Universidade Federal de Juiz de Fora
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Thiago Pereira da Silva

BACTERIA FROM FRESHWATER ECOSYSTEMS: STRUCTURAL ASPECTS AND PROGRAMMED CELL DEATH

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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 em Ecologia Aplicada a Conservação e Manejo de Recursos Naturais.

Orientadora: Profª. Drª. Rossana Correa Netto de Melo

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À família, irmãos de sangue e vida,
Eu dedico este trabalho.

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Abstract

Bacteria are important components of the food web structure in aquatic ecosystems in which they influence the flow of carbon and energy. Populations of bacteria in these ecosystems comprise a diverse spectrum of individual cells able to respond to many factors such as nutrient supply, temperature and virus infection, which regulate bacterial life and death. Bacterial death is a key cellular event involved in the control and production of bacteria in aquatic ecosystems with functional meaning in the carbon and nutrient cycles. Therefore, the study of bacterial structural features and cellular mechanisms underlying bacterial death is crucial to understand processes affecting the entire population. However, both bacterial structure and cellular events of death in aquatic ecosystems are still poorly understood. In the present work, we used single cell approaches to study the structural organization of bacteria as well as to characterize cellular processes of death in these organisms. First, by using fluorescence and transmission electron microscopy (TEM), we provided a general panorama of how microscopy techniques, especially TEM, are powerful tools to understand bacterial structure and their responses to environmental stresses. We showed that bacteria from aquatic ecosystems have remarkable ultrastrutural diversity with components such as bacterial envelope of individual cells differing in structure within the same population. Second, we sought to identify and characterize mechanisms of bacterial cell death. Because our TEM analyses revealed morphological signs of apoptosis, a type of program cell death (PCD), in aquatic bacteria directly collected from natural ecosystems, we applied different techniques to detect apoptosis in bacteria cultured from natural samples. We used TEM as well as different probes to detect this type of PCD in cultured bacteria exposed to increased temperature and viral infection, which are recognized inducers of bacterial death. TEM showed, in both situations, ultrastructural changes indicative of apoptosis, such as cell retraction and condensation, similar to those reported for eukaryotic cells. Assays for membrane permeability, DNA fragmentation, phosphatidilserine exposition and caspase activation were significantly increased in treated bacteria compared to the control group. Altogether, our data demonstrate, for the first time, that PCD occur in aquatic bacteria, and that this event may be a basic mechanism for regulation of bacterial communities in these ecosystems.

Keywords: Bacteria from freshwater ecosystems. Ultrastructure. Programmed cell death. Apoptosis. Electron microscopy. Flow cytometry.

Abbreviations

DAPI	4,6-diamidino-2-phenylindole
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
EM	Electron microscopy
LPS	lipopolysaccharide
NCCD	Nomenclature Committee on Cell Death
OMV	Outer membrane vesicle
PCD	Programmed cell death
POC	Particulated organic carbon
PS	Phosphatidylserine
ROS	Reactive oxigen species
TdT	deoxinucleotidil transferase terminal
TEM	Transmission electron microscopy
UV	Ultraviolet
UVR	Ultraviolet radiation
VBR	Virus-bacteria rate
VLP	Virus like particles

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1. GENERAL INTRODUCTION

In aquatic ecosystems, bacteria play important roles during biogeochemical cycles in which they hold an unique position acting in the transformation of organic substrates and nutrient re-mineralization within microbial food webs (Fuhrman and Azam, 1980; Azam et al., 1983; Pomeroy and Darwin, 2007). Bacterial metabolism plays a key role for cycling of dissolved organic carbon (DOC) in marine and freshwater systems (Azam et al., 1983). The bacterial use of DOC for growth represents a point of entry for organic carbon into aquatic food webs (Azam, 1998; Jansson et al., 2007) and bacterial respiration results in the production of CO_2 which is largely returned to the atmosphere (Del Giorgio et al., 1997; Cole et al., 2007).

The knowledge of the bacterial functional capabilities and behavior in microbial food webs as well as their potential to cause human diseases is largely dependent on methods applied to the direct visualization of them during different conditions. The use of microscopy techniques is important to understand bacterial abundance, heterogeneity, structural organization, growth and division, cell viability, and changes in response to environmental stresses. However, while light microscopic assessment has been widely used for qualitative and quantitative studies of planktonic bacterial communities in water samples (reviewed in Soares et al., 2011; Fuhrman and Caron, 2016), application of electron microscopy methods has been limited and the ultrastructure of these microorganisms remains poorly understood.

Populations of bacteria comprise a diverse spectrum of individual cells that may differ widely from each other in terms of their genetic composition, physiology, biochemistry, or behavior (Koch, 1996; Schuster et al., 2000). This heterogeneity has important practical consequences for a number of ecological processes and functionalities (Gasol et al., 1999; Martins and Locke, 2015). The study of these microorganisms at high resolution by transmission electron microscopy (TEM) is crucial to understand individual cell features that underline processes affecting the entire population. Single-cell imaging provides unprecedented information on how individual microorganisms respond to their environment, interact with each other or undergo complex process (Brehm-Stecher and Johnson, 2004), such as cell death.

The physiological state of bacteria is a classic parameter for understanding the dynamics of the bacterial community in freshwater aquatic ecosystems. Currently, it is recognized that bacterial communities of aquatic ecosystems present a continuum

of physiological states, ranged from living to dead bacteria (Del Giorgio and Cole, 1998; Smith and del Giorgio, 2003). Bacteria in aquatic ecosystems are controlled by several factors, such as (i) quality and availability of organic substrate, which acts on bacterial growth and respiration; (ii) availability of nutrients, which influence bacterial growth and its interactions with other organisms; (iii) temperature, a factor that regulates bacterial metabolism; (iv) predation by protozoa and/or metazoan; and (v) virus infection, phenomenon consistently involved in bacterial death (Coveney and Wetzel, 1988; Fuhrman and Noble, 1995; Del Giorgio and Cole, 1998). The balance between these parameters leads to both the maintenance of the bacterial community and bacterial death.

Bacterial mortality can be described as a process of morphologic and functional loss, including cell lysis and destruction of genetic material (Servais et al., 1985). A large amount of degraded cellular compounds after cell death and lysis can be incorporated into dissolved organic carbon (DOC) and might be used by the bacterial community itself. Thus, cell death is a key event that not only regulates bacterial abundance but also contributes to the carbon and nutrient cycle in aquatic ecosystems (Lindeman, 1942; Pomeroy, 1974; Azam et al., 1983).

1.1 HOW BACTERIA FROM AQUATIC ECOSYSTEMS DIE?

Under an ecological perspective, the definition of bacterial life/death in aquatic ecosystems relies mostly on cell viability and growth analyses (Haglund et al., 2003; Signoretto et al., 2005; Hammes et al., 2010; Foladori et al., 2015). However, bacterial death is a much more complex event.

In general, cell death can occur through distinct mechanisms associated with different morphological and biochemical features. The mechanisms of cell death, such as programmed cell death (PCD), have been characterized in eukaryotic cells but are much less clear in bacteria, especially in bacteria from aquatic ecosystems. PCD is a genetically controlled process that directs cells to death. In eukaryotes, the most frequently documented PCD mechanism is apoptosis, which play important roles in the development, homeostasis and survival of multicellular eukaryotic organisms (Kroemer et al., 2005; Galluzzi et al., 2012). PCD is also described in bacteria and seems to serve for eliminating cells during developmental processes and stress situations. Although not fully understood, PCD in bacteria also plays a

critical role in population survival (Lewis, 2000; Engelberg-Kulka et al., 2006; Bayles, 2014). In bacteria from freshwater ecosystems, however, PCD remains to be characterized.

1.2 OBJECTIVES

The present work addresses two topics: i) applications of light and TEM techniques to understand the structure and function of microorganisms from freshwater ecosystems; and ii) identification of PCD in bacteria from aquatic ecosystems.

In the first section of this thesis, we present the results that contributed to the understanding of the structural complexity of freshwater bacteria linked to their roles in ecological processes. By using different approaches, we provide a general panorama of how microscopy techniques, especially TEM, are powerful tools to study structure and function of microorganisms from aquatic ecosystems. In the second section, we present the results on mechanisms of bacterial death. We demonstrate, for the first time, that apoptosis occurs in bacteria from aquatic ecosystems and discuss how this highly regulated cell mechanism could affect the social structure of microbial communities in ecological processes.

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2. APPLICATIONS OF LIGHT AND TRANSMISSION ELECTRON MICROSCOPY TECHNIQUES TO UNDERSTAND THE STRUCTURE AND FUNCTION OF MICROORGANISMS FROM FRESHWATER ECOSYSTEMS.

This section is composed of three publications:

1) SILVA, T. P. et al. Visualizing aquatic bacteria by light and transmission electron microscopy. Antonie van Leeuwenhoek, v. 105, n. 1, p. 1-14, 2014. ISSN 0003-6072.

2) SILVA, T. P.; GAMALIER, J. P.; MELO, R. C. N. TEM as an Important Tool to Study Aquatic Microorganisms and their Relationships with Ecological Processes. In: JANECEK, D. M. (Ed.). Modern Electron Microscopy in Physical and Life Sciences: InTech, 2016. chapter. 10.

3) SILVA, T. et al. Microscopy techniques applied to the study of cell death in bacteria from freshwater ecosystems. In: MÉNDEZ-VILAS, A. (Ed.). Microscopy and imaging science: practical approaches to applied research and education, 2017. p. 253-259.

The paper entitled *Visualizing aquatic bacteria by light and transmission electron microscopy* describes a new method for direct visualization and counting of bacteria by light microscopy. This technical approach was tested in a variety of freshwater ecosystems. In parallel, the ultrastructure of bacteria from an impacted Amazonian ecosystem was analyzed in detail by TEM.

The book chapter entitled *TEM as an important tool to study aquatic microorganisms and their relationships with ecological processes* highlights ultrastructural aspects of freshwater bacteria underlying their functional capabilities in aquatic environments. Lastly, the book chapter entitled *Microscopy techniques applied to the study of cell death in bacteria from freshwater ecosystems* is focused on the use of light microscopy and TEM to study cell viability and events of cell death in bacteria from freshwater ecosystems.

2.1 PUBLICATION 1

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Visualizing aquatic bacteria by light and transmission electron microscopy

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Abstract The understanding of the functional role of aquatic bacteria in microbial food webs is largely dependent on methods applied to the direct visualization and enumeration of these organisms. While the ultrastructure of aquatic bacteria is still poorly known, routine observation of aquatic bacteria by light microscopy requires staining with fluorochromes, followed by filtration and direct counting on filter surfaces. Here, we used a new strategy to visualize and enumerate aquatic bacteria by light microscopy. By spinning water samples from varied tropical ecosystems in a cytocentrifuge, we found that bacteria firmly adhere to regular slides, can be stained by fluorochromes with no background formation and fast enumerated. Significant correlations were found between the cytocentrifugation and filter-based methods. Moreover, preparations through cytocentrifugation were more adequate for bacterial viability evaluation than filter-based preparations. Transmission electron microscopic analyses revealed a morphological diversity of bacteria with different internal and external structures, such as large variation in the cell envelope and capsule thickness, and presence or not of thylakoid membranes. Our results demonstrate that aquatic bacteria represent an ultrastructurally diverse population and open avenues for easy handling/quantification and better visualization of bacteria by light microscopy without the need of filter membranes.

Keywords Aquatic bacteria · Ultrastructure · Fluorescence microscopy · Aquatic ecosystems · Cell viability · Electron microscopy

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Introduction

Bacteria are an important component of aquatic food webs (Azam et al. 1983; Pomeroy 1974). They are key components of the microbial loop, an alternative route of dissolved organic matter and nutrient transfer to metazoan trophic levels and consequently influence the flow of carbon and energy within an ecosystem (Pomeroy 1974; Pomeroy et al. 2007). Direct visualization and accurate estimates of bacterial abundance from environmental samples are thus essential to our

understanding of aquatic biology, including processes of biogeochemical cycles and global carbon budget.

Although other techniques such as flow cytometry (Gasol et al. 1999; del Giorgio et al. 1996; Wang et al. 2010) have increasingly been applied to evaluate aquatic bacteria abundance, microscopic assessment is still widely used and fundamental for qualitative studies of planktonic bacterial communities (Soares et al. 2011; Romanova and Sazhin 2011). Light and electron microscopy techniques are important to investigate not only bacterial abundance but also to understand heterogeneity, structure and functioning of these organisms. Moreover, individual imaging of bacteria is valuable to recognize bacterial viability and their physiological functions at single-cell level (Joux and Lebaron 2000).

Over the past three decades, epifluorescence microscopy became the standard method for counting aquatic bacteria collected on polycarbonate filters and stained with fluorescent probes such as acridine orange (Hobbie et al. 1977), 4′,6-diamidino-2-phenylindole (DAPI) (Porter and Feig 1980; Kepner and Pratt 1994) and HOECHST (Paul 1982). Because bacterial lysis is a key process in aquatic ecosystems, fluorescence microscopy in combination with viability stains has also been used to evaluate damaged or dying bacteria in these systems (Decamp and Rajendran 1998; Boulos et al. 1999). Despite the fluorescent marker used, most of these methods rely on the use of filter membranes, i.e., because of the small size of aquatic bacteria, routine evaluation of these organisms by light microscopy requires staining with fluorochromes and sample filtration to enable direct observation on the filter surface. This procedure offers some disadvantages such as fluorescence background formation and a high cost of membrane filters.

While observation of aquatic bacteria by light microscopy is an approach extensively used in studies of planktonic bacteria, the ultrastructure of these organisms is not completely understood. Some studies have used transmission electron microscopy (TEM) as a powerful tool to study structural aspects of bacteria in both natural environments and cultures (Moriarty and Hayward 1982; Hoppert and Mayer 1999; Kerfeld et al. 2010). However, little is known on the ultrastructure of aquatic bacteria from tropical ecosystems (Barros et al. 2010).

Here, we investigated aquatic bacteria collected from varied tropical field sites by both light and transmission electron microscopy. We introduce a new methodological strategy for direct visualization and counting of these organisms using samples prepared through cytocentrifugation and observed by fluorescence microscopy. By comparing cytocentrifugation and filter-based methods, we found that bacteria firmly adhere to regular slides (without the need of filters), can be stained by different fluorochromes with no background formation (a common problem induced by filter surfaces) and fast enumerated. Evaluation of these cells by TEM revealed a morphological diversity of bacteria associated with the presence of different internal and external bacterial structures. Our findings help to advance methodological approaches for studies of aquatic bacteria and to understand their ultrastructural organization.

Materials and methods

Sampling

Samples ($n = 47$) from different Brazilian ecosystems (rivers, coastal lakes, Amazonian systems, reservoirs and semi-arid reservoirs) were collected directly from subsurface (0.5 m) of all systems (Table 1) and immediately fixed with free-particle 37 % formaldehyde (<0.2 μm filtered) to a final concentration of 2 %. Fixed samples were kept in the dark at room temperature for further processing and enumeration.

