendo assim usada como uma evidência adicional para a vesiculação celular (AKUTHOTA et al., 2016; GONZÁLEZ-CANO et al., 2010). Aqui, os filamentos de *R.raciborskii* sob condições de estresse têm maior proporção de células positivas para fosfatidilserina em comparação com as células que crescem em condições de controle (Fig. 11). A externalização da fosfatidilserina também é um evento comumente associado à apoptose em células procarióticas (KASUBA; VAVILALA; D'SOUZA, 2015; ZHENG et al., 2013) e eucarióticas (SUZANNE; STELLER, 2013). No entanto, de maneira geral, os resultados indicam que a externalização da fosfatidilserina pode ser considerada mais uma evidência para a vesiculação por *R.raciborskii*, em vez de um indicativo de morte celular. Primeiro, usando um marcador de viabilidade celular, células não viáveis/mortas e viáveis/vivas avaliadas após exposição a UV ou interação interespecífica mostraram externalização de fosfatidilserina (Fig. 11). Segundo, a MET, considerada técnica padronizada para detectar apoptose, revelou ausência de características morfológicas típicas ou semelhantes da apoptose nas células de *R.raciborskii* após os dois tratamentos, como a ocorrência de células vazias com envelope celular intacto e/ou células citoplasmáticas condensação/retração (SILVA, T. et al., 2017). Portanto, as análises de fosfatidilserina corroboram nossos resultados de MET mostrando vesiculação amplificada de *R.raciborskii* em resposta a condições de estresse.

Nossos achados de fosfatidilserina também destacam um importante aspecto biológico de *R.raciborskii*: essa cianobactéria parece alterar a

composição / organização de seus lipídios da membrana em resposta a alterações no ambiente, conforme documentado para outras bactérias (SOHLENKAMP; GEIGER, 2016). De fato, diferentes espécies bacterianas são capazes de alterar a composição / organização / fluidez dos lipídios da membrana para sobreviver em condições desfavoráveis ou mesmo para se adaptar a uma nova situação, como privação de nutrientes ou aumento / diminuição da temperatura (SOHLENKAMP; GEIGER, 2016). Além de sustentar o mecanismo de produção de VMEs, a modificação lipídica da membrana em *R.raciborskii* poderia permitir uma resposta rápida às mudanças nas condições ambientais, como observado para outras espécies bacterianas (SOHLENKAMP; GEIGER, 2016).

7 CONCLUSÕES

Tomados em conjunto, nossas descobertas identificam, pela primeira vez, que *R.raciborskii* (CYRF-01) secreta VMEs durante o crescimento normal e que a liberação dessas vesículas no ambiente circundante aumenta em resposta à radiação UV ou à interação interespecífica com *M. aeruginosa*. O papel potencial dessas VMEs para interagir com células vizinhas ou promover a adaptação de cianobactérias aguarda novas investigações.

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ANEXOS

Durante o desenvolvimento do trabalho foi publicado o artigo intitulado: "The Cyanobacterium *Cylindrospermopsis raciborskii* (CYRF-01) Responds to Environmental Stresses with Increased Vesiculation Detected at Single-Cell Resolution" como primeiro autor. Além disso, outro artigo, com participação na autoria também foi publicado com o título: "Increased production of outer membrane vesicles by cultured freshwater bacteria in response to ultraviolet radiation". Os dois encontram-se na íntegra nas próximas páginas.



The Cyanobacterium *Cylindrospermopsis raciborskii* (CYRF-01) Responds to Environmental Stresses with Increased Vesiculation Detected at Single-Cell Resolution

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Secretion of membrane-limited vesicles, collectively termed extracellular vesicles (EVs), is an important biological process of both eukaryotic and prokaryotic cells. This process has been observed in bacteria, but remains to be better characterized at high resolution in cyanobacteria. In the present work, we address the release of EVs by *Cylindrospermopsis raciborskii* (CYRF-01), a filamentous bloom-forming cyanobacterium, exposed to environmental stressors. First, non-axenic cultures of *C. raciborskii* (CYRF-01) were exposed to ultraviolet radiation (UVA + UVB) over a 1 h period, which is known to induce structural damage to this species. Second, *C. raciborskii* was co-cultured in interaction with another cyanobacterium species, *Microcystis aeruginosa* (MIRF-01), over a 24 h period. After the incubation times, cell density and viability were analyzed, and samples were processed for transmission electron microscopy (TEM). Our ultrastructural analyses revealed that *C. raciborskii* constitutively releases EVs from the outer membrane during its normal growth and amplifies such ability in response to environmental stressors. Both situations induced significant formation of outer membrane vesicles (OMVs) by *C. raciborskii* compared to control cells. Quantitative TEM revealed an increase of 48% (UV) and 60% (interaction) in the OMV numbers compared to control groups. Considering all groups, the OMVs ranged in size from 20 to 300 nm in diameter, with most OMVs showing diameters between 20 and 140 nm. Additionally, we detected that OMV formation is accompanied by phosphatidylserine exposure, a molecular event also observed in EV-secreting eukaryotic cells. Altogether, we identified for the first time that *C. raciborskii* has the competence to secrete OMVs and that under different stress situations the genesis of these vesicles is increased. The amplified ability of cyanobacteria to release OMVs may be associated with adaptive responses to changes in environmental conditions and interspecies cell communication.

Keywords: outer membrane vesicles, extracellular vesicles, cyanobacteria, *Cylindrospermopsis raciborskii*, ultraviolet radiation, interspecific interaction, transmission electron microscopy, phosphatidylserine

INTRODUCTION

The bloom-forming cyanobacterium *Cylindrospermopsis raciborskii* has attracted considerable attention due its widespread distribution and its potential ability to produce diverse toxins, such as hepatotoxins and neurotoxins that can cause detrimental impacts on the environmental health (Molica et al., 2002; Carneiro et al., 2013). Some cyanotoxins are directly associated with fish, domestic livestock, and even human mortalities (Codd et al., 2005; Dittmann and Wiegand, 2006; Svircev et al., 2016). The mechanisms that govern the geographic expansion and bloom formation of *C. raciborskii* involve its high plasticity and physiological tolerance to a wide range of environmental conditions, such as light, nutrients and temperature, as well as its antagonistic interactions with other phytoplankton species (Padisák, 1997; Beamud et al., 2016; Burford et al., 2016). Furthermore, production of allelochemicals by *C. raciborskii* has been proposed as an additional advantage to the dominance of this species via inhibition of other cyanobacteria competitors, including *Microcystis aeruginosa* (Figueredo et al., 2007; Mello et al., 2012).

Secretion of membrane-limited vesicles, collectively termed extracellular vesicles (EVs), is an important cellular event of both eukaryotic and prokaryotic cells. The capacity of bacteria to release EVs has been exponentially demonstrated (reviewed in Kulp and Kuehn, 2010; Jan, 2017). These nano-scale vesicles are extruded from the outer membrane of gram-negative bacteria and have been associated with fundamental biological processes such as pathogenesis (Kolling and Matthews, 1999; Rivera et al., 2010); cellular defense (Manning and Kuehn, 2011; Baumgarten et al., 2012), cell communication (Mashburn-Warren et al., 2008) and DNA transfer (Rumbo et al., 2011). In marine microbial communities, cyanobacteria and heterotrophic bacteria-derived vesicles were reported to be abundant in coastal and open-ocean seawater samples and implicated in marine carbon flux (Biller et al., 2014). More recently, it was indicated that the cyanobacterium *Synechocystis* PCC6803 produces EVs in a manner similar to gram-negative bacteria, that is, from the outer membrane (Pardo et al., 2015). However, little is still known on the ability of cyanobacteria to secrete outer membrane vesicles (OMVs), their biogenesis and potential functions in aquatic biology.

It has been reported that bacteria produce EVs in freshwater ecosystems (Silva et al., 2014) and that ultraviolet (UV) radiation, an environmental stressor, elicits increased vesiculation in heterotrophic bacteria from freshwater (Gamalier et al., 2017). The amplified release of EVs might be a regulated response offering an effective adaptive mechanism under natural and diverse stress conditions (reviewed in Jan, 2017). EVs could pack a variety of cargos related to population survival and persistence, removal of undesirable envelope proteins (McBroom and Kuehn, 2007) or self-defense molecules (Rivera et al., 2010; Manning and Kuehn, 2011).

In the present work, we tested the hypothesis that the cyanobacterium *C. raciborskii* is also able to produce EVs and increases this ability as an adaptive mechanism to underlie the responses to different kinds of environmental stressors such as

UV radiation and interaction with *M. aeruginosa*. UV radiation affects *C. raciborskii* cells leading to molecular and structural changes (Noyma et al., 2015) while the interspecific interaction between *C. raciborskii* and *M. aeruginosa* promotes mutual antagonistic effects through allelopathy (Figueredo and Giani, 2009; Mello et al., 2012; Rzymyski et al., 2014).

The use of transmission electron microscopy (TEM) enables unambiguous visualization of EVs in both eukaryotic and prokaryotic cells, and their genesis during different situations (Akuthota et al., 2016; Gamalier et al., 2017). By performing a comprehensive TEM study, we demonstrated, for the first time, that *C. raciborskii* produces EVs, which were clearly characterized as typical nanovesicles shedding out from the outer membrane. Quantitative TEM revealed that both environmental UV and interspecific interaction were able to positively influence the biogenesis of OMVs by this cyanobacterium.

MATERIALS AND METHODS

Cyanobacterial Strains and Stock Cultures

Strains of *C. raciborskii* (CYRF-01) and *M. aeruginosa* (MIRF-01) were obtained from the cyanobacteria culture collection of the Laboratory of Cyanobacterial Ecophysiology and Toxicology, Federal University of Rio de Janeiro (LETC) (Brazil). Both strains were isolated from Funil Reservoir (Brazil) in 2005, where these species act as natural competitors and show seasonal dominance (Soares et al., 2009). In cultures, *C. raciborskii* (CYRF-

01) grows as filamentous colonies while *M. aeruginosa* (MIRF-01) grows predominantly as single cells (Bolch and Blackburn, 1996). Usually, CYRF-01 produces saxitoxins, whereas MIRF-01 produces microcystins (Bláha et al., 2009; Ferrão-Filho et al., 2009; Mello et al., 2012). Both strains were maintained in sterile ASM-1 growth medium (Gorham et al., 1964) in 300 mL Erlenmeyer flasks placed in a climate-controlled room at 25°C,

35 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$, with a photoperiod of 12:12 h (Mello et al., 2012).

UV Exposure

In order to evaluate the production of EVs by *C. raciborskii* in response to UV radiation, we exposed cultures to artificial UV radiation (UVA UVB, 280–400 nm) as described in a previous work (Noyma et al., 2015). Briefly, samples of *C. raciborskii* from cultures in exponential growth phase were re-suspended in 40 mL of fresh ASM-1 medium (Gorham et al., 1964) at an initial concentration of 10^6 cells/mL and were exposed to artificial UV radiation supplied by UVA (TL 40/05; Philips; emission peak at 365 nm) and UVB (TL 20/01; Philips; emission peak at 312 nm) lamps (Noyma et al., 2015). The UV doses used in the experiment were 254.88 KJ m² (UVA) and 11.66 KJ m² (UVB) while the intensities were 11.8 W m⁻² (UVA) and 0.54 W m⁻² (UVB). The control treatment was performed in quartz flasks (40 mL) under photosynthetically active radiation (PAR; 400–700 nm) at 90 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$. Treatments were performed during 6 h at room temperature (RT; 20 ± 1°C)

and all samples were carefully homogenized prior to subsequent analyses. All experiments were performed in triplicate.

Cyanobacteria Species Interaction

To investigate the potential vesiculation process in *C. raciborskii* in response to an interspecific interaction, we used as a model the co-culture of the strains CYRF-01 and MIRF-01. Monocultures of CYRF-01 and mixed cultures with 50% CYRF-01/50% MIRF-01 at a concentration of 10^6 cells/mL were established from stock cultures as before (Mello et al., 2012). Mono and mixed cultures were maintained in 125 mL Erlenmeyer flasks containing 40 mL of ASM-1 growth medium at the same controlled conditions (25°C and 55 μ mol photon $m^{-2}s^{-1}$, photoperiod 12:12 h). There was no resource limitation during the experiment period. After 24 h, aliquots from *C. raciborskii* monocultures (controls) and mixed cultures were taken for subsequent analyses. All experiments were performed in triplicate.

Cell Density

Samples were taken from each experimental group for cell density determination as before (Sipaúba-Tavares and Rocha, 2003). Samples were fixed with lugol solution for preservation and the cells were counted on a light microscope (B 41, Olympus) at 400 magnification by using an improved Neubauer hemocytometer (Sipaúba-Tavares and Rocha, 2003). Analyses were performed at the beginning (0 h) and at the end of UV (6 h) and interaction (24 h) experiments.