Bacteria quantification

Bacteria were stained with 4′,6′-diamidino-2-phenylindole (DAPI); 0.01 μg mL^{-1} final concentration (Porter and Feig 1980) or acridine orange 0.01 % final concentration (Hobbie et al. 1977).

Table 1 Number of water samples collected from different Brazilian aquatic ecosystems

Aquatic ecosystems	*n* (samples)
Amazonian systems	6
Costal lakes	8
Reservoirs	7
Rivers	10
Semi-arid reservoirs	16
Total	47

For bacteria enumeration using filters, samples (1 mL) plus 10 µL of DAPI or acridine orange were incubated for 10 min, placed in a vacuum pump and filtered onto a black 25-mm, 0.2 µm Millipore polycarbonate membrane (Hobbie et al. 1977) with 1.2 µm supporting filter (Millipore, USA) on a maximum vacuum of 100 mmHg. Filters were dried, placed between glass slides and mounted with a small drop of mineral oil.

For bacteria enumeration using cytocentrifugation preparations, samples (1 mL) plus 10 µL of DAPI or acridine orange were placed in mega funnels (Shandon Mega funnel, Thermo, UK) for immediate centrifugation in a cytocentrifuge (Shandon Cytospin 4, Thermo, United Kingdom) (Fig. 1), at 452*g* and high acceleration for 10 min. Acceleration and speed were established as the procedures for medical microbiology provided by the Cytospin manufacturer manual. Cytocentrifugation was done by using regular slides without any coating.

For each filter or slide, bacteria were directly counted in 20 fields at 1,000× magnification. The fields were selected randomly. A minimum of 200 cells were counted to reduce the standard deviation and provide a trusty mean (Kirchman 1993). Analyses were performed on a fluorescence microscopy (BX-60, Olympus, Melville, NY, USA) using U-MWU2 filter (330–385 nm excitation wavelengths) for DAPI counts and U-MWB2/UMWG-2 (460–490/510–550 nm excitation wavelengths) filters for acridine orange counts. The relationship between two methods was determined using last square regression. Statistical analyses were performed using JMP statistical software (version 5.0.1, SAS Institute, USA) adopting $p < 0.05$ as a threshold level for acceptance.

Bacteria viability

For evaluation of aquatic bacteria viability, a total of 10 samples from lakes and reservoirs were prepared for both cytocentrifugation and filter-based methods using the LIVE/DEAD BacLight kit (Molecular Probes) which enable differentiation between bacteria with intact and damaged cell membranes (Boulos et al. 1999; Freese et al. 2006). This kit contains a mixture of fluorescent stains (SYTO® 9 and propidium iodide) that differ both in their spectral characteristics and their ability to penetrate healthy bacterial cell membranes. SYTO® 9 stains viable cells whereas propidium iodide stains non-viable cells. Bacteria were stained by adding 1 mL of each sample to 3 µL of BacLight. After 20 min in the dark, samples were placed in megafunnels (Shandon Mega funnel, Thermo, UK) for immediate centrifugation in a Cytospin (Shandon Cytospin 4, Thermo, United Kingdom), at 452*g* and high acceleration for 10 min. Samples were prepared by using regular slides without any coating.

Analyses were performed on a fluorescence microscope (BX-60, Olympus, Melville, NY, USA) using U-MWB filter (450–480 nm excitation wavelengths) which allows simultaneous visualization of both markers. Bacteria were directly counted in 10 random fields at 1,000× magnification. It was established the average percentage of live/dead bacteria for each ecosystem.

Cell preparation for TEM

A one-liter sample was collected from Amazonian systems and filtered through a 30 µm membrane mesh to remove large particles. Four hundred millilitres of filtered sample was centrifuged at 3,000*g* for 10 min. They 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), washed twice in the same buffer at 3,000*g* for 10 min, and stored at 4 °C for subsequent use. After fixation, agar embedding was performed as before (Melo et al. 2005), so that uniformly distributed specimens could be processed as easily handled blocks of tissue. Samples were centrifuged at 1,500*g* for 1 min. They then were resuspended in molten 2 % agar in 0.1 M sodium cacodylate buffer, pH 7.4, and quickly recentrifuged. The resulting agar pellets were kept in the same buffer at 4 °C for further processing. Agar pellets containing water specimens were processed as described previously (Melo et al. 2005). Samples were postfixed in 1 % osmium tetroxide in Sym-Collidine buffer, pH 7.4, for 2 h at RT. After being washed with sodium maleate buffer, pH 5.2, they were stained en bloc in 2 % uranyl acetate in 0.05 M sodium maleate buffer, pH 6.0, for 2 h at RT and washed in the same buffer as before, prior to dehydration in graded ethanol and infiltration and embedding with a propylene

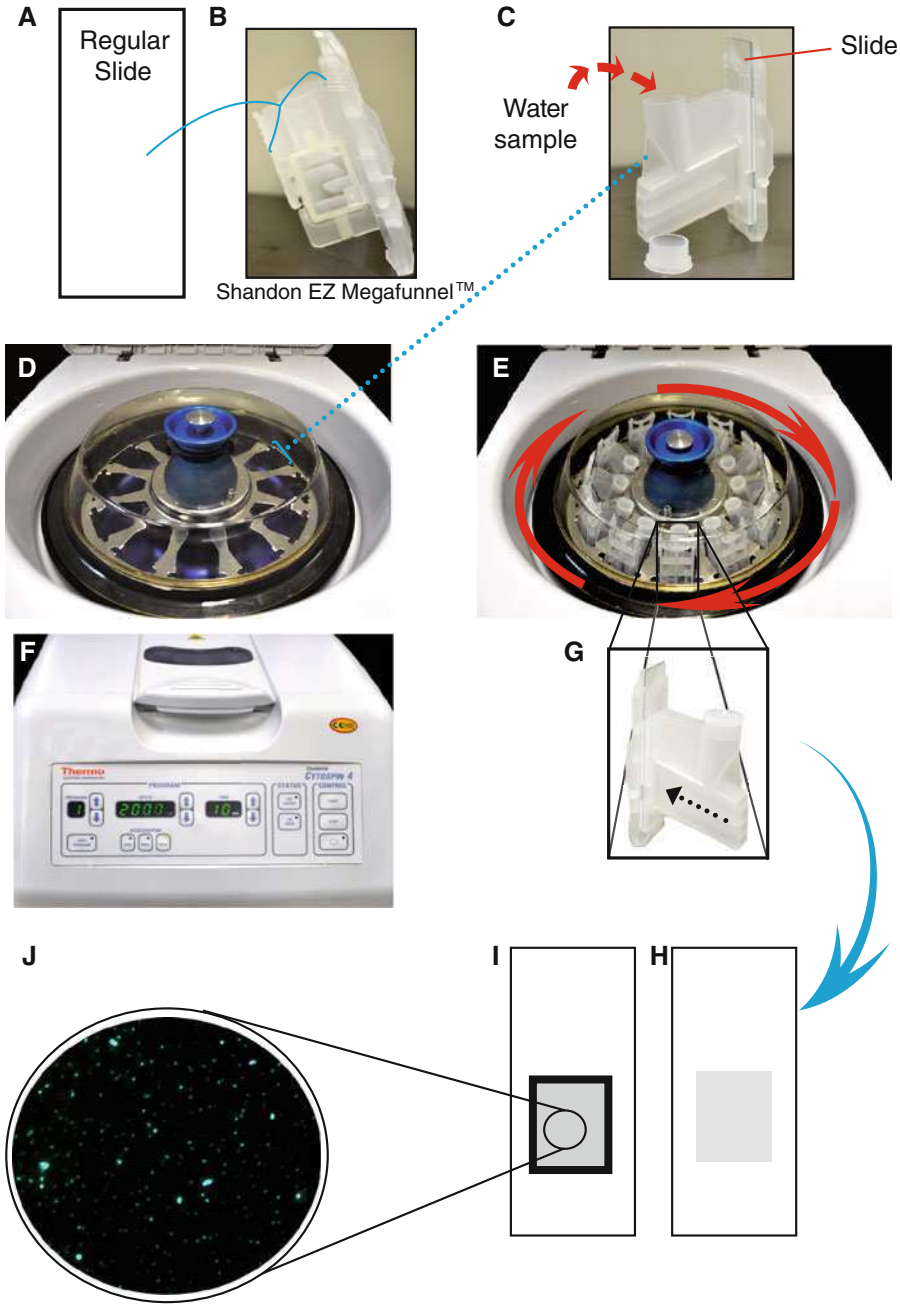

Fig. 1 Cytocentrifugation method for environmental water samples. **a, b** Step 1: A regular glass slide is coupled to a Shandon EZ Megafunnel™. **c** Step 2: After fixation and staining (see "Materials and methods" section), each water sample (1 mL) is placed into the Megafunnel sample chamber. **d** Step 3: The Megafunnel is then loaded into indicated spot inside the cytocentrifuge. Several samples can be processed at the same time **e, f** Step 4: After closing the external lid, the spinning parameters are set in the control panel. Time, speed and acceleration are set according to "Materials and methods" section. In **g**, is seen the Megafunnel in the upright position (*swing position*). The centrifugal force (*arrow*) allows the cell to be spun onto the slide. This effectively by-passes the difficulties normally associated with depositions obtained by direct smear or filtration. **h** Step 5: The slide is uncoupled from megafunnel and a monolayer of cells can be observed in a defined area on the slide. **i, j** Step 6: After waiting few minutes (3–5) to dry, the slide is mounted and it is ready to be analyzed under fluorescence microscopy

oxide-Epon sequence (Eponate 12 resin; Ted Pella, Redding, CA). After polymerization at 60 °C for 16 h, thin sections were cut using a diamond knife on an LKB ultramicrotome (LKB Instruments, Gaithersburg, MD). Sections were mounted on uncoated 200-mesh copper grids (Ted Pella) before being stained with lead citrate. Organisms were examined using a transmission electron microscope (P300; Philips, Eidhoven, Netherlands) at 60 kV.

Quantitative TEM

To study details of the bacterial ultrastructure, electron micrographs from aquatic bacteria were randomly taken at magnifications of 30,000–75,000× The following parameters were evaluated: cell diameter, thickness of the bacterial cell envelope, number of bacteria with substratum particles adhered to their capsules and number of bacteria showing cytoplasmic thylakoid membranes. Cell measurements were evaluated using the software Image J 1.41 (National Institutes of Health, Bethesda, MD, USA). At least 40 cells were analyzed.

Results

Visualization and quantification of aquatic bacteria by light microscopy

First, staining of bacterial samples with acridine orange and DAPI enabled clear imaging of these organisms that were seen as fluorescent, very small structures with different shapes, (Fig. 2a, c respectively). Samples prepared by cytocentrifugation showed bacteria uniformly distributed and firmly adhered to the slides (Fig. 2b, d). In contrast with samples prepared through the use of membranes, cytospin preparations had no background formation (compare Fig. 2a, c with Fig. 2b, d).

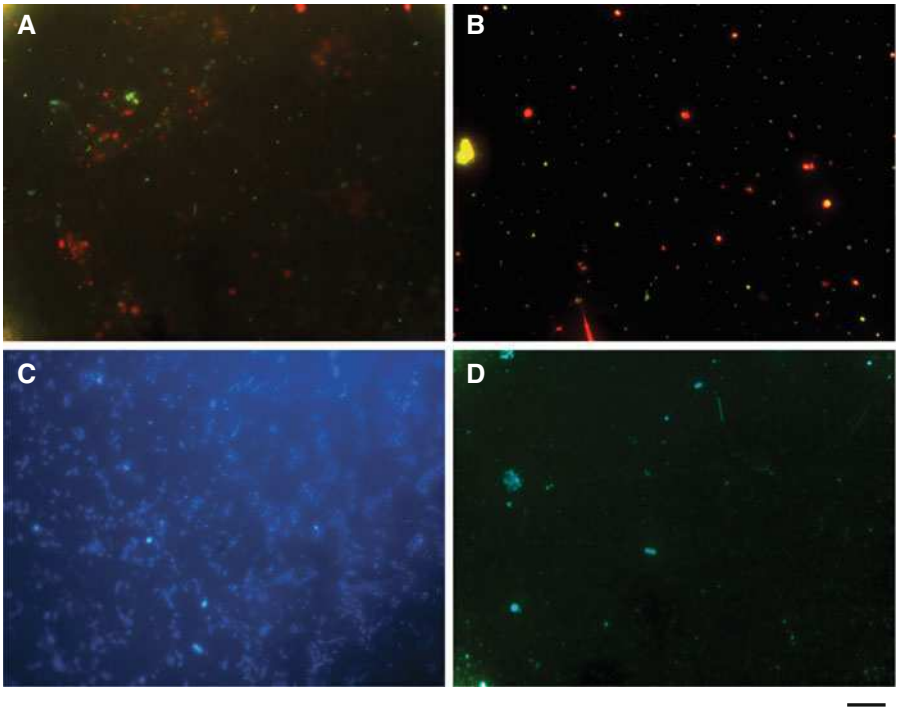

Fig. 2 Aquatic bacteria observed by fluorescence microscopy using membrane filter (**a, c**) or cytocentrifugation (**b, d**). Bacteria appear as homogeneously distributed, very small structures stained in *green/orange* by acridine orange (**a, b**) or *blue* by DAPI (**c, d**). Cytospin preparations enabled a direct bacteria observation on the surface of the slides with no background formation after staining (**b, d**) while preparations using filter membranes show background (**a, c**). Samples were collected, fixed and stained as before (Porter and Feig 1980; Hobbie et al. 1977). Pictures from an entire field were taken using a BX-51 microscope (Olympus) at 1,000× and reproduced without any editing procedure. *Scale bar* 10 μm

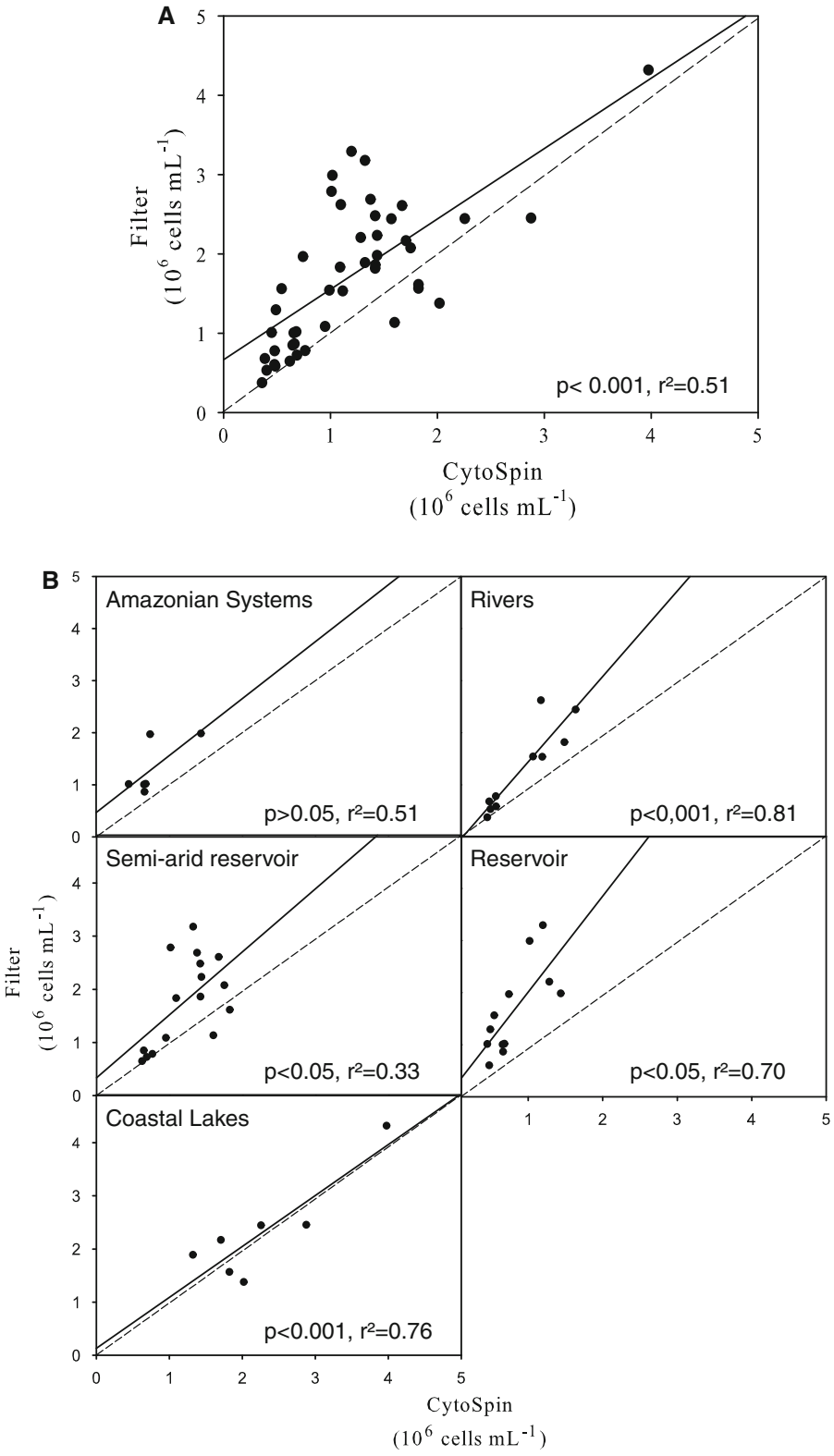

Fig. 3 Regression analyses of bacteria quantitated through the membrane filter method versus the cytocentrifugation method. **a** Total bacteria countings (considering all samples) were a significant statistically regression when the methods were compared ($r^2 = 0.51$, $p < 0.001$). **b** Different correlation coefficients were found in each group of aquatic ecosystems. A total of 47 samples were counted as described in "Materials and methods" section

Next, bacteria were quantitated using a total of 47 samples prepared from both methods and the results were compared. Our quantitative analyses showed a statistically significant regression between heterotrophic bacterial counts using membrane filter and cytocentrifugation methods ($r^2 = 0.5$, $p < 0.001$) (Fig. 3). Total bacterial cell number varied from 0.37×10^6 to 4.31×10^6 cells mL^{-1} using membrane filters and from 0.36×10^6 to 3.90×10^6 cells mL^{-1} using the cytocentrifugation method. The correlation between the two methods was also determined for each group of aquatic ecosystem (Fig. 3b). Different coefficients of determination were found when the aquatic systems were analyzed separately. The highest values were found in rivers ($r^2 = 0.81$, $p < 0.001$) whereas semi-arid reservoirs showed the lowest values ($r^2 = 0.33$, $p < 0.05$). However, the Amazonian system did not show a satisfactory coefficient determination ($r^2 = 0.51$, $p > 0.05$). In summary, the overall results indicate a significant global (with all ecosystems) linear model between the methods. Only the coastal lakes group presented a very tight relationship between the two methods, while all the others followed the global model tendency.

Viable and non-viable bacteria are better observed through cytocentrifugation

Cytocentrifugation preparations enabled a clear imaging of live/dead bacteria as well as observation of other aquatic microorganisms (Fig. 4a) while the visualization of bacteria on the surface of membranes was much less clear with accentuated background formation (Fig. 4c). The sharp visualization of live/dead bacteria on cytocentrifugation preparations allowed precise quantification of these bacteria. Our analyses showed 36.20 % of dead bacteria versus 63.80 % of viable bacteria (Fig. 4d). Quantitative evaluation of live/

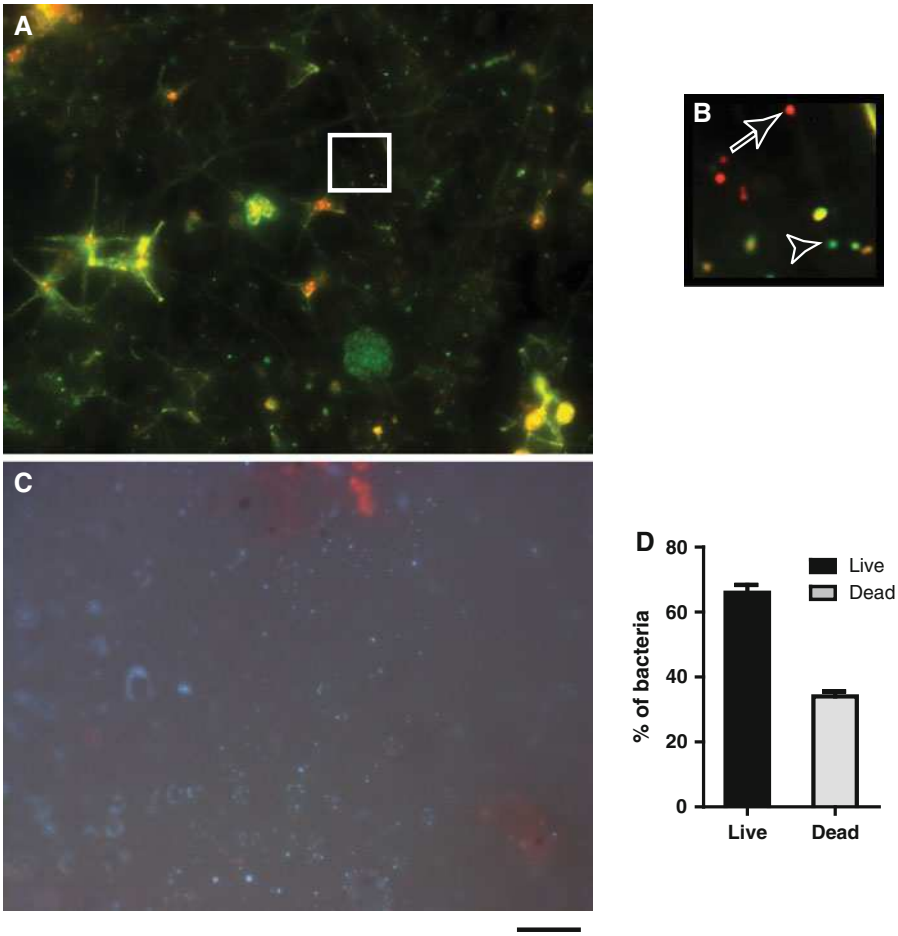

Fig. 4 Viability of aquatic bacteria analyzed through cytospin preparations (**a**) and membrane filters (**c**). Samples ($n = 10$) were collected from 2 lakes (an Amazonian clear water system and a reservoir) and stained with viability markers (Live/Dead BacLight kit). Live/dead bacteria were clearly observed as *green* (**b**, *arrowhead*) or *red* (**b**, *arrow*) structures using the cytocentrifugation method in contrast with the diffuse imaging and accentuated background in membrane filters (compare **a** and **c**). In **d**, quantification of bacteria viability using cytospin preparations (**a**). *Scale bar* 10 μm (**a**, **c**) and 7 μm (**b**)

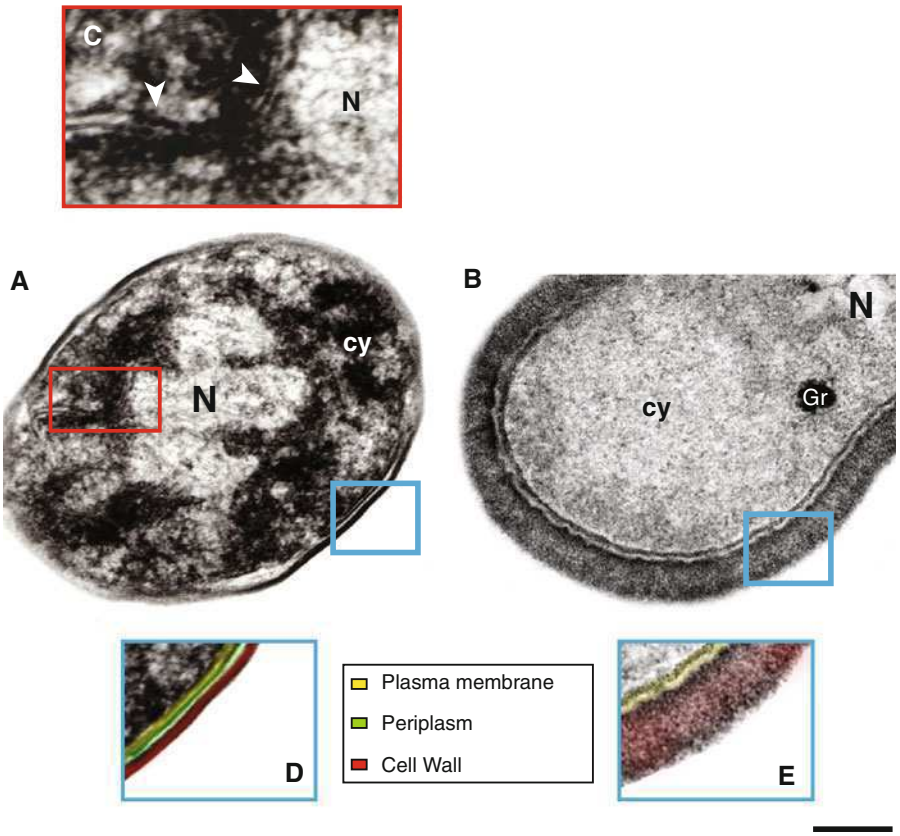

Fig. 5 Transmission electron microscopy of aquatic bacteria from an Amazonian ecosystem. (**a, b**) Bacteria with typical structures as nucleoid (*N*), granule (*Gr*), intracellular membrane system (**c**, *arrowhead*) and cell envelopes showing different thickness and composition (*blue rectangles*). In **d**, a thin cell envelope composed by cell wall, periplasm and plasma membrane is observed in high magnification while in **e** the bacterial cell envelope appear thicker and composed by plasma membrane and cell wall. Note that the cytoplasm (*cy*) of two bacteria show distinct electron-density (compare **a** and **b**). *Scale bar* 240 nm (**a**), 90 nm (**b**), 140 nm (**c**), 180 nm (**d**), and 45 nm (**e**)

dead bacteria using the membrane method was difficult because of the high stain impregnation on the surface of the membrane.

Ultrastructural evaluation of aquatic bacteria

The ultrastructural analyses showed bacteria with different sizes (diameters varying from 0.12 µm to 2.0 µm) and with typical structures, such as nucleoid (Figs. 5a, b, 6a, 7a, b, 8b) cytoplasmic membrane with typical trilaminar organization (Fig. 5), cell wall (Fig. 5a, b) and electron-dense granules (Figs. 5b, 7a). TEM revealed a morphological diversity of bacteria associated with the presence of different internal and external bacterial structures. Internal differences were represented by distinct cytoplasmic electron-density (compare Fig. 5a, b), presence or not of projections of the cell membrane into the cytoplasm (Fig. 5a and boxed area in 5c), and presence or not of thylakoid membranes (Fig. 6). Around 7.5 % of bacteria showed typical thylakoid membrane systems (Fig. 6), indicating that part of the bacterial population was actively involved with primary production in these aquatic ecosystems.

Large variations in the thickness of the bacterial cell envelope (Fig. 5a, b) and capsule were frequently observed (Fig. 7a, b). Our quantitative analyses showed that capsular structures were present in 40 % of the cells with thickness varying between 10.7 nm and 117.0 nm. Interestingly, 32.5 % of bacteria had substratum particles adhered to their capsular structures (Fig. 7).

Remarkably, TEM revealed the presence of membrane-bound vesicles budding off from the bacterium

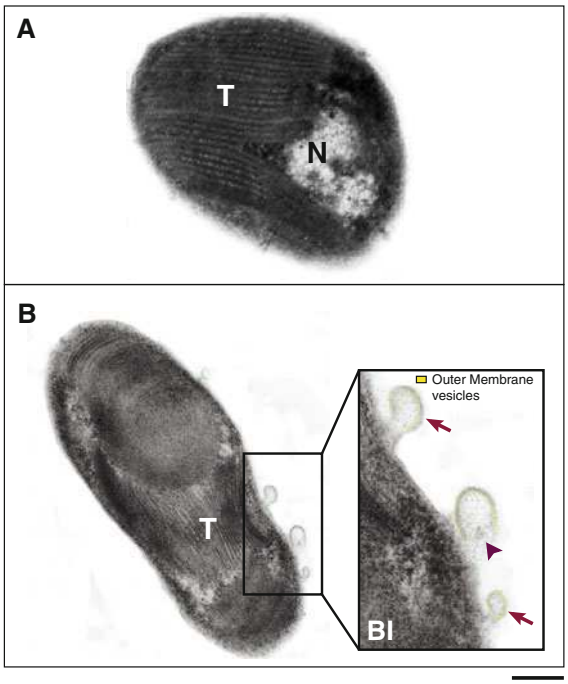

Fig. 6 Ultrastructure of autotrophic aquatic bacteria showing thylakoids membranes and secretory vesicles. (**a, b**) Typical thylakoids (*T*), organized as a system of membranes, are observed in the bacterium cytoplasm. In **c**, note the formation of secretory vesicles from the bacterial outer membrane (*arrow*) and the release of vesicle contents into the extracellular environment (*arrowhead*). The trilaminar aspect of the cellular membrane (highlighted in *yellow*) is clearly observed. *N*, nucleoid. *Scale bar* 165 nm (**a**), 380 nm (**b**), 190 nm (**c**)

outer membrane (Fig. 6b, c), a morphological indicative of a secretory process into the environment. Interaction between bacteria (Fig. 8a) and cells in process of division (Fig. 8c) were also observed.