Cyanobacterial Cell Viability

The formation of EVs may be associated with cell death/viability (Gamalier et al., 2017). Cell membrane integrity was investigated by using a fluorescent probe (Backlight) as an indicator for cell viability (Boulos et al., 1999). Samples were collected from each group and the proportion of live/viable and dead/non-viable cells was determined using LIVE/DEAD BacLight viability kit (Molecular Probes, Inc, Thermo Fisher Scientific, Eugene, OR, United States). This kit contains a mixture of fluorescent dyes, SYTO[®] 9 and propidium iodide, which differ both in their spectral characteristics and their ability to penetrate healthy bacterial cell membranes. Cells with intact membranes (live cells) stain green and those with damaged membranes (dead cells) stain red (Barbesti et al., 2000; Joux and Lebaron, 2000; Hoefel et al., 2003; Berney et al., 2007). A mixture of equal volumes of the stains (total volume of 0.9 μ L) was added to 300 μ L of each sample and incubated for 20 min in the dark. Slides ($n = 3$) for each time point were prepared in a cytocentrifuge (Shandon Cytospin 4, Thermo Electron) as before (Silva et al., 2014; Noyma et al., 2015) at 28 g during 5 min at medium acceleration and evaluated under a fluorescence microscope (BX-60, Olympus, Melville, NY, United States) at 450–480 nm excitation wavelengths, which enable simultaneous visualization of live and dead cells. For each group, 30 filaments were counted and the percentage of live/dead cells determined. Images were taken using Evolution VF (Media Cybernetics, Silver Spring, MD, United States) digital camera and Image Pro-Plus 5.0 software (Media Cybernetics, Silver Spring, MD, United States).

Transmission Electron Microscopy (TEM)

Cylindrospermopsis raciborskii samples collected from treated groups and respective controls were immediately fixed in a mixture of freshly prepared aldehydes (1% paraformaldehyde and 1% glutaraldehyde) in 0.1 M phosphate buffer, pH 7.3, for 1 h at RT before any subsequent centrifugation procedure. Next, samples were washed twice in 0.1 M phosphate buffer, pH 7.3 (3,000 $\times g$ for 10 min), and stored at 4°C for subsequent use. After fixation, agar embedding was performed as before (Silva et al., 2014; Noyma et al., 2015), so that uniformly distributed specimens could be processed as easily handled blocks of cells. Agar pellets containing water specimens were post-fixed in a mixture of 1% phosphate-buffered osmium tetroxide and 1.5% potassium ferrocyanide (final concentration) for 1 h. After washing with 0.1 M phosphate buffer, pH 7.3, pellets were stained *en bloc* in 2% uranyl acetate in 0.1 M phosphate buffer, pH 7.3 at RT and washed in the same buffer prior to dehydration in graded ethanols (70, 95, and 100%), and infiltration and embedding in a propylene oxide-Epon sequence (PolyBed 812, Polysciences, Warrington, PA, United States) (Silva et al., 2014). After polymerization at 60°C for 16 h, thin sections were cut using a diamond knife on an LKB ultramicrotome (LKB Instruments, Gaithersburg, MD, United States). Sections were mounted on uncoated 200-mesh copper grids (Ted Pella) before staining with lead citrate and cyanobacteria were examined using a transmission electron microscope (Tecnaei Spirit G12; FEI Company, Eindhoven, Netherlands) at 80 kV.

Quantitative TEM Analyses

To perform a quantitative analysis of EVs, electron micrographs were randomly taken at magnifications of 30,000–75,000 \times . A total of 122 electron micrographs (UV group: 40 cell sections; interspecific interaction group: 42 cell sections; control groups: 40 cell sections) and a total of 396 OMVs were analyzed. Then, the number of EVs/cell section and the proportion of *C. raciborskii* releasing vesicles were established, as well as the number of OMVs in process of outward budding or closely associated with the cell surface. Additionally, the diameters of EVs were measured and grouped in different ranges (20–80, 81–140, 141–200, 201–260, and 261–320 nm). These analyses were done in clear cell sections exhibiting intact cell envelopes and each cell on a filament was considered separately. All quantitative studies were performed using the *Image J* software (National Institutes of Health, Bethesda, MD, United States).

Annexin V Analysis

In eukaryotic cells, it is recognized that phosphatidylserine is relocated to the plasma membrane leaflet at sites on the cell surface where EV shedding occurs (reviewed in Hugel et al., 2005; Muralidharan-Chari et al., 2010). To detect exposed phosphatidylserine, cells were stained with FITC-conjugated Annexin V, a marker for this molecule (Invitrogen, Carlsbad, CA, United States). Samples (1×10^7 cells/mL) were collected, washed in phosphate-buffered saline (PBS) (0.1 M sodium phosphate, 0.15 M sodium chloride, pH 7.4) and stained with annexin -V - FITC (20 μ M) (Dwyer et al., 2012). After incubation for 20 min,

samples were analyzed by both differential interference contrast (DIC) and fluorescence microscopy (BX-60, Olympus, Melville, NY, United States) at 450–480 nm excitation wavelengths (FITC). For each group, 30 filaments were counted and the percentage of annexin-positive cells determined. Experiments were performed in triplicate.

Statistical Analyses

Data from cyanobacteria cell density and viability analyses were compared using ANOVA, followed by the Turkey's comparison test. OMVs number by TEM analyzes and proportion of annexin-V positive cells by fluorescence microscopy were compared using the Student's *t*-test ($P < 0.05$). Statistical analyses and graphs were performed using the software Prism 6.0.1 (GraphPad software, San Diego, CA, United States).

RESULTS

C. raciborskii Releases EVs

Over the past years, our research group has been studying the ultrastructure of bacteria and cyanobacteria by TEM both *in situ* and in cultures (Silva et al., 2014, 2016; Noyma et al., 2015; Gamalier et al., 2017). Our EM methodology includes prompt aldehyde fixation while the cells are still in suspension and before any subsequent centrifugation procedure, which is important to optimal cell preservation and to capture specific biological events in response to varied stimuli. By examining resulting electron micrographs from *C. raciborskii* growing in control culture conditions, small vesicles were found closely associated with this cyanobacterium (Figure 1). Similar to other cyanobacteria, this species is characterized by an envelope composed of three layers: an inner (plasma membrane), an intermediate (periplasmic space and peptidoglycan layer) and an outer membrane (Figures 2Ai,Aii). Our ultrastructural analyses clearly revealed vesicles budding off from the outer membrane of *C. raciborskii* cells (Figure 2). These OMVs were identified as round vesicles with a trilaminar structure typical of bilayered phospholipidic membranes (Figures 2Bi,Bii). Moreover, secreted vesicles frequently exhibited an external amorphous coating as observed on the surface of the cell envelope (extracellular polymeric substances - EPS) (Figure 2Ai). We found that

89.8 ± 5.9 % (mean ± SEM, $n = 396$ vesicles) of OMVs exhibited

this EPS layer.

± ± =

Production of OMVs by *C. raciborskii* Increases with UV Radiation-Induced Stress

We next investigated the production of OMVs by *C. raciborskii* in response to UV radiation, as observed before for bacteria from aquatic ecosystems (Gamalier et al., 2017). After we treated cultures of *C. raciborskii* with UV as done previously (Noyma et al., 2015), TEM micrographs showing intact plasma membrane were randomly taken and carefully examined. Exposure to this radiation triggered the release of OMVs by *C. raciborskii* (Figure 3A). To quantify the number of OMVs from the

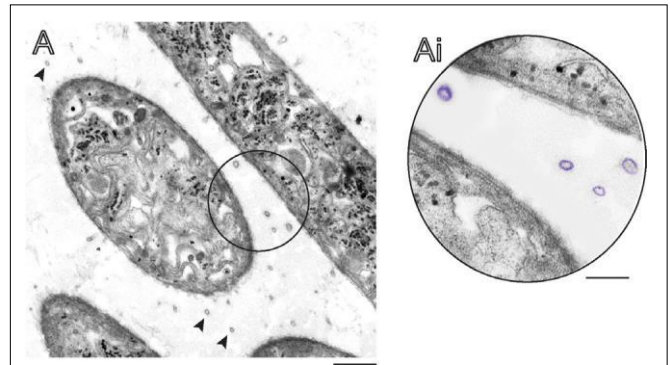


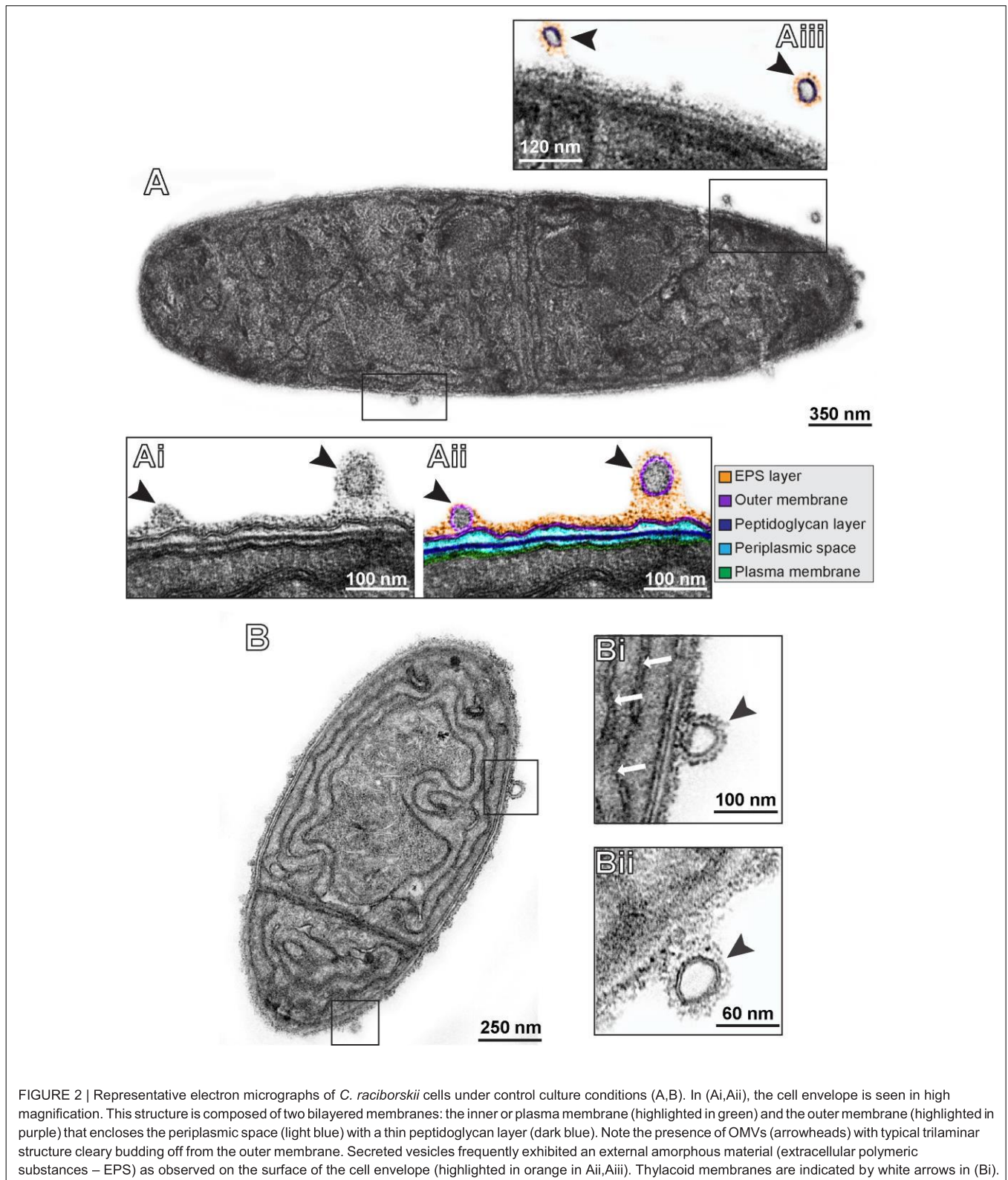
FIGURE 1 | Longitudinal and cross sections of *Cylandrospermopsis raciborskii* growing in control conditions seen by transmission electron microscopy (TEM). (A) Several extracellular membrane vesicles (indicated by arrowheads in A and highlighted in purple in Ai) are observed around cultured cyanobacterial cells. experimental and control groups. *C. raciborskii* cell sections were evaluated ($n = 40$ cells), and a total of 164 OMVs were counted. UV radiation led to a significant increase of OMV production (11.56 ± 3.34 OMVs/cell section,) compared to control cells (5.16 ± 1.67 OMVs/cell section, $P < 0.0001$) (Figure 3B). Moreover, by scoring the number of vesicles, we found that in untreated cells, 50% of OMV-producing cells released 4–6 OMVs/cell section whereas 70% of UV-treated cells produced 7–22 OMVs/cell section (Figure 3C).