Discussion

In the present work, we describe microscopy approaches that can improve direct visualization and studies of aquatic bacteria. We report on the application of a novel strategy to study aquatic bacteria by light microscopy and also reveal structural aspects of these organisms using transmission electron microscopy.

Using samples collected from different aquatic ecosystems, we show that a method based on cytocentrifugation is reliable to direct visualization of these cells and represent a good alternative to the use of filters for bacterial counting. The cytocentrifugation method is based on the transfer of any sedimentable particles, including cells, from liquid suspension onto microscope slides. This technique has been widely used in several biological studies and clinical analysis, especially cytology, hematology, microbiology and analysis of other fluids (Melo et al. 2009; Chapin-Robertson et al. 1992), but has not been applied to limnological evaluations. The principles of centrifugation are well-known (Stokes 2004). Briefly, the principles involve density of particles and speed of the sedimentation through the fluid medium density when a centrifugal force is applied to the particle mass. Particle sedimentation speed increases with the rotational speed of the centrifuge, and it is faster for large or dense particles than for small or light particles. Despite having the same concept of a regular centrifuge, the cytocentrifuge is a device that uses carefully controlled centrifugation to separate and deposit a thin layer of cells on slides while maintaining cellular integrity. It produces better cell capture and good representation of all cell types present in homogeneous liquid samples. Hence, cytocentrifugation of water samples from aquatic ecosystems may be very useful for analysis of cell populations of these systems, including bacteria.

By light microscopy, aquatic bacteria imaging is currently performed on the surface of membrane filters which requires individual, time-consuming sample preparation. In contrast, cytocentrifugation enables a faster sample processing at low cost. This represents a great advantage over the filter-based method considering that limnological studies of bacteria communities generally involve analysis of a high number of water samples. Moreover, our data showed that a significant coefficient of determination (relationship) when the two methods were analyzed by square regression. However, this relationship varied for each aquatic ecosystem group (Fig. 3b). The tighter relationship was found in costal lakes, while the higher correlation values were found in rivers and the lowest in semi-arid reservoirs. This may be explained by the heterogeneity of these systems and presence of factors that may influence bacterial abundance such as predominance of other microorganisms, presence of other particles in suspension and composition of bacterial communities (Seo et al. 2010). In aquatic Amazonian ecosystems, for example, the high concentration of particles in suspension, may affect bacterial abundance determination through fluorescence microscopy (Ferrao-Filho and Esteves 1994; Esteves et al. 1994). In addition, some

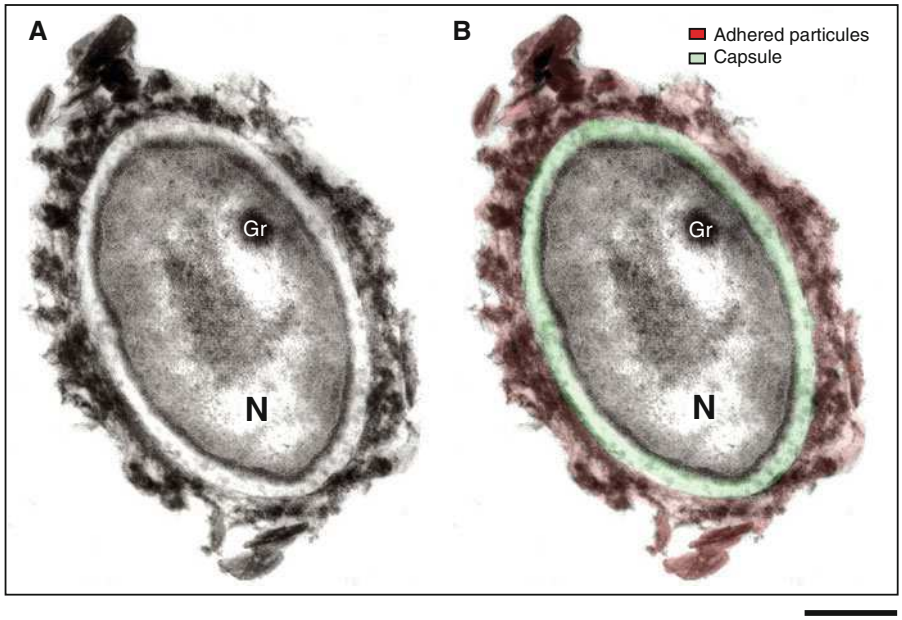

Fig. 7 Capsular ultrastructure of an aquatic bacterium. Substratum particles (**b** highlight in *red*) are adhered externally to the capsule (**b** highlight in *green*). Typical bacterial structures such as nucleoid (*N*) and granule (*Gr*) are observed. *Scale bar* 130 nm (**a**, **b**)

variation in the total number of bacteria, especially in specific aquatic ecosystems, is expected when different quantification approaches are compared (Gasol et al. 1999). Indeed, when we compared the two methods in each system, the filter-based method revealed higher bacterial abundance than that obtained with the cytocentrifugation method. On the other hand, our results also showed that higher quality of bacteria imaging was achieved with cytocentrifugation compared to imaging of these organisms on the filter surfaces. In fact, artifacts such as auto fluorescent particles, detritus and even other autotrophic organisms, for example, algae, was noticeably higher for filters compared to other methods such as direct visualization of bacteria on bioadhesive slides (Franklin et al. 2011). Therefore, the fact that bacterial numbers quantitated with the cytocentrifugation were lower than those observed with the filter-based method may not necessarily mean that the filter method is more accurate for bacteria abundance evaluation. In fact, although membrane filters are currently the most used methods, we demonstrate here that alternative approaches may be more advantageous and also provide reliable results.

Quantification of viable and non-viable bacteria is an important parameter in aquatic ecosystems to understand variations on microbial communities, and their potential impact on the food web and fluctuations in geochemical cycles in which these microorganisms are involved (Decamp and Rajendran 1998). Assessment of bacterial death by light microscopy is still mostly performed indirectly through determination of bacterial abundance. However, some works have highlighted that a large fraction of what were then being counted as heterotrophic bacteria by light microscopy were in fact particles without genome: dead cells, ghosts or large viruses and cell fragments (Zweifel and Hagstrom 1995; Seo et al. 2010; Gasol et al. 1999). Therefore, the use of adequate approaches to direct visualization of bacterial viability is crucial for limnological studies. Our data showed that membrane filters can cause considerable background in light microscopy, particularly when regular fluorescent markers for cell viability (SYTO 9 and propidium iodide) are used (Fig. 4c). On the other hand, the use of cytospin preparations proved to be much more adequate for visualization of viable and non-viable bacteria (Fig. 4a). Viability of other aquatic microorganisms such as cyanobacteria was also better observed on slides prepared through cytocentrifugation (data not published). Indeed, both enumeration and viability investigation of individual physiological groups of bacteria and other microorganisms always

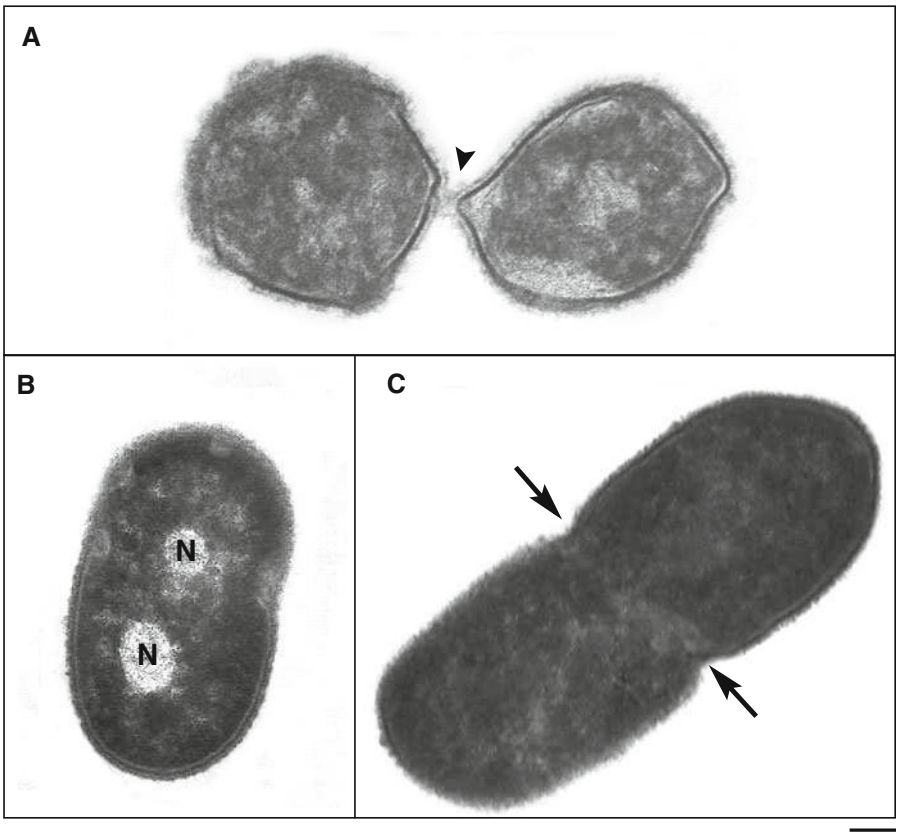

Fig. 8 Ultrastructural views of aquatic bacteria collected from Amazonian systems. In **a**, an interaction between two bacteria (*arrowhead*) is observed. In **b** and **c**, longitudinal sections of bacteria with very electron-dense cytoplasm and typical nucleoid (*N*). In **c**, rod-shaped bacteria in division. Note that the cell splits in the mid line by binary fission forming a septum on each cell side (*arrows*). *Scale bar* 100 nm (**a**) and 190 nm (**b, c**)

remain an urgent problem due to the high heterogeneity and dynamics of microbial communities (Romanova and Sazhin 2011). It is well documented a continuum of physiological status of bacterial life and death in aquatic ecosystems and a great variation in bacterial viability depending on factors such as heterogeneity of bacterial populations, environmental stress, nutrient competition and predation (Joux and Lebaron 2000; Smith and Del Giorgio 2003; Sawstrom et al. 2008; Romanova and Sazhin 2011). In marine ecosystems, bacterial viability may vary from 40 to 85 % (Decamp and Rajendran 1998) while in temperate freshwater ecosystems, the variation range is from 13 to 83 % (Sawstrom et al. 2008). Thus, our results demonstrate that cytocentrifugation is a simple method that can greatly improve the live/dead analysis quality and then, contribute for many studies of bacterial viability and physiological state.

The ultrastructure of aquatic bacteria from Amazonian ecosystems was analyzed by TEM. We used a method of pre-inclusion in agar to obtain optimal preservation and reduction of artifacts caused by mechanic damages and loss of specimens during sample manipulation (Melo et al. 2007, 2009). Our TEM analyses revealed not only typical bacterial structures (Fig. 5), but also a great morphological diversity of these cells represented by the presence of distinct external and internal structures (Figs. 5, 6, 7, 8). Differences in the thickness of cell envelopes likely represent the presence of gram-positive and gram-negative bacteria, both commonly found in aquatic ecosystems (Moriarty and Hayward 1982; Beveridge 1999; Nevot et al. 2006). Our data also showed that 31.4 % of aquatic bacteria had capsular structures. This frequency is higher than that found for marine bacteria (7–27 %) (Heissenberger et al. 1996;

Stoderegger and Herndl 2001; Cowen 1992). Bacterial capsules are important for cell interaction with the environment, enabling not only the absorption and storage of nutrients (Roberts 1996), but also serving as a protection barrier against toxic agents from the medium and predation (Hahn et al. 2004). Moreover, our data revealed substratum particles adhered to the bacterial capsular structures, a sign that indicate a survival strategy for increase efficiency for acquisition of organic matter and protective of predators (Grossart 2010).

An interesting ultrastructural observation was the presence of membranous secretory vesicles projecting from the bacterium external membrane into the extracellular medium (Fig. 6b, c). These secretory vesicles, observed for the first time in aquatic bacteria from tropical ecosystems here, may be important for bacterial survival and inhibition of lysis induced by viral infection (Kulp and Kuehn 2010). In fact, it was demonstrated that there is a strong correlation between virus and bacteria in Amazonian systems indicating that virus-infected bacteria is a common finding in these aquatic ecosystems (Barros et al. 2010). Moreover, these secretory vesicles may have distinct products involved in the formation of the cell wall, inhibition of toxic components present in the surrounding medium (McBroom and Kuehn 2007) and formation of biofilms (Schooling and Beveridge 2006). Bacterial secretion may also be associated with delivery of enzymes important for nutrients acquisition (Thompson et al. 1985) and autolysins for degradation of other bacteria favoring the competition for niches (Li et al. 1998).

TEM also revealed a consistent system of endomembranes (mesosomes and/or thylakoid membranes) in the bacterial cytoplasm (Figs. 5a, c, 6). Mesosomes have been regarded as structures involved in a range of cell functions including chromosome segregation during cell division (Higgins et al. 1971; Ryter 1968) or even structural artifacts (Higgins et al. 1976). More recently, intracellular formation of mesosome-like membranes has been documented in pathogenic bacteria under stress conditions (Santhana Raj et al. 2007; Li et al. 2008; Hartmann et al. 2010).

Thylakoid are important membranes related to metabolic processes, particularly photosynthesis, and are found within cyanobacteria (Liberton et al. 2006) and in a small group of bacteria (Yurkov and Beatty 1998; Miller 1979). Although some distinction among heterotrophic and autotrophic can be seen by light microscopy depending on the type of fluorochrome and microscope filter used, our results highlight TEM as a powerful tool to distinguish these organisms because of the high resolution provided by this technique.

The high complexity and variation in the availability of organic matter in aquatic ecosystems facilitate the evolution of a vast repertoire of physiologic bacteria adaptations to the environment (Grossart 2010; Azam 1998). Therefore, the great ultrastructural bacterial diversity reported here may be related to the functional diversity of these organisms.

In summary, our data demonstrate that aquatic bacteria from tropical systems represent an ultrastructurally diverse and complex community with distinct components likely involved in a range of ecological processes and open avenues for easy handling/quantification and better visualization of bacteria by LM without the need of filter membranes.