C. raciborskii Vesiculation Increases in Response to Interaction with *M. aeruginosa*

We also detected an increased vesiculation by *C. raciborskii* when cultivated with a *M. aeruginosa* strain (Figures 4A,Ai). Quantitative EM analysis showed significant increase in the total numbers of OMVs secreted by *C. raciborskii* in co-cultures (8.27 ± 3.07 OMVs/cell section) compared to controls in monocultures (4.49 ± 1.98 OMVs/cell section, $P < 0.001$) (Figure 4B). By scoring the numbers of OMVs, we found that while 50% of *C. raciborskii* in monocultures released 4–6 OMVs/cell section, 65% of this species in co-cultures produced 7–22 vesicles/cell section (Figure 4C).

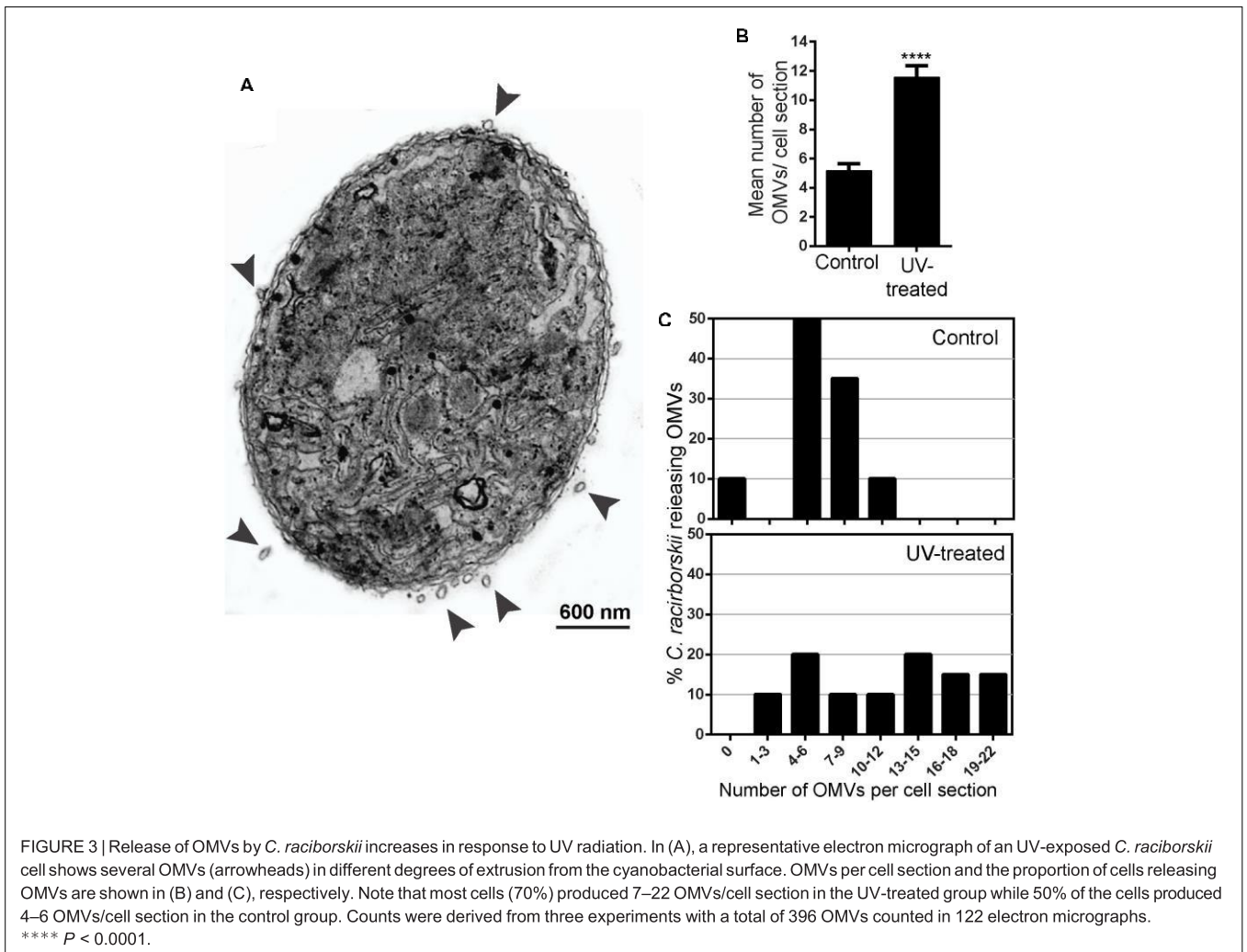
C. raciborskii Vesiculation Is a Dynamic Process

In eukaryotic cells, the genesis of EVs is a rapid event with nascent vesicles being observed by TEM in different stages of outward budding from the plasma membrane and/or completely released at the cell surface (Akuthota et al., 2016). We next studied in more detail the process of vesicle release in *C. raciborskii* by quantitative evaluation of nascent OMVs that were detaching from the outer membrane (budding OMVs) or free at the cell surface (Figures 5A–C). The numbers of budding OMVs in *C. raciborskii* cells were significantly higher in both treated groups (Figures 5D,E) compared to the controls.



In addition, we also established the average size of OMVs released from *C. raciborskii* cells to be 86.23 ± 4.86 nm (mean \pm SEM) in diameter in control conditions and

99.64 ± 4.53 and 101.50 ± 4.62 (mean \pm SEM) nm after UV exposure and interaction with *M. aeruginosa*, respectively. Considering all conditions (control and



treated groups), OMV diameters varied from 20 to 320 nm, with most OMVs showing diameters between 20 and 140 nm (Figures 5F,G). OMVs released in response to stress stimulus (UV exposure and species interaction) were larger compared to controls (Figures 5F,G). Interestingly, our TEM analyses enabled to detect vesiculation by individual cells closely interrelated in the same filament (Figure 6).

Phosphatidylserine Relocates in *C. raciborskii* Outer Membrane

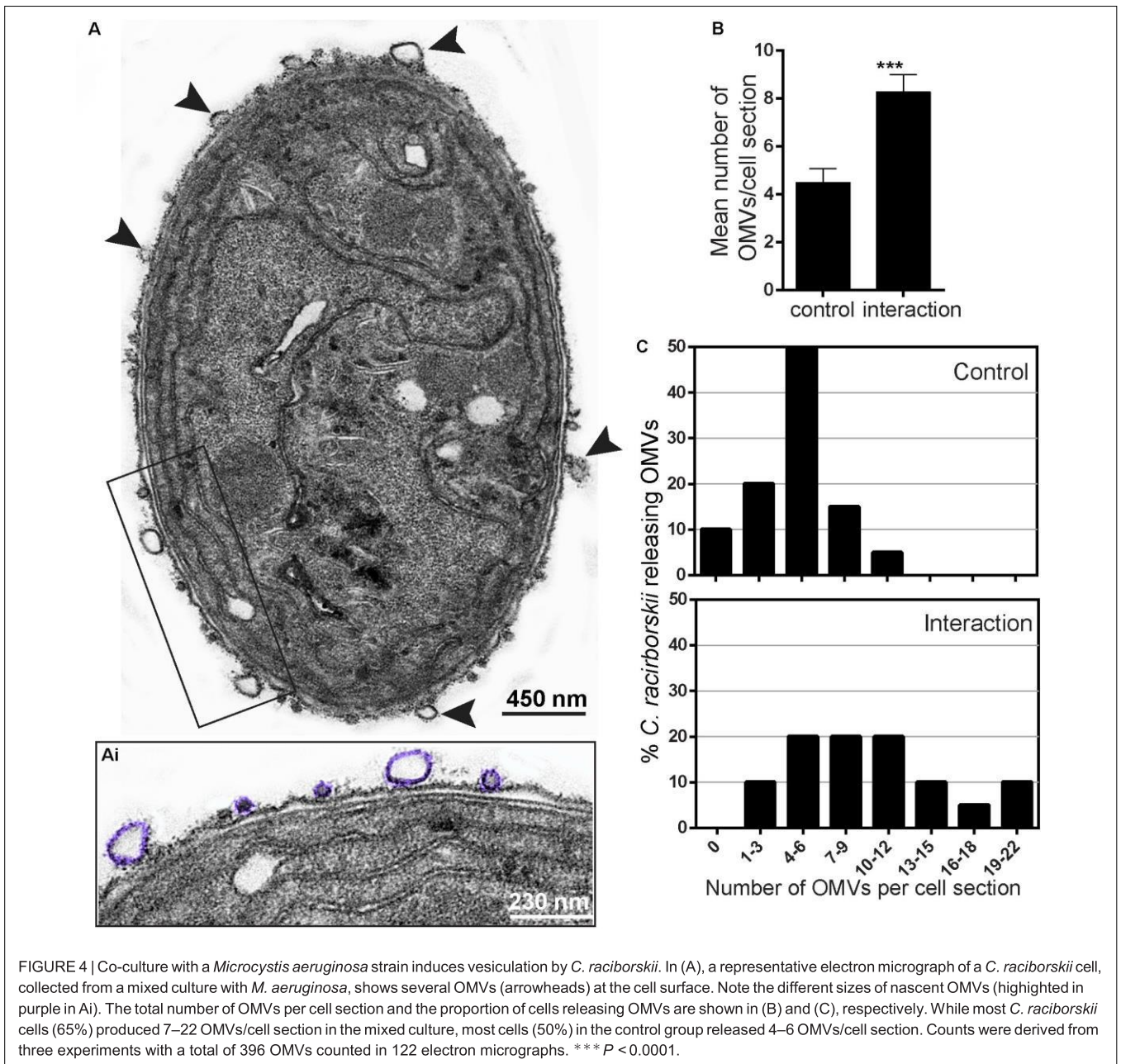
As noted, phosphatidylserine relocation may be associated with EV shedding (reviewed in Hugel et al., 2005; Muralidharan-Chari et al., 2010). Thus, we studied whether the same phenomenon would happen in the outer membrane of cyanobacteria. We stained cells with annexin-V-FITC and samples were analyzed by fluorescence microscopy (Figure 7A). A higher proportion of *C. raciborskii* showed annexin-V-positive staining after both UV treatment and interaction with *M. aeruginosa* compared to control cells (Figure 7B).

Cell Viability and Density of *C. raciborskii* upon UV Radiation Exposure and Interaction with *M. aeruginosa*

Because annexin-V is also used as a marker for cell death, we also evaluated both the cell concentration and cell viability by using classical assessments for cell density (DAPI staining) and membrane viability (baclight) (Joux and Lebaron, 2000; Sipaúba-Tavares and Rocha, 2003). As expected, the cell density was significantly reduced while the proportion of non-viable cyanobacteria significantly increased after UV exposure (Figures 7C,Ci), as previously demonstrated (Noyma et al., 2015). On the other hand, neither reduction of the cell density nor loss of the cell viability was observed as a result of the interaction with *M. aeruginosa* (Figures 7D,Di).

DISCUSSION

The data presented in this work demonstrate, for the first time, that vesiculation is a common phenomenon for *C. raciborskii*. By studying the strain CYRF-01, we showed that this



cyanobacterium constitutively releases OMVs during its normal growth and amplifies such ability in response to environmental stressors, such as UV radiation and interaction with a potential competitor. We thus recognized that *C. raciborskii* has the competence to secrete OMVs and deals with different stress situations with increased genesis of these vesicles. Our data are supported by previous works on other cyanobacterial species and different strains which found production of EVs in response to stressors such as hyperosmotic (hypersaline) conditions (Xu et al., 2013), antibiotic supplementation (Oliveira et al., 2016) or reduced temperature (Oliveira et al., 2016). Moreover, the present work expands our understanding of cyanobacteria as organisms able to actively release EVs (Zheng et al., 2009;

Biller et al., 2014; Oliveira et al., 2015; Pardo et al., 2015; Brito et al., 2017).