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2.2 PUBLICATION 2

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TEM as an Important Tool to Study Aquatic Microorganisms and their Relationships with Ecological Processes

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Additional information is available at the end of the chapter

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Abstract

Microorganisms are critically important for ecological processes in aquatic environments. Bacteria and viruses are key components of the microbial loop and are central for biogeochemical cycles in aquatic ecosystems. Our group has been using transmission electron microscopy (TEM) to study aquatic microorganisms in both natural tropical ecosystems and cultures. In this review, we highlight structural aspects of freshwater bacteria, based on TEM findings that have provided insights into the functional capabilities of these cells in aquatic tropical ecosystems. First, we focus on TEM applied to the study of the ultrastructural diversity and morphological alterations of bacteria in response to environmental stress. Second, we address the relationship between viruses and bacteria in freshwater ecosystems. Third, we demonstrate by TEM that outer membrane vesicles (OMVs), structures associated with cell secretion and cell communication, are released by aquatic bacteria into natural ecosystems and cultures. Thus, TEM has proven to be a powerful technique to study aquatic microorganisms, contributing to the understanding of ecological processes, including regulation of bacterial populations, during different environmental conditions.

Keywords: Transmission electron microscopy, freshwater bacteria, ultrastructure, aquatic ecosystems, cell viability, cell death

1. Introduction

Aquatic microorganisms such as bacteria and viruses are critically important for ecological processes, for example, carbon cycling and energy flow in aquatic environments [1]. Bacteria are key components of the microbial loop in aquatic ecosystems, an alternative route of dissolved organic matter and nutrient transfer to metazoan trophic levels and consequently

influence the flow of carbon and energy within an ecosystem [2, 3]. Viruses are remarkably abundant in aquatic ecosystems and within bacteria play an important role in the aquatic microbial loop. Viruses can infect bacteria and act in their mortality, thus exerting a significant control over aquatic bacterial and phytoplankton communities. Therefore, viruses can impact the pathways of matter and energy transfer in aquatic ecosystems [4, 5].

The understanding of the functional capabilities of microorganisms in microbial food webs and human health issues is largely dependent on methods applied to the direct visualization of them during physiological and environmental stress conditions. For example, individual imaging of bacteria is valuable to recognize bacterial viability and their physiological functions at single-cell level [6]. Our group has been using transmission electron microscopy (TEM) to study aquatic microorganisms, especially bacteria, from tropical ecosystems. The structural organization of these organisms has been investigated in water samples directly collected from natural environmental sites or kept in cultures. In this review, we highlight the ultrastructural aspects of freshwater bacteria, based on TEM findings, which have provided insights into the functional capabilities of these cells in aquatic tropical ecosystems.

2. Ultrastructure of freshwater bacteria: diversity and morphological alterations in response to environmental stress

While observation of aquatic bacteria by light microscopy is an approach extensively used in studies of planktonic bacteria, the ultrastructure of these organisms is not completely understood [7-9]. In the past, bacteria were considered as prokaryotic microorganisms with a very simple ultrastructure. However, improvement of electron microscopy techniques and more refined analyses have revealed well-defined structures and higher levels of cell organization in bacteria [9].

In aquatic ecosystems, short-time physicochemical variations are frequent and affect environmental properties. Thus, bacterial communities need to be able to respond efficiently to fluctuating conditions of the aquatic environment [5, 10, 11]. On the other hand, bacterial cells can exhibit morphological and ultrastructural changes in response to environmental stress.

2.1. Ultrastructural diversity of bacteria from aquatic ecosystems

The morphological diversity of bacteria goes far beyond a simple description of the bacterial shape, as frequently reported by ecological studies [12-16]. Bacteria from aquatic ecosystems have a complex cell ultrastructure with a cell envelope enclosing a cytoplasm with a variety of cell structures and compartments that can serve as organelles [17-19]. Freshwater bacteria, in addition of showing typical structures in the cytoplasm, such as nucleoid, granules, and lipid bodies, can exhibit intracellular membrane systems represented by mesosomes and thylakoid membranes. External structures such as cell envelope with distinct compositions, S-layer, external capsule, and extracellular vesicles, are also found in freshwater bacteria. The main bacterial structures depicted by TEM are listed in Table 1.

Structure	Morphological Description	Functions	Figures
Cell envelope	A complex multilayered structure which envelopes bacteria. Basically, there are two types of bacterial cell envelopes: (1) Gram-positive, composed of plasma membrane and cell wall and (2) Gram-negative, composed of plasma membrane, periplasm and outer membrane. Capsular structures and S-layers may also constitute the cellular envelope.	This structure serves to protect bacteria from their unpredictable and often hostile environment.	1A, 1Ai, 1B, 1Bi, 2A, 2Ai
Plasma membrane	A bilayer membrane seen under TEM as a classical trilaminar structure limiting the cell contents.	Plasma membrane acts as a permeability barrier for most molecules and serves as sites for transport of molecules into the cell. In addition, it is functionally associated with energy conservation as the location in which a proton motive force is generated.	1A, 1Ai, 1B, 1Bi, 2A, 2Ai
Cell Wall	Structural layer adjacent to the plasma membrane that appears as an electron-dense layer composed by peptidoglycans (gram-positive envelope) or a complex formed by periplasm and outer membrane (gram-negative envelope).	Cell wall provides structural integrity to the cell and prevents osmotic lysis.	1A, 1Ai, 1B, 1Bi, 2A, 2Ai
Periplasm (periplasmic space)	The periplasm is a concentrated gel-like matrix in the space between the inner plasma membrane and the bacterial outer membrane in gram-negative bacteria. This space is called periplasmic space. Gram-positive bacteria present this structure as a conspicuous space between the plasma membrane and the cell wall. Periplasm is filled with water and proteins and is therefore somewhat reminiscent of the cytoplasm	Periplasmic proteins have various functions in cellular processes including cell transport, cell degradation and cell motility.	1A, 1Ai, 1B, 1Bi, 2A, 2Ai
S-Layer	Cell surface protein layer that is composed of a two-dimensional array of proteins with a crystalline appearance.	Uncertain functions. It has been suggested that this layer acts as a partial permeability barrier for large substrates and provides resistance, adhesion and stabilization to the cell.	2A, 2Ai

Structure	Morphological Description	Functions	Figures
Capsule	Electron-lucent extracellular layer attached to the cellular envelope. This layer is formed by an exopolymeric matrix of polysaccharides.	Bacterial capsule has important functions related to cell recognition, defense and virulence.	2B, 2Bi
Outer Membrane	Outer bilayer membrane with a typical trilaminar appearance, delimiting gram-negative bacteria, and, for this reason, is a distinguishing feature of these bacteria. It is adjacent to the periplasmic space. The outer membrane composition differs from that of the inner plasma membrane, being composed of glycolipids, mainly lipopolysaccharides, which are located at its outer leaflet.	This membrane acts as a permeability barrier despite containing many passive transport channels. In addition, contributes for the increase of bacterial virulence.	3A
Outer Membrane vesicles (OMVs)	Spherical or rod-shaped vesicles, which are released from the outer membrane. OMVs are delimited by a bilayer membrane with typical trilaminar aspect and variable electron-density. OMVs can vary in size from 20 to 300 nm in diameter.	OMVs may contain proteins and other molecules that are related with cellular communication, defense, biofilm formation and DNA transfer.	3A, 3B, 3Bi
Mesosomes	Folded invaginations of the plasma membrane, which appear as tubular, vesicular or lamellar sacs.	Uncertain functions. It seems associated with cell division.	1A
Thylakoid membranes	System of lamellar membranes located in a large area of the cell cytoplasm.	These membranes serve as sites for the photosynthetic apparatus, enzymatic systems and electron transfer chains.	3A
Granules	Appear as spherical electron-dense structures in the bacterial cytoplasm.	These structures store a variety of organic and inorganic compounds.	1B, 2B, 2Bi
Nucleoid	Non-delimited electron-lucent areas in the cytoplasm. It is composed of DNA with a small amount of RNA and proteins.	Regulator center of cellular activities and cell replication.	1A, 1B, 2B, 2Bi
Gas Vesicles	Cylindrical tubes closed by conical end caps with perimeter size varying from 45 to 200 nm. They are mostly restricted to planktonic microorganisms (cyanobacteria and some bacterial species).	Gas vesicles promote cell buoyance in aquatic environments and enable vertical migration of cyanobacteria.	-

Structure	Morphological Description	Functions	Figures
Lipid Bodies	Electron-dense or electron-lucent spherical organelles surrounded by a half-unit membrane.	Lipid bodies store lipophilic compounds that are used as metabolic energy. However, they might be related with other more complex yet unclear functions in prokaryotes and may have associated proteins.	-
Flagella	Tubular filamentous structures attached to the cell surface. It is better observed by TEM when samples are negatively stained.	The flagellar filament is rotated by a motor apparatus in the plasma membrane and allows the motility of the cell in aquatic environments.	-

Table 1. Main ultrastructural components of freshwater bacteria

TEM has been helping to understand the ultrastructural diversity among bacteria from aquatic ecosystems, associated with the presence of different internal and external structures. Our studies from tropical aquatic ecosystems have shown that ultrastructural diversity is an important aspect to be considered for better understanding of the role of these microorganisms. For example, variations in the cytoplasmic electron density (Figures 1 and 2) are frequently observed in freshwater bacteria and might reflect different stages of metabolism and/or differential molecular compositions. We also found a substantial variation in the bacterial cell envelope thicknesses (Figure 1) and compositions (Figures 1 and 2A), which are related to the presence of gram-positive and gram-negative bacteria, both commonly found in aquatic ecosystems. Our quantitative TEM analyses revealed a significant proportion of bacteria with a limiting capsule (Figure 2B and 2Bi). Our data showed that 31 % of freshwater bacteria had capsular structures [20]. This frequency is higher than that found in marine bacteria (7–27 %) [21-23]. This structural component is important for multiple functions, such as cell interaction with the environment, absorption and storage of nutrients, barrier against toxic agents from the medium and predation, and protection from viral infection and biofilm formation. Moreover, some bacteria showed particles adhered to the bacterial capsular structure (Figure 2v and 2Bi), which may be indicative of a survival strategy important for acquisition of organic or inorganic nutrients and protection against predators [24]. The well-defined coating formed by particles around bacteria, revealed by TEM observations, may act as an important micro-environment that is not identified by other techniques and open new frontiers in the understanding of bacterial ecology [25].

An interesting ultrastructural observation is the presence of membranous secretory vesicles projecting from the bacterium outer membrane into the extracellular medium in samples from

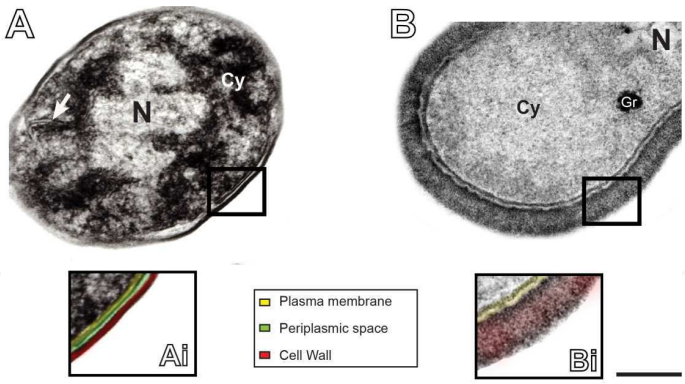

Figure 1. Ultrastructural views of aquatic bacteria collected from an Amazonian ecosystem. (A and B) In the cytoplasm (Cy), observe typical compartments and structures, as nucleoid (N), mesosomes (arrow), and granule (Gr). In (Ai) and (Bi), note the cell envelopes with different thicknesses and composed of plasma membrane (highlighted in yellow) and cell wall (red), with (Ai) and without (Bi) periplasmic space (green). Bacteria also show the cytoplasm with distinct electron-density. Reprinted from ref. [20] with permission. Scale bar: 160nm (A), 60nm (B), 120nm (Ai), and 30nm (Bi).

both natural environments and cultures (Figures 3A and 3B). This particular aspect is discussed in more detail in Section 3.0.

TEM also revealed that bacteria from aquatic ecosystems may exhibit a consistent system of endomembranes, — mesosomes and/or thylakoid membranes in the bacterial cytoplasm. Mesosomes (Figure 1A, arrow), considered as artifacts in the past, have, more recently, been receiving increasing attention because of their association with some cell functions, such as chromosome segregation during cell division. Intriguingly, mesosomes have been documented in bacteria in response to stress conditions [26]. The presence of thylakoids is a distinct morphological feature, found in cyanobacteria and a small group of bacteria [27] (Figure 3B). These endomembranes have a crucial function related to metabolic processes, particularly photosynthesis. Because thylakoids are unambiguously identified in high resolution by TEM, this technique is a reliable tool to distinguish between heterotrophic and autotrophic aquatic prokaryotes in environmental samples. Routine evaluation of these types of organisms currently relies on the use of light microscopy and appropriate fluorochromes, which do not enable detailed visualization of the thylakoids.

Our TEM data reinforce the fact that bacteria constitute structurally complex organisms and denote the functional complexity of these microorganisms, likely related to their metabolic and adaptive diversity [2, 24].

2.2. Ultrastructural alterations and death of bacteria in response to environmental stress

The physiological state of bacteria is an important parameter in aquatic ecosystems to understand variations on microbial communities and their potential impact on the food web and fluctuations in geochemical cycles in which these microorganisms are involved. Bacterial communities from aquatic ecosystems cannot be restrictively categorized as active or inactive,

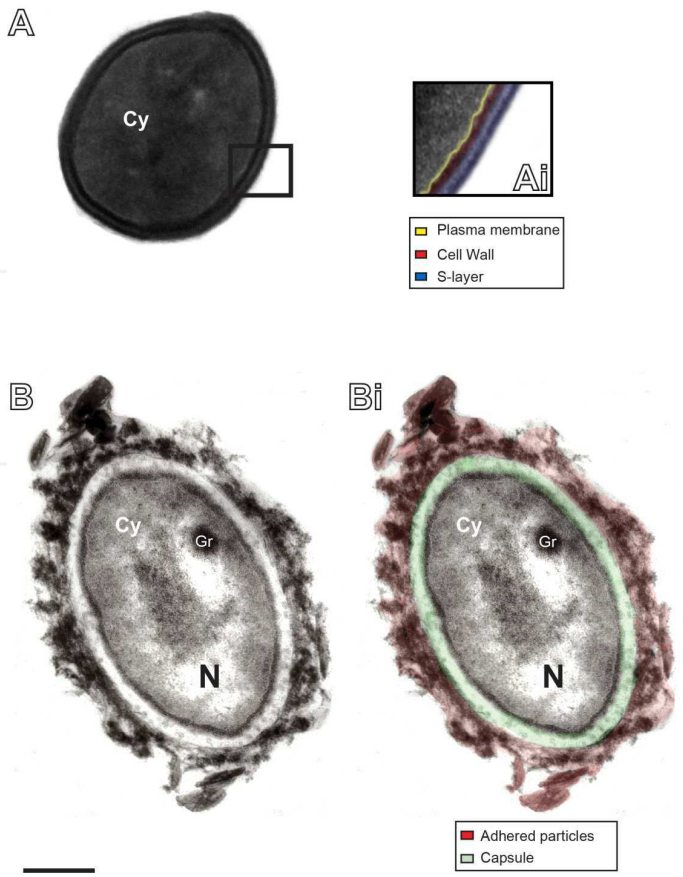

Figure 2. Ultrastructural components of freshwater bacteria. (A and Ai) A cultured bacterium shows the cell envelope composed by plasma membrane (highlighted in yellow), cell wall (red, and S-layer (purple). (B and Bi) Substratum particles (highlighted in red in Bi) are seen as an adhered coating localized externally to the capsular structure (highlighted in green in Bi) of a bacterium collected from a natural environment. Typical bacterial structures such as nucleoid (N) and granule (Gr) are observed in the cytoplasm (Cy). Figure 2B was reprinted from ref. [20] with permission. Scale bar: 130 nm.

since these cells present a continuous variation of their physiological state. From an ecological point of view, bacteria can be distinguished within microbial communities as viable/live cells, which play a functional role and participate in the production of biomass or dead cells, which no longer play a role in secondary production [28]. It is well documented a continuum of physiological status of bacterial life and death in aquatic ecosystems and a great variation in bacterial viability depending on factors such as heterogeneity of bacterial populations, environmental stress, nutrient competition and predation [6, 28-30].