To identify and characterize EVs, we used TEM, a technique that distinguishes EVs from non-membranous particles of similar size (Lotvall et al., 2014). This is because EVs are nanostructures delimited by a phospholipid membrane, which can be unambiguously imaged by TEM as a “trilaminar” structure in which the hydrophilic phosphate “heads” are electron-dense and the hydrophobic fatty acids “tails” are electron-lucent (Bozzola and Russell, 1999; Lodish et al., 2016). For this reason, the use of TEM provides the most direct evidence for EV production. Indeed, TEM is considered a gold standard technique to characterize individual EVs (Akuthota et al., 2016;

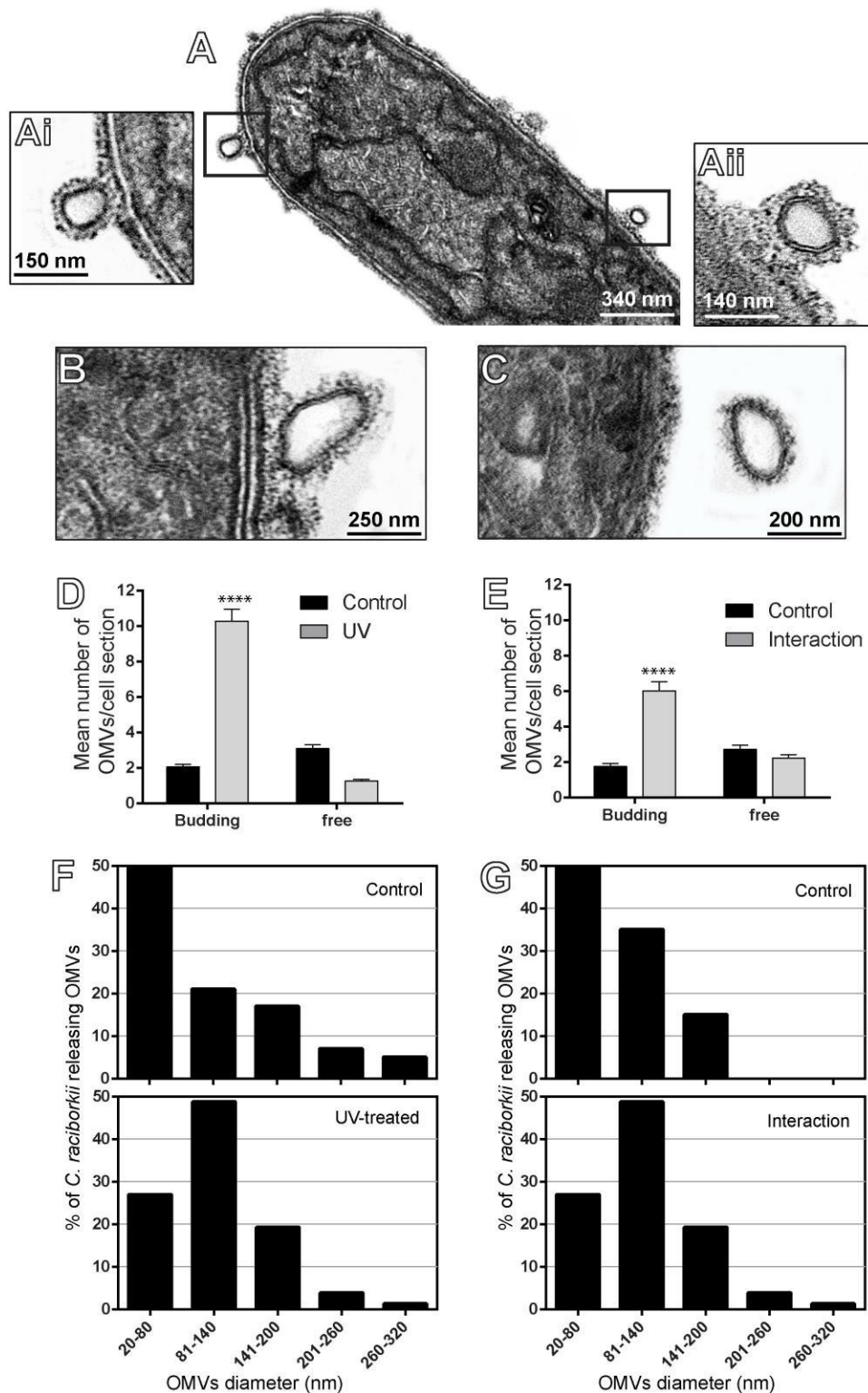


FIGURE 5 | Characterization of nascent OMVs and budding rate from *C. raciborskii* cells. In (A–C), OMVs with different sizes and in progressive outward budding from the cell surface are observed. (Ai,Aii) Are boxed areas of (A) seen at higher magnification. (D,E) The numbers of budding OMVs significantly increased in response to both UV radiation (D) and interaction with *M. aeruginosa* (E), compared with respective controls. (F,G) Both treatments elicited secretion of OMVs with higher size in comparison with controls. Counts were derived from three experiments with a total of 396 OMVs counted in 122 electron micrographs. **** $P < 0.0001$.

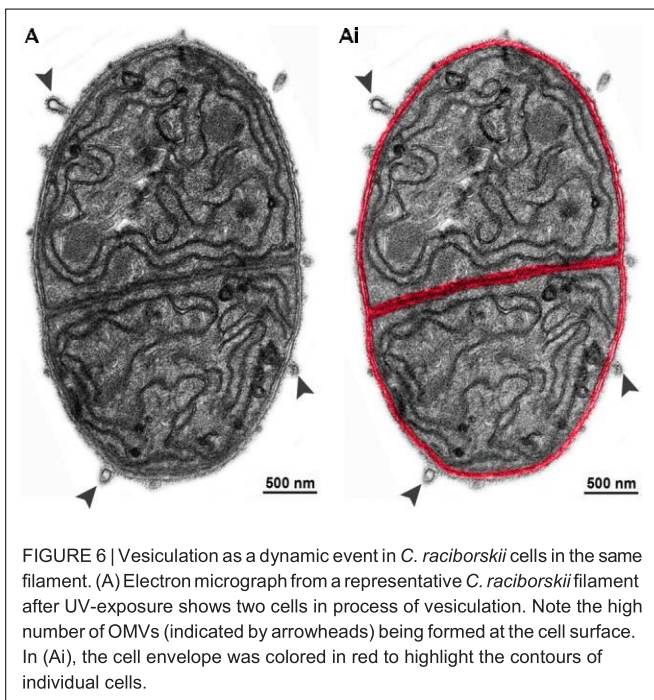


FIGURE 6 | Vesiculation as a dynamic event in *C. raciborskii* cells in the same filament. (A) Electron micrograph from a representative *C. raciborskii* filament after UV-exposure shows two cells in process of vesiculation. Note the high number of OMVs (indicated by arrowheads) being formed at the cell surface. In (Ai), the cell envelope was colored in red to highlight the contours of individual cells.

Lawson et al., 2016; Gamalier et al., 2017) as endorsed by the International Society for Extracellular Vesicles (ISEV) (Lotvall et al., 2014). Moreover, the ISEV recommends that, for better characterization of the vesiculation event, TEM images should show a “wild field” encompassing multiple vesicles in addition to close-up images of single vesicles at cell surface (Lotvall et al., 2014). Our TEM analyses included a quantitative approach, which enabled clear size characterization and quantification of many nascent EVs at the entire cell surface (Figures 2–4, 6), providing conclusive evidence for the production of EVs by *C. raciborskii*. We also provided, for the first time, direct evidence that EVs released by this cyanobacterium are formed by fission from the outer membrane, similar to the process described for liberation of OMVs by gram-negative bacterial species (reviewed in Kulp and Kuehn, 2010; Jan, 2017). That cyanobacteria vesiculate similar to gram-negative bacteria was suggested by previous studies (Zheng et al., 2009; Oliveira et al., 2015; Brito et al., 2017). In a study using *Synechocystis* PCC6803, for example, proteins and lipids derived from the outer membrane were detected in cell-free supernatants derived from *Synechocystis* (Pardo et al., 2015). Additionally, our high-resolution approach enabled imaging of the EPS layer in most nascent OMVs. This means that OMVs present a layer of polysaccharidic nature, which might provide structural integrity and protection against UV radiation, important for persistence of OMVs in the environment (Pereira et al., 2009). Other potential roles for the EPS layer in OMVs include serving as an alternative storage for carbon compounds (Pereira et al., 2009; Pannard et al., 2016). In fact, sugars are molecules previously hypothesized to be transported by OMVs in cyanobacteria (Xu et al., 2013).

Our study also demonstrated that the number of OMVs in process of outward budding from the outer membrane, that

is, still physically attached to this membrane, increased in cells under stress compared to cells in normal growth (Figure 5). The higher rate of OMV production after exposure to the stress conditions possibly enabled us to capture more frequently the different degrees of vesicle budding off from the outer membrane. Moreover, our findings revealed that nascent OMVs show varied sizes (range of 20–320 nm, Figure 5), indicating that there are morphologically different populations of OMVs secreted by *C. raciborskii*. We also captured the vesiculation event by individual cells in the same filament (Figure 6) indicating that different cells are able to respond collectively to the stressor agents.

To explore the link between vesicle release and environmental stress, we exposed *C. raciborskii* to different conditions, such as UV radiation, which is known to promote clear alterations in the density, viability and photosynthetic structures of this cyanobacterium (Noyma et al., 2015) as well as damage in other species of cyanobacteria (reviewed in Singh et al., 2010). Our findings showed an increased production of OMVs in response to UV radiation. Remarkably, our analyses at high resolution also showed that vesiculation by UV-treated cyanobacteria were not a result of cell lysis. Although most cells were non-viable, as detected by a marker of membrane permeability (Figure 7), nascent OMVs were released from structurally intact cyanobacteria (Figures 3, 5). The same phenomenon was observed for gram-negative bacteria exposed to UV radiation (Gamalier et al., 2017) indicating that damaged cells release OMVs before cell lysis likely as a protective mechanism against UV radiation to increase cell survival since cell compounds such as DNA could be preserved within these nanovesicles.

When in co-cultures with *M. aeruginosa*, *C. raciborskii* can inhibit the growth of *M. aeruginosa* strains (Figueredo et al., 2007; Mello et al., 2012; Rzymyski et al., 2014). Allelochemicals secreted by *C. raciborskii* are likely mediating this action (Figueredo et al., 2007; Rzymyski et al., 2014). Because EVs constitute a way by which both eukaryotic and bacterial cells secrete products to the extracellular medium, we hypothesized that *C. raciborskii* would be able to increase vesiculation upon interaction with

M. aeruginosa. Our results clearly demonstrated such capacity. Although the chemical identity of the cargo within the vesicles remains to be established, it is clear that *C. raciborskii* responds to the interaction with production of secretory vesicles, which might be involved in the mechanism for *M. aeruginosa* growth inhibition.

Phosphatidylserine is an anionic glycerophospholipid present in the membranes of both eukaryotic (reviewed in Kay and Grinstein, 2011) and some prokaryotic cells (reviewed in Sohlenkamp and Geiger, 2016). In eukaryotes, phosphatidylserine is located on the internal leaflet of the plasma membrane and its externalization, that is, its exposure on the outer leaflet of this membrane, has been reported to be a distinct molecular event during formation of EVs (reviewed in Hugel et al., 2005; Muralidharan-Chari et al., 2010), being thus used as an additional evidence for cell vesiculation (Gonzalez-Cano et al., 2010; Akuthota et al., 2016). Here, we demonstrated that *C. raciborskii* filaments under stress