Live/dead bacteria can be characterized by: (i) presence/absence of structures, (ii) genetic parameters, (iii) metabolism or functional activity, and (iv) reproduction and growth viability

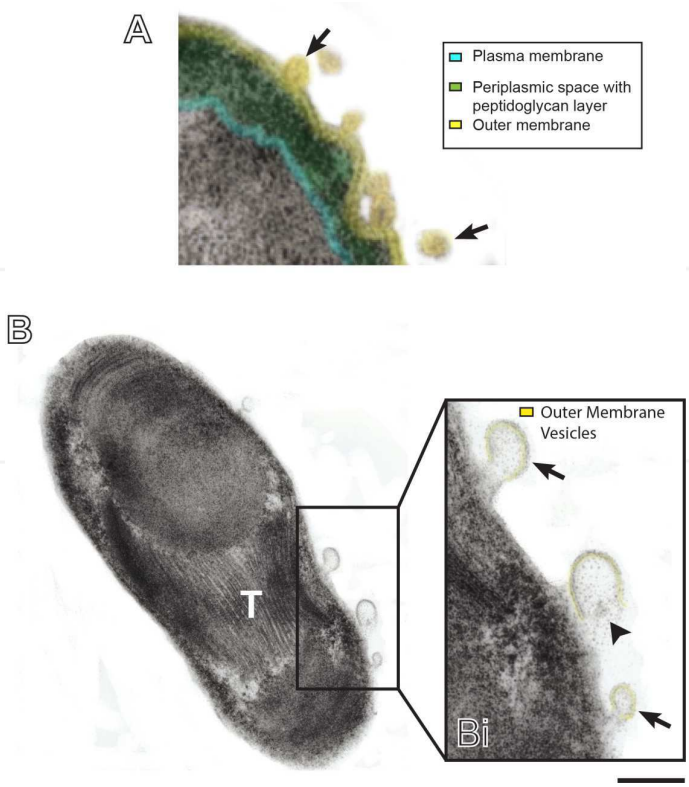

Figure 3. Ultrastructure of gram-negative bacteria from aquatic ecosystems. Observe in (A), a typical gram-negative envelope of a bacterium exhibiting plasma membrane (highlighted in blue), periplasmic space with periplasmic layer (green) and outer membrane (yellow). In (B), an autotrophic aquatic bacterium shows thylakoids membranes (T), organized as a system of membranes in the bacterium cytoplasm. Note in (A) and (Bi) the formation of secretory vesicles from the bacterial outer membrane (arrows) and the release of vesicle contents into the extracellular environment (arrowhead in Bi). The trilaminar aspect of the outer membrane (highlighted in yellow) is clearly observed in high magnification in (Bi). Figure 3B was reprinted from ref. [20] with permission. Scale bar: 80 nm (A, Bi), 200 nm (B).

[31]. Yet, under an ecological perspective, the definition of bacterial life/death in aquatic ecosystems relies mostly on cell viability and growth analyses [32-36].

Although epifluorescence microscopy became the standard method for evaluating environmental bacteria death through indirect quantification of bacterial concentration (35-37), this approach, which is based on the use of routine fluorochromes such as DAPI and Acradine Orange, do not enable accurate assessment of the viability state of bacterial cells and may highlight other particles that are not necessarily bacteria (38). Moreover, this technique does not consider physiological aspects of bacterial cells [37-39]. More recently, other bacterial counting methods, which use more specific fluorescent dyes that consider the physiological aspects of bacterial cells, have been described [40]. However, TEM is the only technique with sufficient resolution to reveal morphological aspects indicative of cell viability and physiology,

enabling the detection of cell alterations that occur even before cell lysis. Therefore, bacteria with intact structures and bacteria presenting damaged cellular structures can be considered live or in process of death, respectively.

By studying impacted freshwater ecosystems in Brazil: Batata Lake (Amazonian region) that received tremendous amounts of bauxite tailings from a mining operation [41], and Funil Reservoir (Rio de Janeiro state) that received industrial, domestic, and erosive process effluents [42], we found several ultrastructural aspects indicative of bacterial cell death. The most frequent bacterial changes in response to environmental stress were: clumped granules (Figure 4A), cytoplasmic condensation (Figure 4B), structural damage of the cell envelope (Figure 4B), loss of cell shape (Figure 4C), and cell elongation (Figure 4D). Bacteria lacking internal structures known as "ghost bacteria" [43] were also observed (Figure 4A). Therefore, ultrastructural analyses were revealing in clarifying the effects of environmental stress on bacterial cell structures and bacterial dynamics in aquatic ecosystems.

2.3. Visualizing virus-infected bacteria in aquatic ecosystems

Viruses are the smallest biological entities known. They are intracellular parasites, which can infect prokaryotic or eukaryotic cells. Viruses are ubiquitous in aquatic ecosystems, and increasing attention has been paid on their role in aquatic food webs since it was discovered that they are the most abundant aquatic components. Because viruses play an important biogeochemical function by releasing dissolved organic matter and nutrients through host cell lysis, they can affect various ecological factors, such as ecosystem respiration, primary production, genetic transfer between microorganisms, and species distribution [5].

TEM studies frequently report the occurrence of viral particles infecting bacteria termed bacteriophages [4, 29, 44]. Viruses are seen by TEM as small electron-dense particles with varied shapes and perimeter size varying from 20–200 nm (Figure 5). Viruses consist of genetic material (DNA or RNA, single- or double-stranded) surrounded by a protein coat (some also have lipids) [45]. They act on the control of bacterial population and are responsible for 40% of bacterial mortality in aquatic ecosystems [4, 46].

Bacteriophages have basically two different life cycles considering the onset of a viral infection until lysis of host cell: (1) Lytic cycle: viruses attach to host bacteria and inject their genetic material (DNA or RNA) into the cell, then they drive the host to produce numerous progeny viruses leading to bacterial cell burst and infection spreading to other cells. (2) Lysogenic cycle: viral genome integrates the genome of host bacteria and reproduces as genetic material without cell lyses. In this case, stress to the host bacteria can trigger a switch to lytic infection (lysogenic ↔ lytic) [5].

Our group has been investigating the relationship of virus—bacteria by TEM and has demonstrated an important correlation among free-living bacteria and virus in an Amazonian ecosystem (Batata Lake) [44]. Although there is a growing body of research on aquatic viral ecology, little is known about viral function in tropical ecosystems, particularly in Amazon environments [44, 47]. TEM revealed the occurrence of viruses with nearly spherical heads and without tails (Figure 5). The structure of the virus capsid with its repetitive morphological

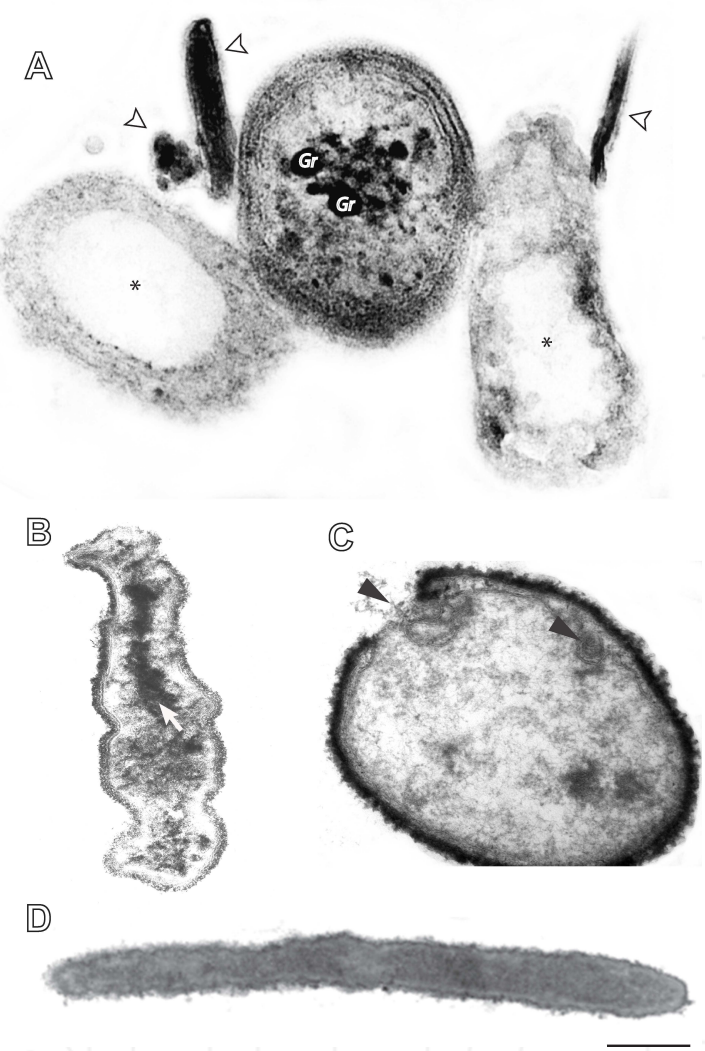

Figure 4. Bacteria from impacted aquatic environments show clear ultrastructural alterations. In (A), a damaged bacterium with clumped granules (Gr) is seen between two "ghost" bacteria, characterized by the presence of an empty cytoplasm (*). Bacteria-associated cellular debris is observed (A, white arrowhead). In (B), note bacterial cytoplasmic condensation (arrow) and loss of cell shape while in (C) a clear structural damage of the cell envelope is observed (arrowheads). Cell elongation is shown in (D). Water samples were collected from Batata Lake (Amazonian region, Brazil), immediately fixed and processed for TEM as ref. [20]. Scale bar: 180 nm (A, B, and C) and 350 nm (D).

units occasionally could be observed in some cells (Figure 5, boxed area). On the other hand, some infected bacteria lacked an intact cell membrane or were partially empty. This morphological aspect indicates that viruses can induce bacterial cell death, which is associated with the lytic cycle of the virus in aquatic environments.

Our data demonstrated that a variable number of phages are present within virus-infected bacteria. TEM quantitative analyses showed that 34.2% of bacteria had viruses in the cytoplasm (Figure 5), with 10.0 ± 3.5 (mean ± SEM) phages per cell-section. Additionally, we have found virus-infected bacteria in cultured samples from Funil Reservoir, indicating that the presence of viruses in tropical ecosystems is a broad event.

Several environmental factors, including solar radiation and temperature, can influence viral abundance. Exposure to UV radiation decreases viral abundance, while low temperatures decrease their capability of infection in aquatic ecosystems [48-50]. It is also described that the increase of organic matter and anthropogenic pollutants increase the abundance of viral particles in water environments [44, 47, 51].

Altogether, our ultrastructural data showed a variable number of viruses within the bacterial cytoplasm, which demonstrates a clear interaction between these organisms. Assessment of viral production and virally caused mortality of bacteria are crucial parameters to understand the detailed role of viruses in food webs.

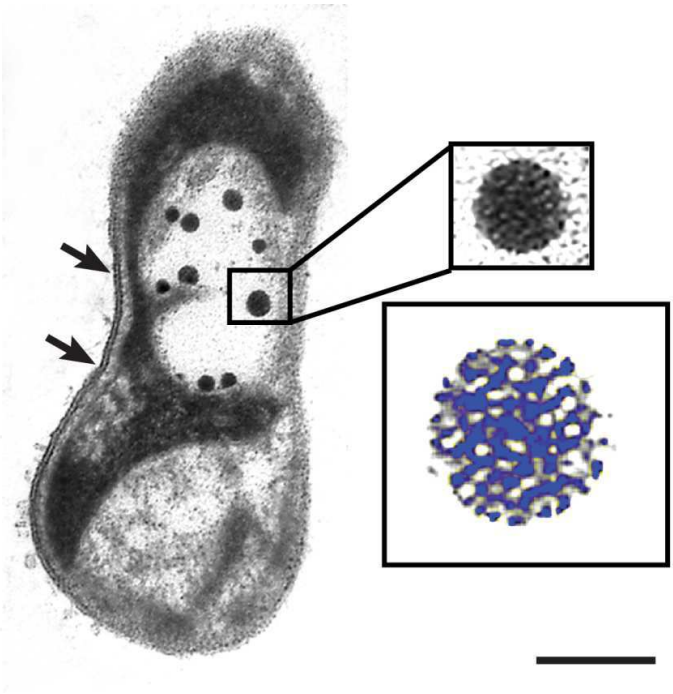

Figure 5. A virus-infected bacterium from an aquatic ecosystem shows several phages. The boxed area shows the virus capsid structure at high magnification. Note that the capsid is composed of repetitive morphological units (highlighted in blue at a higher magnification). The trilaminar structure of the plasma membrane is partially observed (arrows). Scale bar: 266 nm, 80 nm (Box, virus at high magnification), and 40 nm (Box, highlighted in blue). Reprinted from ref. [44] with permission.

3. Production of outer membrane vesicles by freshwater bacteria

In recent years, the extracellular release of membrane-bound vesicles by prokaryotic cells has become the subject of great interest. In prokaryotes, these vesicles are frequently extruded from the outer membrane (OM) of gram-negative bacteria and cyanobacteria, and, for this reason, they are known as outer membrane vesicles (OMVs). By TEM, OMVs appear as spherical or rod-shaped vesicles enveloped by a double membrane with variable electron-density content and diameter size varying from 20 to 300 nm [52-54] (Figure 3).

OMVs have been shown to contribute to diverse bacterial processes, such as pathogenesis [55, 56], cellular defense [53, 57], cell-to-cell communication [58], and DNA transfer [52, 59]. OMVs are able to store and transport a broad range of cargo repertory from bacterial periplasm and cytoplasm, that can explain the variable electron-density observed by TEM. Thus vesicular transport represents a relevant signal trafficking system in prokaryotes (reviewed in [54, 60]). Despite the numerous ways in which vesicles may affect microbial communities, their abundance and potential functions in aquatic ecosystems remain unknown. Recently, these vesicles were recognized as abundant and important to carbon flux in marine ecosystems [61]. Vesicle release occurs during the normal growth of many species, and although growth conditions, stressors, and membrane structure can influence the number of vesicles produced, the regulation of vesicle production is still unclear.

By studying microorganisms from freshwater ecosystems in both natural environmental and cultures through TEM, we have identified a consistent production of OMVs by bacteria [20]. These vesicles were round, delimited by classical membrane with trilaminar appearance, and exhibited morphology similar to those described on the surfaces of other bacterial species [52] (Figure 3). They appeared attached to the outer membrane of the bacteria with typical gram-negative envelope or free in the extracellular environment (Figure 3).

Our data from samples collected from Batata Lake suggest that OMV-mediated secretion is an important cell process of freshwater bacteria (Figure 3). Although the function of OMV remains to be defined, these secretory vesicles, observed for the first time by us in aquatic bacteria from a tropical ecosystem [20], may be important for bacterial survival and inhibition of lysis induced by viral infection that is relevant in this ecosystem [44], as mentioned before. Moreover, OMVs may be relevant in the transport of products involved in the formation of the cell wall, inhibition of toxic components present in the surrounding environment, and formation of biofilms. They may also be associated with delivery of enzymes for nutrients acquisition and autolysins for degradation of other bacteria favoring the competition for niches. All these environmental factors may be more prominent in an impacted ecosystem.

4. Concluding remarks and perspectives

Although our understanding of the biological aspects of bacteria from aquatic ecosystems has advanced significantly, our knowledge of the structural organization of these ecologically

important microorganisms is still incomplete. It is unknown how bacteria differ in their cellular architecture and respond at the structural level to abiotic and biotic stress in aquatic environments. This knowledge is essential for an integrative understanding of the bacterial physiology and ecology. TEM has helped to elucidate the internal organization of aquatic bacteria at the nanometer scale. Earlier views of the ultrastructure of these microorganisms, considered in the past as cells with a very simple structure, are now being expanded to encompass a new understanding of their multifunctional activities and cellular complexity. Our results from environmental and culture-based TEM studies have revealed an ultrastructurally diverse population of bacteria in freshwater ecosystems, characterized by distinct cytoplasmic and external structures. The recognition that these microorganisms have cytoplasmic membranes and are able to release membrane-bound vesicles may be crucial to the understanding of their functional capabilities. Several aspects of the bacteria life remain to be defined. For example, it is not understood how bacteria interact with each other in aquatic ecosystems. Is there a regulated vesicular transport-mediated secretion from/to bacterium? If yes, can this pathway be blocked or stimulated by a cell stressor? These and other aspects, including the bacterial responses to several environmental stresses, mechanisms of bacterial cell death and the bacteria—viruses interaction, need to be investigated in more detail so that the functional significance of bacteria and other microorganisms from aquatic ecosystems can be fully appreciated as critical regulators of ecological processes.

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2.2 PUBLICATION 3

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Microscopy techniques applied to the study of cell death in bacteria from freshwater ecosystems

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Bacterial cell death has key functional implications in bacterial dynamics and biogeochemical cycles in aquatic ecosystems. In this review, we highlight the use of light microscopy and transmission electron microscopy (TEM) to study cell viability and events of cell death in bacteria from freshwater ecosystems. First, we focus on the applications of fluorescence microscopy to visualize changes of membrane permeability. Second, we address the applications of TEM, including quantitative TEM, to the study of morphological alterations of freshwater bacteria, with detailed analysis of bacterial structures. Finally, we discuss the occurrence of different death mechanisms in bacteria from aquatic ecosystems, especially programmed cell death detected by microscopy techniques.

Keywords: Bacteria; aquatic ecosystems; light microscopy; transmission electron microscopy; ultrastructure; cell death; cell viability.

1. Introduction

Bacterial cell death is an important event that regulates bacterial abundance and contributes to the carbon and nutrient cycles in aquatic ecosystems [1, 2]. Bacterial mortality has been described as a process of morphologic and functional loss of bacterial cell integrity, including cell lysis and destruction of genetic material [3]. Therefore, it is important to recognize a "dead bacterial cell" as a final time point from a death process, which is associated with various biotic and abiotic factors in aquatic ecosystems, such as nutrient availability, predation, viral infection, temperature changes and environmental stress. In this sense, the physiological state of bacteria is a classical parameter to understand variations on microbial communities in freshwater ecosystems [4]. In fact, aquatic ecosystems are characterized by a continuum of bacterial physiological states in which a large proportion of bacteria can be considered dead or inactive [4, 5].

Microscopy approaches are considered powerful tools to provide an accurate view of the bacterial structure and have been successfully used to study bacterial viability/death at single cell level. For example, light microscopy with the use of fluorescent probes, such as propidium iodide and Syto 9, enables detection of bacteria in process of death before cell lysis while transmission electron microscopy (TEM) precisely identifies subcellular changes associated with viability loss and cell death [6, 7]. Our group has been using fluorescence microscopy (FM) and TEM to study viability and death of bacteria from freshwater ecosystems directly collected from natural environmental sites or kept in cultures [6-9]. In this review, we highlight the application of FM and TEM for understanding processes and mechanisms of cell death in planktonic bacteria. These findings have provided insights into the functional role of bacterial death in aquatic ecosystems.

2. Bacterial viability and death

Cell viability can be understood as a physiological state that indicates the ability or not of a cell to reproduce, grow and be metabolically active thus predicting cell life or death. Therefore, the presence of reproducible and growing viable cells in an environment may be considered the primary measure of bacterial viability. Classical methods for the determination of bacterial viability rely just on the ability of cells to actively grow and form visible colonies on solid media. Because of this, the terms 'viability' and 'culturability' were often associated in many microbiological studies [10-12]. However, by using traditional culture methods, bacterial death can only be observed in retrospect, that is, after complete cell inactivation. These methods are hardly capable to detect intermediate physiological states in which intact cells are metabolically inactive and considered "dead" thus limiting the observation of a more complete panorama of the cell death process. Moreover, considering that bacteria can be live and viable but not cultivable and the fact that most bacterial species in aquatic environment cannot be cultivable [10, 13-16], accurate determination of live and dead bacteria has been a challenge to aquatic microbiologists.

Currently, it is largely accepted that bacteria in aquatic environments present a continuum of physiological states ranging from live active to dead bacteria [17]. Therefore, the use of adequate approaches that enable direct visualization and quantification of bacterial viability is central for limnological studies to understand the biology of

microbial communities [18-22]. While routine assessment of bacterial death in freshwater ecosystems is still mostly performed indirectly through determination of bacterial abundance by light microscopy [8, 23-25], approaches have been used to detect bacterial viability at the single-cell level without the need for cultivation. These approaches are based mostly on fluorescent probes, which can be observed under fluorescence microscopy or flow cytometry and reflect different levels of cellular integrity or functionality [reviewed in 26, 27].

Evaluation of bacterial viability is mainly based on plasma membrane integrity, which is an important structural and physiological feature of the bacterial cell. Loss of membrane integrity represents a significant damage to bacteria since multiple functions of these organisms such as respiratory activity and permeability are linked to the plasma membrane [13]. Assuming that damaged membrane may not maintain an electrochemical gradient and deactivates the ability of division/growth, bacteria with non-intact membrane can be considered dead [13]. In this sense, commercially available kits using florescent markers for detecting integrity of the bacterial plasma membrane have increasingly been used to understand cell death in bacteria from aquatic ecosystems [reviewed in 27]. These approaches have been applied for the diagnosis of natural bacterial assemblages from water samples especially for enumeration of live/dead bacteria in environments [17, 18, 28, 29]. For example, the LIVE/DEAD® BacLight™ kit consists of two stains, propidium iodide (PI) and SYTO 9, which differ both in their spectral characteristics and in the ability to penetrate in bacterial membranes. Green-fluorescing SYTO 9 is able to enter all cells, whereas red-fluorescing PI enters only cells with damaged plasma membranes. Thus, the observation of bacteria concomitantly stained with these two stains by FM enables the differentiation between viable/live and non-viable/dead bacteria [18]. By studying samples collected from different freshwater environments in Brazil by FM after BacLight staining, we were able to observe the general occurrence of live and dead bacteria (Fig. 1) [6]. Quantification analyses showed that the proportion of dead bacteria varies in a broad range. In fact, it has been recognized that a great proportion of bacteria can be found dead in freshwater environment and this aspect may be related to limnological conditions of each ecosystem [29].

Environmental stresses lead to a decrease of bacterial density, therefore, indirectly indicating the occurrence of cell death [8, 23, 30-33]. Indeed, by using viability markers that directly provide the distinction of live/dead bacteria, we confirmed that cultured bacteria from freshwater ecosystems die under different stress conditions, such as ultraviolet radiation (UVR) [9] and temperature increase (personal data) (Fig. 1). This fact may be related to several structural changes induced by the harmful conditions, since these environmental factors play a recognizably role in the control of bacterial communities of aquatic ecosystems. Thereby, the knowledge on bacteria viability and quantification of live/dead bacteria are important parameters to better understand abundance variations of planktonic bacteria in freshwater ecosystems [18].

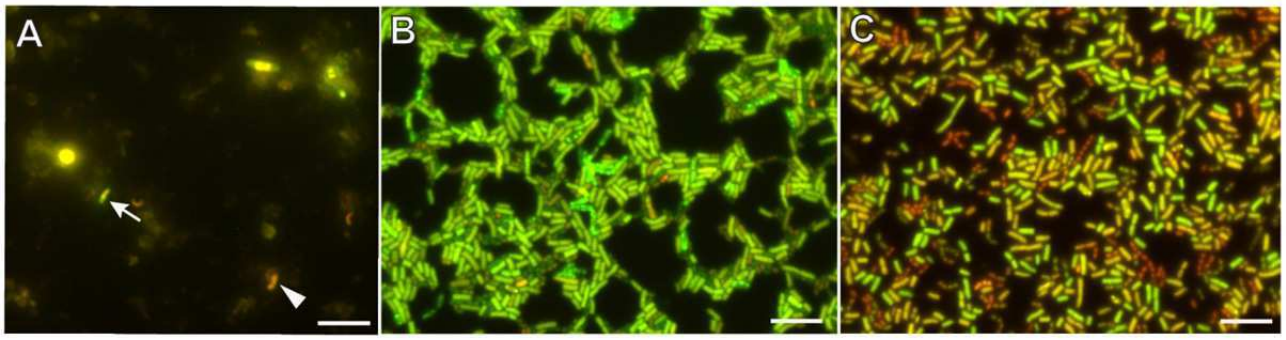

Figure 1 – Bacteria from freshwater ecosystems seen under fluorescence microscopy after staining with a cell viability probe. (A) Freshwater bacteria from a natural ecosystem appear as very small structures stained in green (live bacteria, arrow) or red (dead bacteria, arrowhead). In (B and C), cultured bacteria are observed. While most untreated bacteria (B) stain in green, ultraviolet radiation (UVR)-treated bacteria (C) show differential staining in red. Samples were collected from freshwater Brazilian ecosystems; cultures were established in Luria Bertani (LB) broth media and submitted to UVR treatment for 3h. Bacteria from natural and culture samples were stained with LIVE/DEAD® BacLight™, and observed under fluorescence microscopy after cytocentrifugation, as before [6, 9]. Scale bar = 4 μm.

3. Ultrastructural features of freshwater bacteria

Bacteria from freshwater ecosystems have a complex structural organization with a cell envelope enclosing a cytoplasm and a variety of cell structures and compartments that can serve as organelles such as lipid bodies and mesosomes [34, 35]. A viable bacterium shows intact typical structures, such as the nucleoid, irregularly shaped regions in which most of the genetic material is located (Fig. 2A, 2B), and the cell envelope (Fig. 2A), a complex structure with multiple metabolic functions in bacteria. The structure of the cell envelope (Fig. 2) differs among bacterial groups and it is usually composed of a plasma membrane, periplasmic space and outer membrane (Fig. 2Ai). Also, capsular components

(2Ai) can also integrate some cell envelopes. Dividing bacteria with a typical symmetric constriction (Fig. 2B) are frequently observed in samples collected from freshwater ecosystems. Because TEM is the only technique that enables clear visualization of these subcellular structures at high resolution, application of this technique has been crucial to understand bacterial structural organization and to detect ultrastructural changes associated with bacterial degeneration and death [7, 9, 36, 37].

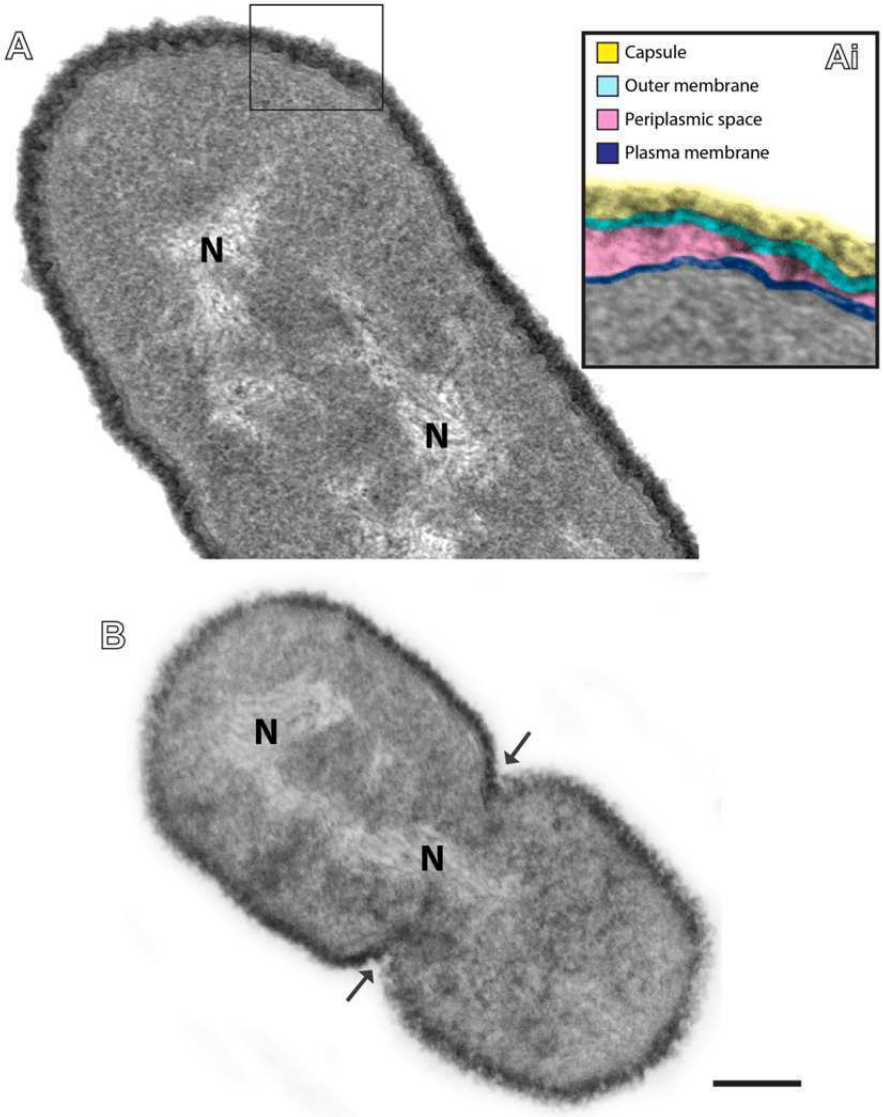

Figure 2 – Ultrastructural aspects of intact bacteria from freshwater ecosystems. In (A), observe bacterial typical structures, such as cell envelope and nucleoid (N). In the detail (Ai), note a preserved cell envelope composed of plasma membrane (dark blue), periplasmic space (pink), outer membrane (cyan) and capsule (yellow). In (B), a diving bacterium with a symmetric constriction (arrows). Freshwater cultured bacteria were fixed in a mixture of freshly prepared aldehydes and prepared for TEM. Scale bar, 50 nm.