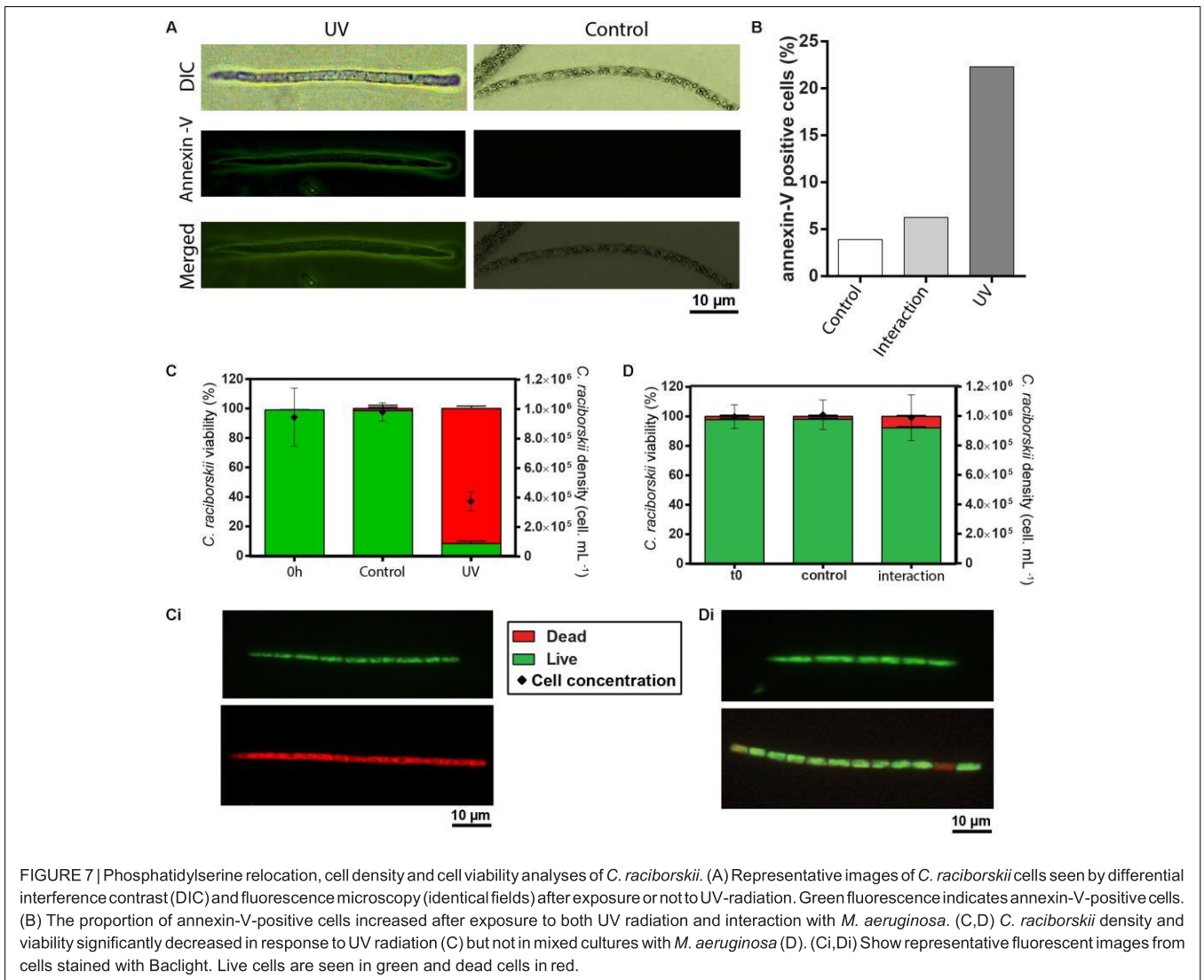


FIGURE 7 | Phosphatidylserine relocation, cell density and cell viability analyses of *C. raciborskii*. (A) Representative images of *C. raciborskii* cells seen by differential interference contrast (DIC) and fluorescence microscopy (identical fields) after exposure or not to UV-radiation. Green fluorescence indicates annexin-V-positive cells. (B) The proportion of annexin-V-positive cells increased after exposure to both UV radiation and interaction with *M. aeruginosa*. (C,D) *C. raciborskii* density and viability significantly decreased in response to UV radiation (C) but not in mixed cultures with *M. aeruginosa* (D). (Ci,Di) Show representative fluorescent images from cells stained with Baclight. Live cells are seen in green and dead cells in red.

conditions have higher proportion of phosphatidylserine-positive cells compared to cells growing in control conditions (Figure 7). Phosphatidylserine externalization is also an event commonly associated with apoptosis in both prokaryotic (reviewed in Zheng et al., 2013; Kasuba et al., 2015) and eukaryotic (reviewed in Suzanne and Steller, 2013) cells. However, overall, our findings indicate that phosphatidylserine externalization can be mostly considered another evidence for *C. raciborskii* vesiculation instead of an indicative of cell death. First, by using a marker for cell viability, both non-viable/dead and viable/live cells, evaluated after UV exposure or interspecific interaction showed phosphatidylserine externalization (Figure 7). Second, TEM, which is considered a gold standard to detect apoptosis, revealed absence of typical or similar morphological features of apoptosis in *C. raciborskii* cells after both treatments, such as the occurrence of empty cells with intact cell envelope and/or cells with cytoplasmic condensation/retraction (reviewed in Silva et al., 2017). Therefore, the phosphatidylserine analyses corroborate our TEM

results showing amplified *C. raciborskii* vesiculation in response to stress conditions.

Our phosphatidylserine findings also highlight an important biological aspect of *C. raciborskii*: this cyanobacterium appears to change the composition/organization of its membrane lipids in response to alterations in the environment as documented for other bacteria (reviewed in Sohlenkamp and Geiger, 2016). In fact, different bacterial species are able to change membrane lipid composition/organization/fluidity to survive under unfavorable conditions or even to adapt to a new situation such as nutrient deprivation or increase/decrease of temperature (Sohlenkamp and Geiger, 2016). In addition to underlie the mechanism of OMV production, membrane lipid modification in *C. raciborskii* could allow a rapid response to changes in environmental conditions, as observed for other bacterial species (Sohlenkamp and Geiger, 2016).

Taken together, our findings identify, for the first time, that *C. raciborskii* (CYRF-01) secretes OMVs during normal growth and that the release of these vesicles to the surrounding

environment increases in response to UV radiation or interspecific interaction with *M. aeruginosa*. The potential role of these OMVs to interact with neighboring cells or to promote cyanobacterial adaptation awaits further investigations.

AUTHOR CONTRIBUTIONS

RM provided study guidance, mentorship and critical editing of the manuscript. VZ, TS, NN, JG, and MME performed the experiments, acquired and analyzed the data. VZ, TS, and NN performed the TEM analyses. MMA contributed with cyanobacteria strains and cultures. All authors contributed in part to writing and editing the manuscript and approved the final version.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Increased production of outer membrane vesicles by cultured freshwater bacteria in response to ultraviolet radiation



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Secretion of membrane vesicles is an important biological process of both eukaryotic and prokaryotic cells. This process has been characterized in pathogenic bacteria, but is less clear in non-pathogenic bacteria from aquatic ecosystems. Here, we investigated, for the first time, the process of formation of outer membrane vesicles (OMVs), nanoscale vesicles extruded from the outer membrane (OM) of gram-negative bacteria, in cultures of freshwater bacteria after exposure or not to ultraviolet radiation (UVR) as an environmental stressor. Non-axenic cultures of freshwater bacteria isolated from a Brazilian aquatic ecosystem (Funil reservoir) were exposed or not to UVR (UVA + UVB) over a 3 h period, during which cell density, viability and ultrastructure were analyzed. First, we showed that UVR induce bacterial death. UVR triggered significant negative effect on cell density after 3 h of UVR treatment. This decrease was directly associated with cell death as revealed by a cell viability fluorescent probe that enables the distinction of live/dead bacteria. Transmission electron microscopy (TEM) revealed changes indicative of cell death after 3 h of UVR exposure, with significant increase of damaged cells compared to the control group. Second, we demonstrated that gram-negative bacteria release OMVs during normal growth and after UVR exposure. OMVs were clearly identified as round, membrane-bound vesicles budding off from the bacterial OM as isolated or clustered vesicles or free in the extracellular medium. Remarkably, quantitative TEM analyses showed that bacteria respond to UVR with increased formation of OMVs. Moreover, while OMVs numbers per intact or damaged cell did not differ in the untreated group, UVR led to a higher vesiculation by bacteria in process of death. This means that degenerating bacteria release OMVs before lysis and that this secretion might be an adaptive/protective response to rapid changes in environmental conditions such as UV radiation.

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1. Introduction

In recent years, the extracellular release of membrane-bound vesicles by prokaryotic cells has become the subject of great interest. These nanoscale vesicles (20–300 nm) are extruded from the outer membrane (OM) of gram-negative bacteria and have been associated with varied biological processes that resemble those used by higher organisms, such as pathogenesis (Kolling and Matthews, 1999; Rivera et al., 2010), cellular defense (Manning and Kuehn, 2011; Baumgarten et al., 2012), cell communication (Mashburn and Whiteley, 2005) and DNA transfer (Rumbo et al.,

2011; Pérez-Cruz et al., 2013). It is now recognized that outer membrane vesicles (OMVs) can store and transport a broad repertoire of cargo derived from bacterial periplasm and cytoplasm. Thus, vesicular transport represents a relevant signal trafficking system in prokaryotes (reviewed in Kulp and Kuehn, 2010; Haurat et al., 2015).

The current knowledge on the biogenesis of OMVs and their proposed roles in bacteria are mostly based on different models of pathogenic species (reviewed in Kulp and Kuehn, 2010; Haurat et al., 2015). Much less known are the ability of bacteria from aquatic ecosystems to secrete OMVs, their abundance and potential functions in aquatic biology. In marine microbial communities, bacteria-derived vesicles were reported to be abundant in coastal and open-ocean seawater samples and implicated in marine carbon flux (Biller et al., 2014). Recently, we have provided the first evidence that bacteria from freshwater ecosystems produce OMVs

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in their natural environment (Silva et al., 2014). In studying the ultrastructure of aquatic bacteria from water samples collected from varied tropical freshwater sites, we identified typical bilayer membrane vesicles budding off from the bacteria OM, a morphological indicative of a distinct secretory process into the environment (Silva et al., 2014).

Here, we investigated the process of OMV formation in cultures of freshwater bacteria after exposure to the ultraviolet radiation (UV) as an environmental stressor (Häder and Sinha, 2005; Kultz, 2005). It has been demonstrated that UV induces adverse effects on the dynamics of aquatic bacteria (Paul et al., 2012; Hunting et al., 2013; Garcia-Corral et al., 2014) and lead to damages on their cellular structures and DNA (Santos et al., 2013; Strickler et al., 2015). The UVA and UVB radiation spectra (UVR) are the most significant to the bacterial alterations on Earth, since UVC spectrum is totally absorbed by ozone layer on atmosphere (Diffey, 1991). Recently, the UV effects on aquatic ecosystems have been considered more prominent due to the ozone depletion and other factors associated with climate changes, such as increasing temperatures and precipitation (reviewed in Häder et al., 2011; Häder et al., 2015).

By using transmission electron microscopy (TEM), including quantitative TEM and approaches for cell density and viability evaluations, we demonstrated, for the first time, that OMVs are naturally secreted by freshwater bacteria into the culture medium during normal growth and that the release rate of OMVs significantly increased in response to cell viability changes induced by UVR. The amplified bacterial ability to release OMVs may be associated with adaptive responses to rapid changes in environmental conditions.

2. Materials and Methods

2.1. Aquatic bacteria cultures

Cultures of aquatic bacteria were established from water samples collected directly from the subsurface (0.5 m; pH 7.4; 28 °C) of the *Funil* reservoir, an aquatic ecosystem located in southwest of Brazil (Supplementary Fig. 1). Funil Reservoir is a hydropower reservoir 22° 30' S, 44° 45' W, altitude 440 m, Cwa climate (i.e. warm temperature, dry winter, hot summer in the Köppen climate classification system) with 16 800 km² of catchment area, a surface area of 40 km², mean and maximum depths of 22 and 70 m (total depth at the sampling station 50 m), respectively, a total volume of 890 × 10⁶ m³ and a retention time of 25 to 80 d. Funil Reservoir was constructed at the end of the 1960s by damming the *Paraíba do Sul* River (Soares et al., 2009). No specific permissions were required for the water samples collected from this reservoir. Our field studies did not involve endangered or protected species.

Briefly, serially diluted (1:10, 1:100, 1:1000, 1:10000) water samples were spread onto two different non-selective solid culture media: Reasoner's 2A agar (R2A, Himedia®, Mumbai, India) and trypticase soy agar (TSA, BD, Sparks, MD), and incubated at 28 °C. This temperature was chosen based on the temperature assayed at the sampling point. R2A is a nutrient-poor medium, whereas TSA is a nutrient-rich medium. The use of culture media with different nutrients concentration enables the recovery of a broad heterotrophic bacteria variety from aquatic environments (Rice et al., 2012). After visible growth, individual colonies morphotypes were then harvested from plates and transferred onto a new plate. This process was repeated two to three times in order to isolate individual bacterial colonies (Scott et al., 2012). Bacterial isolates from both culture media were transferred to trypticase soy broth media (TSB, BD, Sparks, MD) and incubated at 28 °C. After incubation (24 h), aliquots (1.5 mL) from growth cultures were taken and mixed in glycerol (final concentration of 30%) to constitute stock

bacterial isolates that were stored at 80 °C for further use in the experiments as below.

2.2. UV exposure

Prior to experiments, bacterial frozen isolates were thawed and streaked onto a plate containing TSA. Solid cultures were grown at 28 °C until observation of visible growth. After, colonies were extracted from plates and grown in TSB liquid media overnight at 28 °C with 225 rpm shaking. For all experiments the cultures were re-suspended in 40 mL of TSB medium at an initial concentration of 10⁶ cells/mL. The UV exposure was performed using the same intensity and procedures as previous work (Noyma et al., 2015). Briefly, cultures in borosilicate glass Erlenmeyers were submitted to artificial UVR (UVA + UVB, 280–400 nm) supplied by UVA (TL 40/05; Philips; emission peak at 365 nm) and UVB (TL 20/01; Philips; emission peak at 312 nm) lamps. The UV intensities used in experiments were 11.8 W m⁻² (UVA) and 0.54 W m⁻² (UVB). The UV intensity used was based on natural solar radiation measurements taken during May, 2009 in Juiz de Fora City (21°45'51" S), in southeast, Brazil (Noyma et al., 2015). UVR-treatment was performed during 3 h and control cultures were maintained in the dark, both at 28 °C. Experiments were performed in triplicates and all samples were carefully homogenized prior to analysis.

2.3. Bacteria quantification

For cell quantification, bacteria were stained with 4',6'-diamidino-2-phenylindole (DAPI); 0.01 µg mL⁻¹ final concentration (Porter and Feig, 1980). Analyses were performed during different time points of UV exposure (0, 1, 2 and 3 h). Samples were fixed with free-particle 37% formaldehyde (0.2 µm filtered) to a final concentration of 2%. After, samples (1 mL) plus 10 µL of DAPI were placed in megafunnels (Shandon Mega funnel, Thermo, UK) for immediate centrifugation in a cyto centrifuge (Shandon Cytospin 4, Thermo, United Kingdom), at 452 g and high acceleration for 10 min (Silva et al., 2014). Acceleration and speed were established as the procedures for medical microbiology provided by the Cytospin manufacturer manual. Cyto centrifugation was done by using regular slides without any coating. Analyses were performed on a fluorescence microscope (BX-60, Olympus, Melville, NY, USA) and U-MWU2 filter (330–385 nm excitation wavelengths). Bacteria were directly counted in 10 random fields at 1000x magnification using an ocular graticule grid.

2.4. Bacteria viability

Cell membrane viability was investigated using the LIVE/DEAD® BacLight™ kit (Molecular Probes Eugene, inc, ThermoFisher Scientific, OR, USA). This kit contains a mixture of fluorescent stains (SYTO 9 and propidium iodide) that differ both in their ability to penetrate healthy bacterial cell membranes and allows differentiation between bacteria with intact and damaged cell membranes (Boulos et al., 1999; Freese et al., 2006). Cells with intact membranes (viable cells) stain green and those with damaged membranes (non-viable) stain red. Bacteria were stained by 1 mL of each sample to 3 µL of *BacLight* and slides (n = 9) for each time point (0, 1, 2 and 3 h) were prepared in a cyto centrifuge (Shandon Cytospin 4, Thermo Electron Corporation, Madison, WI, USA) as previously described (Silva et al., 2014). Analyses were performed under a fluorescence microscope (BX-60, Olympus, Melville, NY, USA) at 450–480 nm excitation wavelengths, which enable simultaneous visualization of *BacLight* stains. Bacteria were directly counted in 10 random fields (at an ocular graticule grid) at 1,000 x

magnification. The average percentage of live/dead bacteria was established for each slide sampled.

2.5. Transmission electron microscopy

Bacteria samples collected after 3 h of UVR exposure and not-exposed controls were immediately fixed in a mixture of freshly prepared aldehydes (1% paraformaldehyde and 1% glutaraldehyde) in 0.1 M phosphate buffer, pH 7.3, for 1 h at room temperature (RT). Next, samples were washed twice in 0.1 M phosphate buffer, pH 7.3 (3,000 g for 10 min), and stored at 4 °C for subsequent use. After fixation, agar embedding was performed as before (Barros et al., 2010; Silva et al., 2014), so that uniformly distributed specimens could be processed as easily handled blocks of cells. They then were re-suspended in molten 2% agar (Merck, Darmstadt, Germany) for further processing. Agar pellets containing specimens were post-fixed in a mixture of 1% phosphate-buffered osmium tetroxide and 1.5% potassium ferrocyanide (final concentration) for 1 h prior to dehydration in graded ethanols and infiltration and embedding in a propylene oxide-Epon sequence (PolyBed 812, Polysciences, Warrington, PA, USA) (Silva et al., 2014). After polymerization at 60 °C for 16 h, thin sections were cut using a diamond knife on an LKB ultramicrotome (LKB Instruments, Gaithersburg, MD, USA). Organisms were examined using a transmission electron microscope (Tecnai Spirit G12, FEI, Netherlands) at 80 kV.

2.6. Quantitative TEM analyses

To study morphological aspects of the bacterial cells and OMV formation, electron micrographs were randomly taken at magnifications of 30,000–75,000 x and a total of 165 electron micrographs from gram-negative bacteria were analyzed (n = 55 from control cells and n = 110 from UV-exposed cells). The numbers and diameter of OMVs were determined using the software *Image J 1.41* (National Institutes of Health, Bethesda, MD, USA). A total of 386 OMVs were counted and the number of vesicles per cell section was determined. Bacteria were also categorized according to their ultrastructural integrity (Heissenberger et al., 1996) as: (i) *Intact bacteria* in which an intact plasma membrane, cell envelope and a fully developed (not degraded) cytoplasm can be observed and (ii) *Damaged bacteria* which lack at least one of the above criteria. The number of intact and damaged bacteria releasing OMVs was also established.

2.7. Statistical analyses

Data from bacteria quantification by light microscopy, viability analysis and ultrastructural evaluation of bacteria integrity were compared using ANOVA, followed by the Turkey's comparison test. OMVs number and diameter by TEM analyses were compared using the Student's t Test ($P < 0.05$). Statistical analyses and graphs were performed using the software Prism 6.0.1 (GraphPad software, San Diego, CA).

3. Results

3.1. UV radiation affects cell density and leads to bacterial death

First, we investigated if the UV treatment would induce changes in bacterial density. Quantitative analyses by fluorescence microscopy after DAPI staining demonstrated a progressive and significant decrease ($P < 0.05$) of cell numbers during the period of 1 to 3 h of UVR exposure compared to the control group (Fig. 1A). We next wondered if the cell density reduction would be resultant from UVR-induced bacterial death. By using a probe for membrane integrity (Baclight), which detects non-viable/dead cells in red and

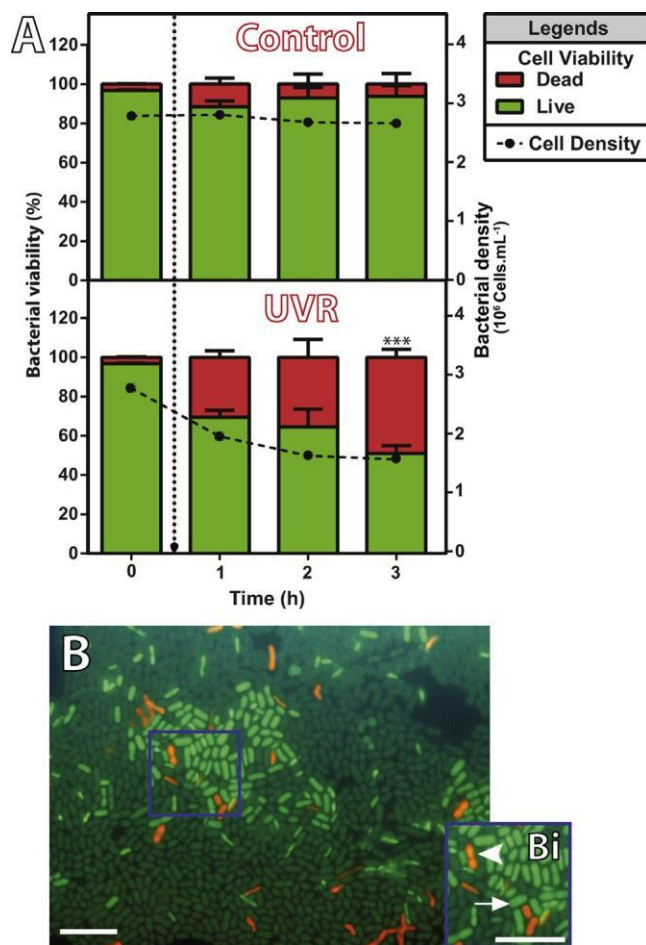


Fig. 1. Bacterial density and viability.

(A) UVR treatment induced both decrease of cell density and increase of cell death compared to untreated and 0 h groups. Cultured bacteria were stained with DAPI or LIVE/DEAD[®] BacLight[™] and counted under fluorescence microscopy for evaluation of cell density and viability, respectively. Data represent the mean \pm SEM of bacteria counted from 10 randomly selected fields/slide (n = 9 slides/time point) from 3 independent experiments ($***P < 0.001$ versus all other groups). (B) and (Bi), viable/live (green, arrow) and non-viable/dead (red, arrow head) bacteria are observed after 3 h of UVR exposure. Scale bar = 5 μ m (B) and 3 μ m (Bi). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

viable/live cells in green (Fig. 1B and Bi), we identified a parallel increase in the percentage of dead cells during the period of UVR exposure (Figs. 1A and 2). This increase was significant after 3 h of UVR treatment ($P = 0.001$) (Fig. 1A).

3.2. Ultrastructural imaging of intact and dying bacteria

To investigate ultrastructural alterations underlying cell death, cultured bacteria were then prepared for TEM after 3 h or in absence of UVR treatment. Our EM analyses showed clear ultrastructural changes indicative of cell death after UVR exposure. While in the control group there was predominance of intact cells with typical morphology and well-preserved cell envelope (Fig. 3), the UVR-treated group exhibited damaged cells with cytoplasmic condensation (Fig. 4A) or rarefaction (Fig. 4B), cell envelope disarrangement (Fig. 4B), loss of cell shape (Fig. 4A and B) and cell elongation (Fig. 4B). To quantitate the number of bacteria exhibiting UVR-induced morphological changes, gram-negative bacteria were categorized in intact or damaged as before (Heissenberger et al., 1996). TEM quantitative analyses revealed a significant increase of damaged cells after UVR treatment (Fig. 4C).

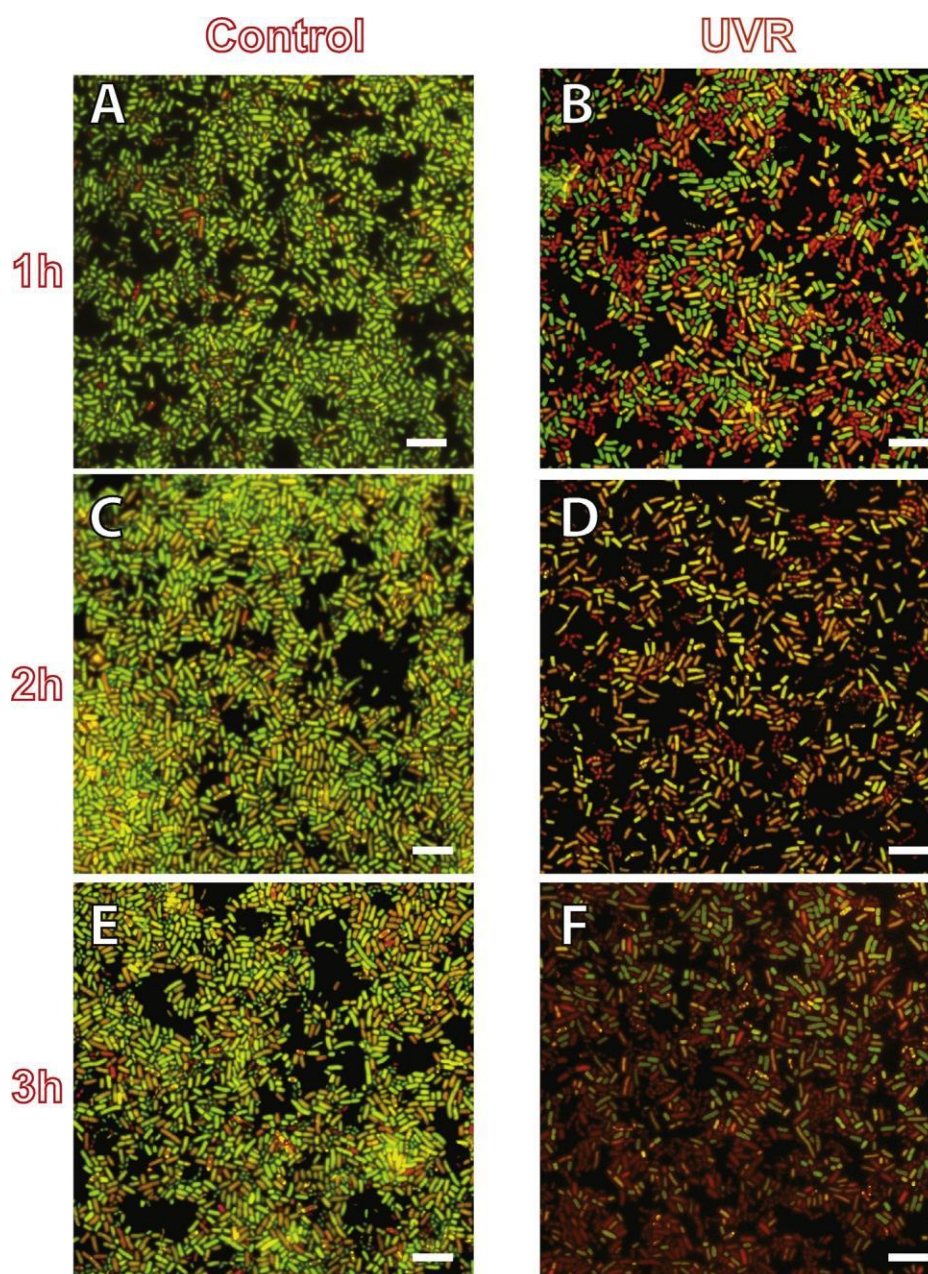


Fig. 2. Viable (green) and non-viable (red) freshwater bacteria visualized along 3 h of exposure to UV radiation. Untreated (A, C and E) and UVR-treated (B, D and F) cultured freshwater bacteria are observed under fluorescence microscopy after staining with a cell viability probe (LIVE/DEAD[®] BacLight[™]). Note that the number of red cells increases in parallel with the exposure time. Scale bar = 10 μ m (A–F). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.3. Bacteria release membrane vesicles during normal growth and after UVR exposure

The cell envelope structure of gram negative bacteria is constituted of two bilayered membranes: the inner or plasma membrane and the OM that encloses the periplasmic space and a thin peptidoglycan layer (Fig. 3Ai) (Silhavy et al., 2010). Interestingly, our ultrastructural analyses revealed the presence of membrane vesicles budding off from the OM of these bacteria in both, untreated (Fig. 5A and C) and UVR-treated groups (Fig. 4A and B). OMVs were clearly identified as round, membrane-bound vesicles (Figs. 4 and 5), with morphology similar to those described on the surfaces of other bacterial species (Beveridge, 1999; Kulp and Kuehn, 2010). OMVs were frequently found attached to bac-

terial cell envelope as single (Figs. 4Ai, 5A, Ai, Aii and C) or clustered vesicles (Figs. 4Ai, B and Bi). Free OMVs were also detected around bacteria (Figs. 3Aii and 5Aii) in association (Fig. 3Aii) or not (Fig. 5Aii) with amorphous material.

3.4. OMV production increases in response to UVR

To study the process of OMV formation and investigate whether UVR induced bacteria vesiculation, we performed quantitative ultrastructural studies in 165 electron micrographs from gram-negative bacteria. A total of 386 OMVs were counted and the number of vesicles per cell section was determined. Exposure to UVR led to significant increase of OMV production compared to untreated cells (control = 5.95 ± 1.17 OMVs/cell section, UVR-

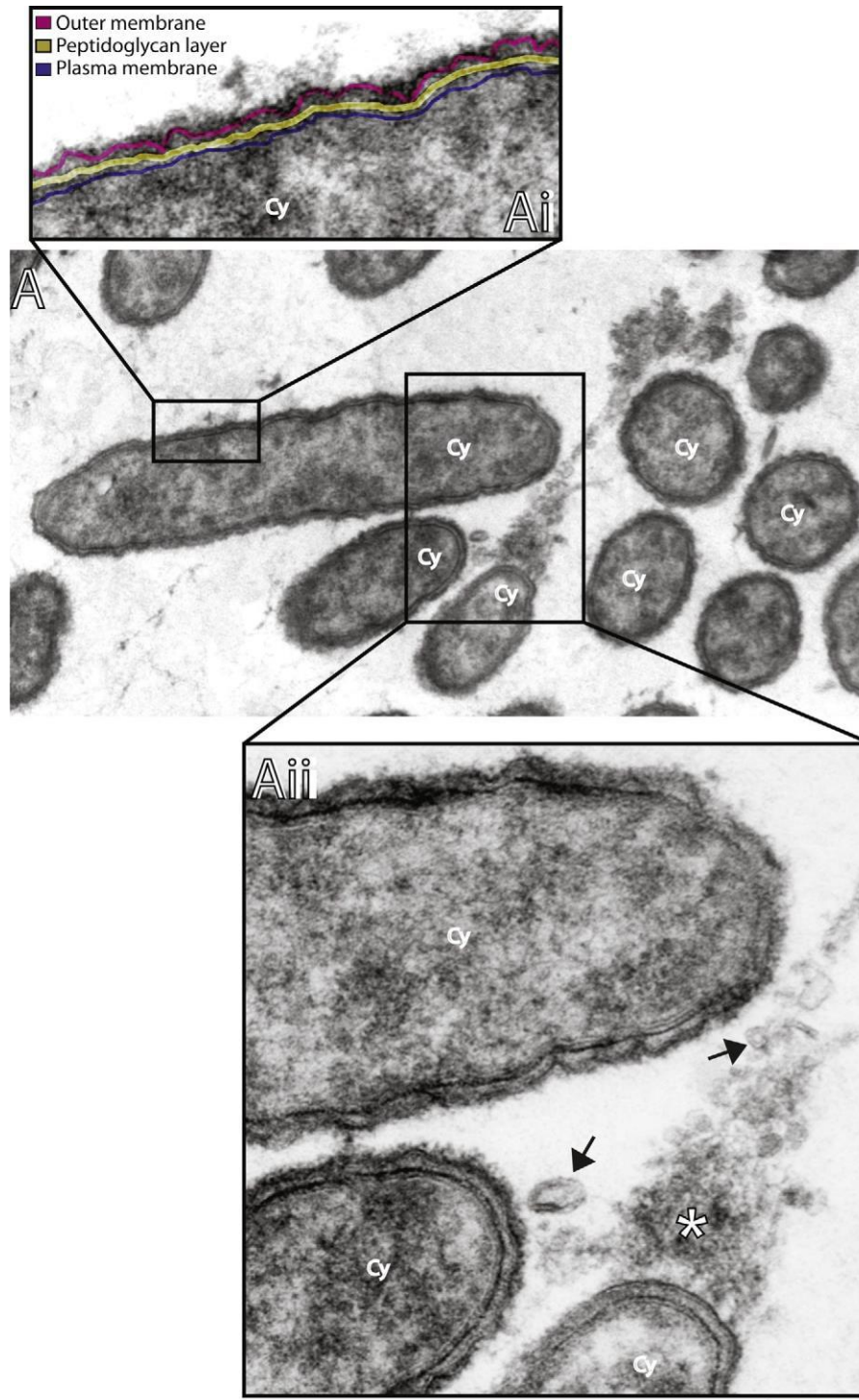


Fig. 3. Ultrastructure of freshwater bacteria growing in culture.

(A) Longitudinal and cross sections of several bacteria show a well-preserved cell envelope and cytoplasm (Cy). In (Ai), the cell envelope is seen in high magnification. This structure is composed of two bilayered membranes: the inner or plasma membrane and the outer membrane that encloses the periplasmic space and a thin peptidoglycan layer. In (Aii) observe free extracellular vesicles (arrows) in association with amorphous material (asterisk) nearby bacteria. Cultured cells were fixed in a mixture of freshly prepared aldehydes and prepared for transmission electron microscopy. Scale bar = 165 nm (A); 25 nm (Ai), 50 nm (Aii). (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article).

treated = 13.01 ± 3.35 OMVs/cell section, mean SEM, $P < 0.0198$, Fig. 6A).

We also determined the average size of OMVs to be 78.77 ± 6.00 nm (mean \pm SEM) in diameter in control cells and 45.26 ± 3.90 (mean \pm SEM) nm in UV radiation-treated bacteria

(Fig. 6B). Thus, OMVs formed by UVR-exposed bacteria had lower size compared to the controls (Fig. 6B, $p < 0.0001$). By analyzing OMVs diameters ranges we noticed that the UV radiation-treated group presented larger proportion of vesicles concentrated in lower ranges, while the control group showed larger proportion concen-

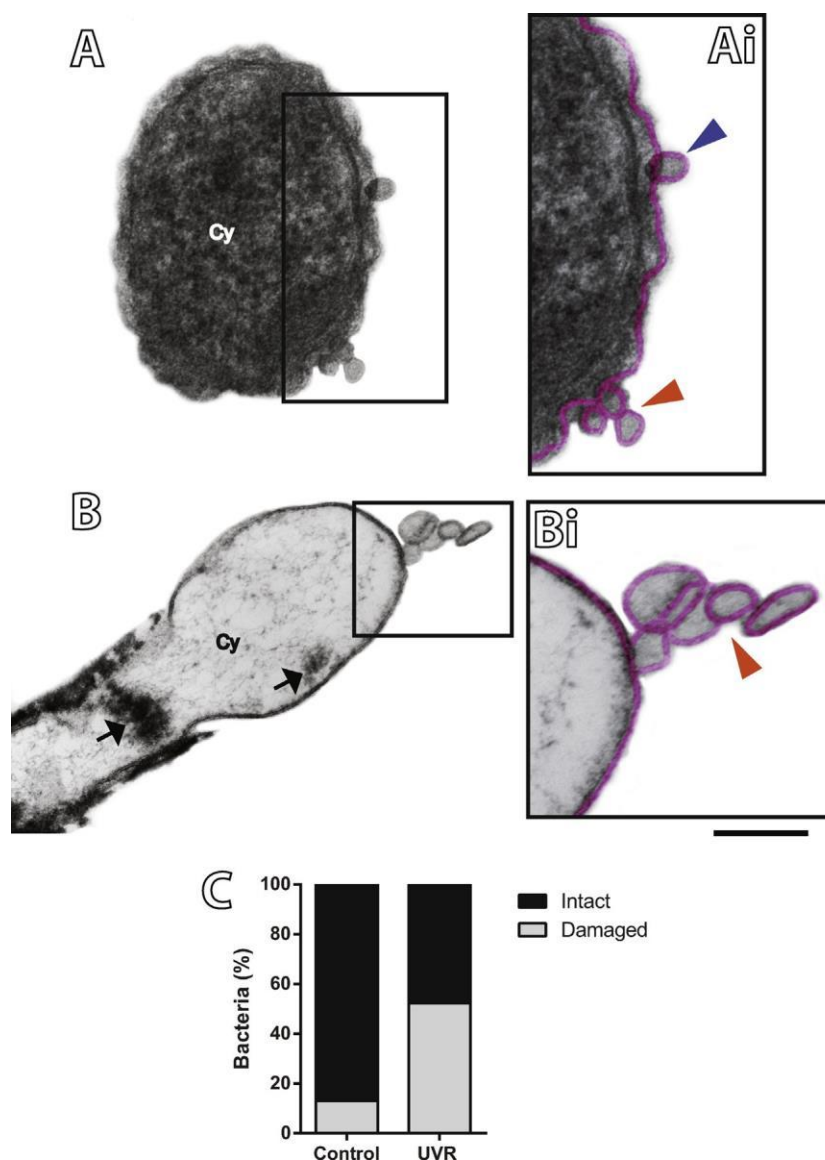


Fig. 4. UVR induces ultrastructural changes in freshwater bacteria.

UVR-exposed bacteria show loss of cell shape (A and B), cell envelope disarrangement (B) and cell elongation (B). Changes in the bacterial cytoplasm (Cy) such as condensation (A) or rarefaction (B) with condensed areas (arrows) were frequently observed. Damaged bacteria release membrane-bound vesicles from the outer membrane (highlighted in pink in Ai and Bi). Note that vesicles are secreted as single (blue arrowhead) or clustered vesicles (red arrowheads). In (C), the proportion of damaged and intact bacteria from UVR-treated and untreated groups are shown. Cultured cells were fixed in a mixture of freshly prepared aldehydes and prepared for transmission electron microscopy. A total of 165 electron micrographs were analyzed and the number of intact and damaged bacteria established. Scale bar = 100 nm (A), 75 nm (Ai), 350 nm (B) and 180 nm (Bi). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

trated in higher ranges (Fig. 6C). For example, the proportions of OMVs with diameters between 10–25 nm were 15% and 40% of the total OMVs number for control and UVR-treated bacteria, respectively. OMVs with diameters over 75 nm corresponded to 30% of the total OMVs number in control cells whereas in the UVR group this range was 15% (Fig. 6C).

Finally, we wondered if the increased production of OMVs in response to UVR was associated or not with ultrastructurally damaged bacteria. For this, the numbers of intact and damaged bacteria releasing OMVs were established. Our data showed that while the mean number of OMVs per intact or damaged cell did not differ in the control group ($p = 0.9976$), UVR radiation-exposed cells had a higher number of OMVs associated with damaged bacteria (Fig. 6D, $p = 0.005$). This means that cells in process of death had an increased rate of OMV release.

4. Discussion

Secretion of membrane vesicles is an important biological process of both eukaryotic and prokaryotic cells. This process has been characterized in pathogenic bacteria (Rivera et al., 2010; Tashiro et al., 2012; Pérez-Cruz et al., 2013), but is less clear in non-pathogenic bacteria from aquatic ecosystems. Here, we identified, for the first time, that gram-negative freshwater bacteria are able to release OMVs during their growth in cultures and react to an environmental stress (UV radiation) with increased formation of these structures. In addition, we demonstrated that the amplified rate of OMVs secretion, induced by UVR, occurs in bacteria undergoing viability and ultrastructural changes indicative of death. We thus recognized an active vesicular traffic operating in freshwater bacteria under normal and stress conditions.

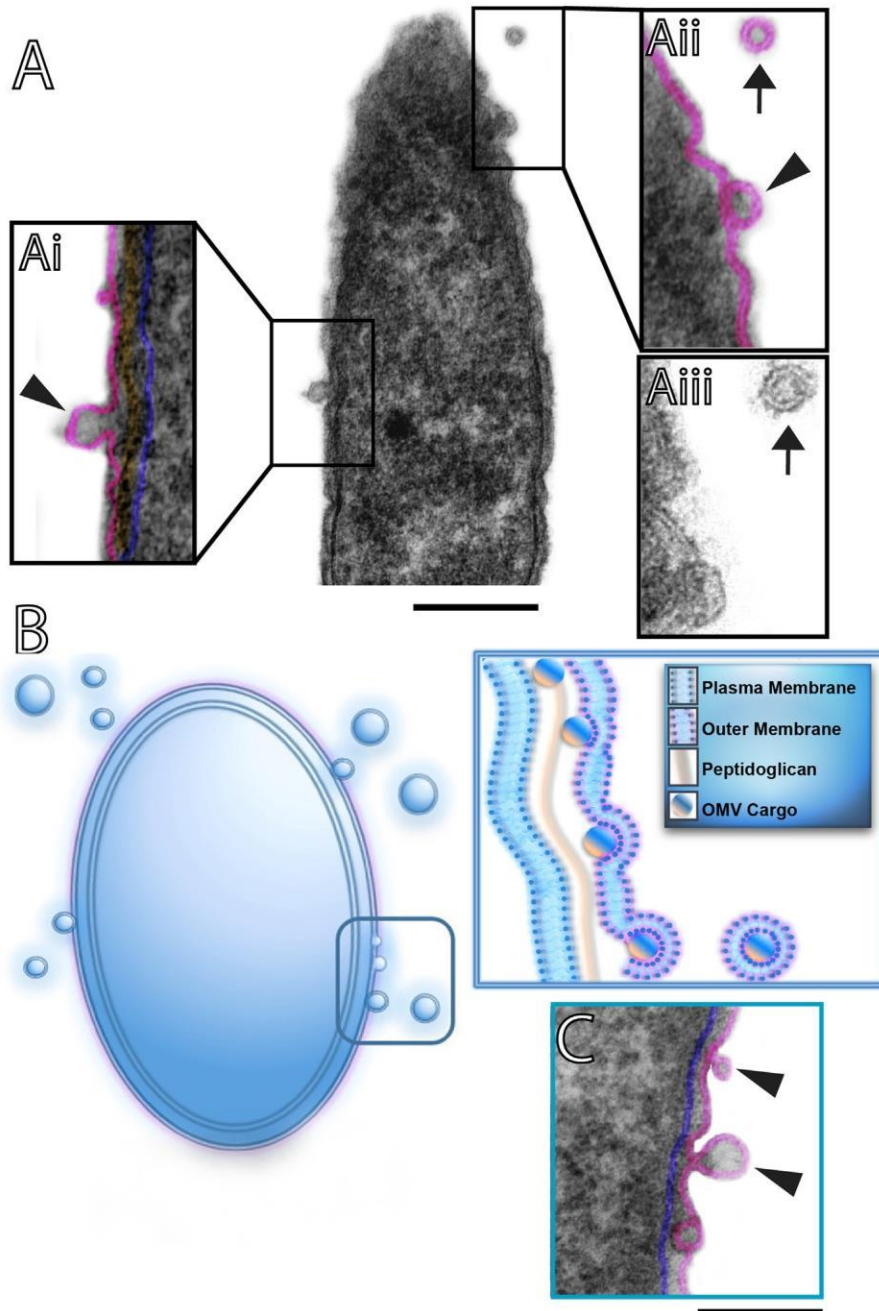


Fig. 5. Transmission Electron Microscopy (TEM) reveals OMVs release from growing freshwater bacteria.

(A) Representative electron micrograph of an intact freshwater gram-negative bacterium growing in culture. Note the presence of OMVs (arrowheads) budding off from the bacterial OM (highlighted in pink in Ai and Aii). The plasma membrane is highlighted in blue in (Ai). A free vesicle in the extracellular medium is indicated (arrow) in (Aii) and (Aiii). Note in (Aiii), the OMV trilaminar structure. In (B), a schematic representation of the cell envelope structure and OMV biogenesis from a typical gram-negative bacterium. In (C), the plasma membrane and the OM were highlighted in blue and pink, respectively in an electron micrograph taken from a growing bacterium in process of vesiculation (arrowheads). Cultured cells were fixed in a mixture of freshly prepared aldehydes and prepared for TEM. Scale bar = 120 nm (A), 40 nm (Ai and Aii), 30 nm (Aiii) and 50 nm (C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

In physiological normal conditions, bacterial cells have to be growing in order to secrete OMVs (reviewed in [Schwechheimer and Kuehn, 2015](#)). Because of the small size of OMVs, precise observation of these structures was achieved with TEM, which allows high resolution imaging of the vesicles. Here, we provided a comprehensive investigation of OMVs biogenesis at the EM level. After optimal fixation, we used a method of pre-inclusion in agar to avoid artifacts caused by mechanic damages and loss of specimens during sample manipulation ([Barros et al., 2010](#); [Silva et al., 2014](#)). Our studies demonstrated a true vesiculation process detected at the

bacteria OM, with clear identification of the membrane trilaminar structure in secreted vesicles ([Fig. 5Aiii](#)). Vesiculation was identified in freshwater bacteria growing in cultures in accord with recent works showing that both freshwater ([Silva et al., 2014](#)) and marine ([Biller et al., 2014](#)) bacteria release OMVs when growing in their natural environments. Thus, OMVs have likely a yet ill-understood role in homeostasis of the microbiota from aquatic ecosystems.

To induce bacterial stress, we treated the cells with UVR as before ([Noyma et al., 2015](#)), which intensities corresponded well with UVA and UVB intensities on a sunny day in the Brazilian south-

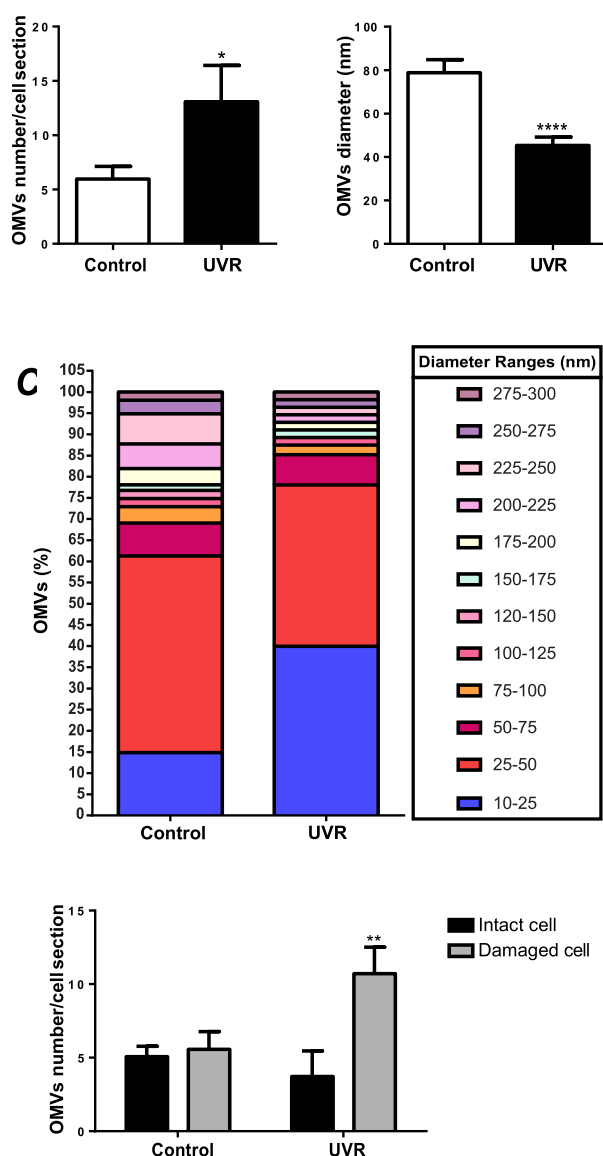


Fig. 6. Morphometric analyses of OMVs from cultured freshwater bacteria exposed to UVR.

(A) The number of OMVs produced by UVR-exposed bacteria is significantly higher compared to control cells. (B) OMVs secreted by bacteria in response to UVR had lower diameter in comparison to growing untreated bacteria. In (C), the graph shows the percentage of OMVs distributed in diameter ranges. (D) UVR-exposed cells had a higher number of OMVs associated with damaged bacteria. Data are expressed as mean \pm SEM. A total of 386 OMVs were counted in 165 electron micrographs and the number of vesicles per cell section was determined. * $P < 0.05$, ** $P < 0.01$ and **** $P < 0.0001$ versus control group. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article).

east region during autumn (UVA: 11.8 W m⁻² and UVB: 0.54 W m⁻²). Therefore, the UV intensities adopted here in our laboratory conditions can be considered moderate intensities when compared to those of summer in regions of high and middle latitudes (UVA: 45–50 W m⁻² and UVB: 7–8 W m⁻²) (Castenholz and Garcia-Pinchel, 2000).

It has been demonstrated in both cultures and natural environments that UV radiation can decrease the number of bacteria from aquatic ecosystems, thus indirectly indicating the occurrence of cell death (Winter et al., 2001; Warnecke et al., 2005; Hörtnagl et al., 2011; Santos et al., 2012). Here, we confirmed that UVR leads to significant reduction of the cell density (Fig. 1A). Additionally, by using viability markers for cell membrane permeability that enable the

distinction of live/dead bacteria under fluorescence microscopy, we provided direct evidence for bacterial death induced by UVR treatment (Figs. 1 and 2). In fact, irradiation with long-wave UV radiation (320–400 nm) can affect the membrane's unsaturated fatty acids, which induces changes in membrane permeability that can lead to death (Bintsis et al., 2000). Indeed, in parallel to an increased number of non-viable bacteria detected after 3 h of UVR-exposure, TEM revealed ultrastructural changes indicative of cell death (Fig. 4), characterized by loss of recognizable and intact cell structures (Heissenberger et al., 1996). Thus, taken together, our data demonstrated that UVR induce bacterial damage and death.

Our present results demonstrated an increase in OMVs production after exposing the bacterial cells to UV radiation. Under other stress conditions, such as rising temperatures or accumulation of undesirable proteins at the cell envelope, pathogenic gram-negative bacteria also increased vesiculation (McBroom and Kuehn, 2007), but this is the first time that increased secretion of OMVs is detected in response to UVR. UVR-induced OMVs showed though lower size compared to OMVs produced in normal conditions (Fig. 6). This might be explained by the rapid production of these membranous structures, which may be affecting membrane replenishment and dynamics required for vesicle formation.

Interestingly, our TEM analyses revealed that the OMVs production by UVR-treated bacteria was not a result of cell lysis. Nascent OMVs were clearly observed at the cell envelope of damaged bacteria without rupture of this structure (Fig. 4) and an amplified rate of OMVs release was detected in these bacteria in response to UVR. This means that damaged bacteria are able to release OMVs before lysis and that this secretion might be an adaptive response to rapid changes in environmental conditions such as UV radiation. Thus, we can speculate that OMVs formation constitute a protective mechanism against UVR.

The demonstration of overvesiculation in response to UVR raises the question of which kind of cargo(s) are packed and transported by these vesicles. Is stress-induced vesiculation a process to export undesirable products or to preserve undamaged material? Different compounds have been found within bacterial OMVs depending on the species/strain and experimental conditions. For example, in pathogenic species such as *Borrelia burgdorferi*, OMVs can deliver lipids that seem to act in immune responses modulation (Crowley et al., 2013). Quorum-sensing signaling molecules involved in cell communication, such as antimicrobial quinolones (Déziel et al., 2004) and virulence factors can be packed within vesicles in *Pseudomonas aeruginosa* during normal growth and exposure to antibiotics (Kadurugamuwa and Beveridge, 1995). In *Escherichia coli* and *Salmonella* sp., misfolded proteins accumulated at the cell envelope were exported within vesicles alleviating damage to the cell (McBroom and Kuehn, 2007). OMV production has also been pointed out as a system for DNA delivery to other bacterial cells. Antibiotic-resistant isolates of *A. baumannii* may release OMVs as a mechanism for horizontal transfer of antibiotic resistance genes to surrounding bacteria (Rumbo et al., 2011). Interestingly, OMVs from an Antarctic psychrotolerant bacterium *Shewanella vesiculosa* M7T can incorporate DNA (Pérez-Cruz et al., 2013). Future studies will be required to explore the cargo composition of OMVs produced in response to UVR as well as the stress response pathways involved in the vesiculation process of freshwater non-pathogenic bacteria.

Taken together, our findings demonstrate, for the first time, that a vesicular trafficking characterized by production of OMVs occurs in cultured freshwater gram-negative bacteria under normal and UVR-exposure conditions and that UVR triggers increased OMVs production in bacteria in process of death. The knowledge of physiological responses at cellular level in bacterial populations affected by UV radiation is important to understand the connec-

tion of environmental changes and persistence of species in aquatic ecosystems (Noyma et al., 2015).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at <http://dx.doi.org/10.1016/j.micres.2016.08.002>.

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