4. Ultrastructural changes associated with bacterial death

TEM has been helpful to identify a range of morphological bacterial alterations associated with degeneration/cell death. Bacteria from aquatic ecosystems have been categorized according to their ultrastructural integrity as: (i) *Intact bacteria* in which an intact plasma membrane, cell envelope and a fully developed (not degraded) cytoplasm can be observed (Fig. 2) and (ii) *Damaged bacteria*, which lack at least one of the above criteria (Fig. 3 and 4) [8, 9, 36]. Ultrastructural changes indicative of cell death include cytoplasmic condensation or rarefaction, cell envelope disarrangement, and loss of cell shape (Fig. 3 and 4). These alterations have been identified in bacteria present in water samples collected from aquatic ecosystems and when these bacteria were kept in cultures. Another ultrastructural change shown by freshwater bacteria is associated with viral infection that cause up to 40% of bacterial mortality in aquatic environments [38]. For example, in an Amazonian freshwater ecosystem, it was demonstrated a clear interaction between virus and bacteria [8,

23]. TEM showed that virus-infected bacteria present a variable number of phages within the bacterial cytoplasm. Moreover, some infected bacteria exhibited plasma membrane damage or were partially empty [8].

A recent work from our group demonstrated that bacteria from aquatic ecosystems exposed to UVR in cultures show not only increased alteration of plasma membrane permeability as detected by fluorescent probes (Fig. 1C) but also clear ultrastructural changes characterized by loss of recognizable and intact cell structures [9] (Fig. 4). Quantitative TEM analyses showed that these morphological changes increase in response to the UVR [9]. Additionally, we identified, for the first time, an amplified bacterial ability to release outer membrane vesicles (OMVs) in response to UVR. Interestingly, damaged UVR-treated bacteria increase the production OMVs indicating an overvesiculation process before cell lysis. This secretory event, characterized by release of membrane-bound vesicles, may be associated with adaptive responses to rapid changes in environmental conditions [9, 39]. Thus, TEM provided direct evidence for bacterial death induced by UVR treatment, an effect that was previously described only indirectly through conventional analyses of the cell density, which is based just on the presence/absence of bacteria [40-43]. In fact, the use of fluorescent markers of plasma membrane viability and TEM reveal that a considerable number of bacteria can be damaged or dead before undergoing lysis, that is, a bacterial population may be misinterpreted as active/alive if just conventional density evaluations are performed.

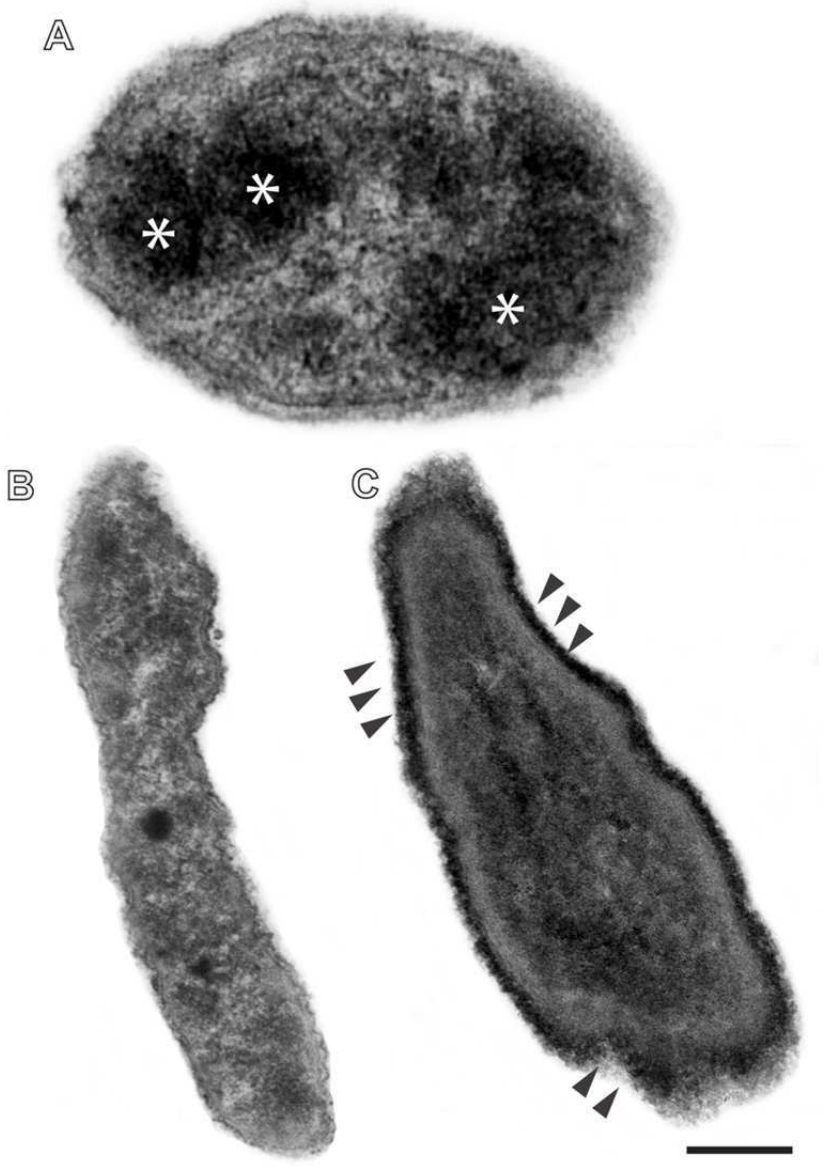

Figure 3 – Damaged bacteria from a freshwater environment. In (A), a bacterium exhibiting condensation seen as electron-dense areas in the cytoplasm (*). In (B, C), observe loss of the cell shape and cell elongation. Regions of cell envelope with retractions are indicated (C, arrowheads). Cultured bacteria collected from a Brazilian aquatic ecosystem were fixed and processed for TEM as before [6]. Scale bar, 90 nm.

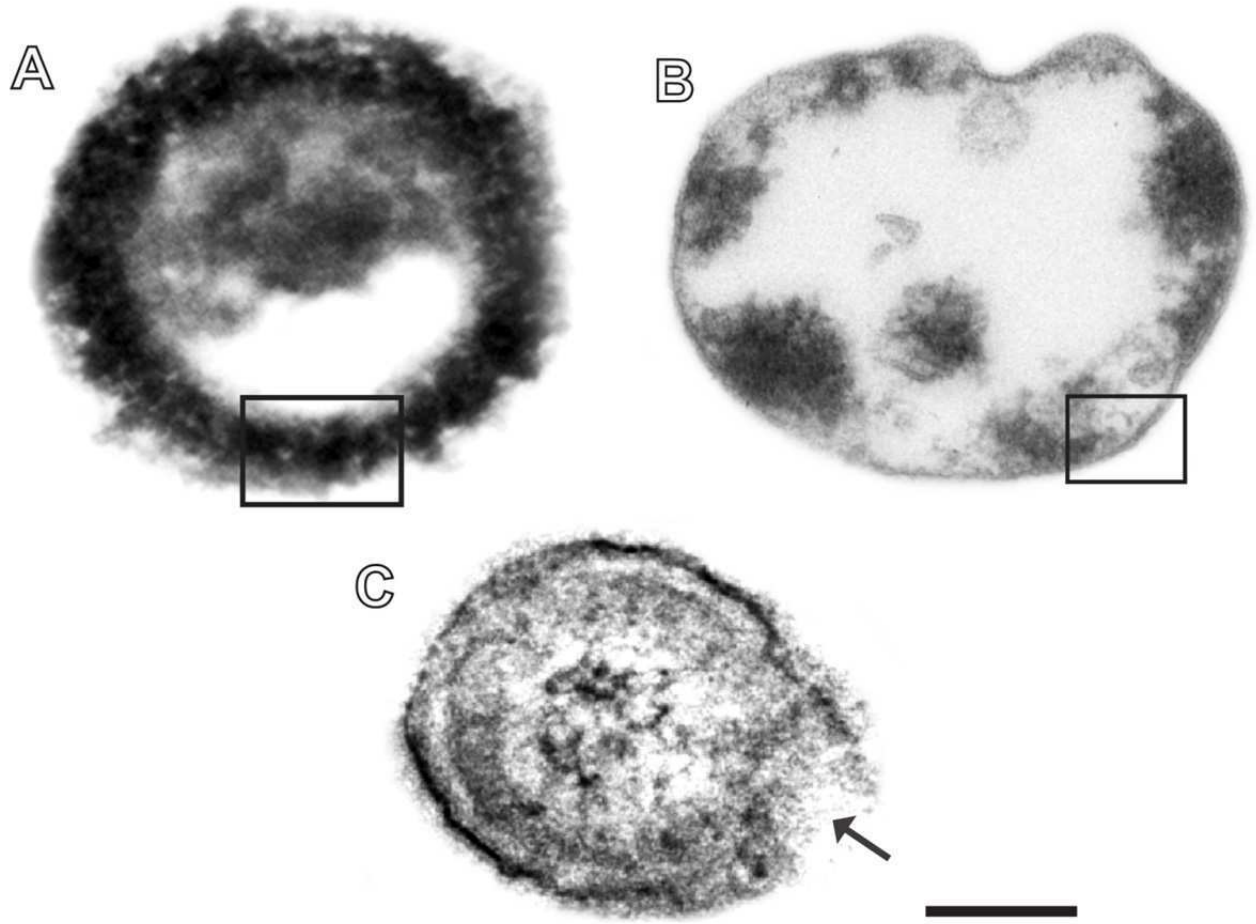

Figure 4 – Ultrastructure of freshwater bacteria in late stages of cell death (A, B, C). Observe damaged bacteria without any recognizable internal structures. In (A, B), cultured bacteria showing a defined cell limitation but with an emptying cytoplasm are seen after treatment with UV radiation. Note in (A) that the cell envelope appears condensed and disarranged (box), while in (B), the plasma membrane is intact (box). In (C), a bacterium shows disruption of the cell envelope and consequent cell burst (arrow). This aspect is frequently referred to as "ghost bacterium". Samples from cultured (A, B) or non-cultured (C) (directly observed from water samples) bacteria were fixed and processed for TEM as before [6]. Scale bar, 90 nm.

5. Bacterial cell death processes

For all cell types, eukaryotic and prokaryotic, cell death may occur through different mechanisms associated with distinct morphological and biochemical features. It has been accepted that the process of bacterial death is not only an ordinary event of lysis but also a process that is fundamental to bacterial physiology and to the understanding of how bacteria develop within complex communities. The first description of death mechanism in bacteria was "autolysis", firstly identified as a bacterial counterproductive and enigmatic process [44]. Autolysis consists of a self-digestion of the bacterial cell wall by peptidoglycan hydrolases called autolysins. Both synthesis and hydrolysis of peptidoglycans are necessary for building the cell wall, and at least some autolysins are part of this normal cell growth activity [45].

Over the past decades, advances in microscopic and biochemical approaches led to improved knowledge on the bacterial cell biology including a better view of death process in these organisms. Although the mechanisms of cell death are not completely elucidated for prokaryotic cells, more complex processes of death have been recognized in bacteria. One of these processes is programmed cell death (PCD), a genetically controlled process that leads to cell death. PCD in eukaryotes comprises several mechanisms such as the well-documented mechanism of apoptosis, which play important roles in the development, homeostasis and survival of eukaryotic multicellular organisms [46, 47]. In bacteria and other single-cell organisms, PCD is considered a process to clear defective or damaged cells from the population, thus playing critical roles in the population survival [48, 49]. However, morphological and biochemical changes indicative of PCD in bacteria have not been fully clarified and are mostly based on eukaryotic PCD alterations.

Some studies have demonstrated PCD in species of cultivable pathogenic bacteria that can be also found in freshwater ecosystems. For example, some genetic modules in *Escherichia coli* are related with PCD occurrence. Biochemical alterations associated with apoptosis were also described for this species, such as DNA fragmentation, phosphatidylserine translocation and caspase activity [50]. Alterations indicative of apoptosis, as chromatin

fragmentation and condensation, changes in cell shape and loss of membrane potential were also described in *Heliobacter pylori* under nutrient stress and *Bacillus subtilis* during sporulation events [51]. These PCD-associated changes were also found in marine and freshwater cyanobacteria [Reviewed in 52].

TEM is considered the "gold standard" for the identification of apoptotic cells. Our ongoing TEM studies have been providing some evidences for the occurrence of PCD in bacteria from freshwater ecosystems. Bacteria showing cytoplasmic condensation (Fig. 3A) and retraction (Fig. 3C) are likely undergoing apoptosis, a process characterized by these ultrastructural features in eukaryotic cells. We have also observed the presence of damaged bacteria (empty) with intact cell envelope (Fig. 5B, 5D). These non-lytic, dead bacteria may represent apoptotic cells in correlation with similar morphological features of apoptosis of eukaryotic cells in which the plasma membrane remains intact until late stages of the death process. However, the identification of a complete PCD scenario in freshwater bacteria awaits further investigation.

6. Concluding remarks and perspectives

The traditional view of bacterial cell death as a passive and insipid process has increasingly been replaced by a more complex and highly regulated event in which different mechanisms take place. Microscopy techniques have been helping to understand structural changes associated with bacterial cell death and provide insights into the mechanisms that govern this event in bacterial communities. It is becoming clear that PCD is a conserved mechanism in bacteria including freshwater bacteria. Future studies are required to understand the complex regulatory signals and pathways that govern bacterial death in aquatic ecosystems.

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3. IDENTIFICATION OF PROGRAMMED CELL DEATH IN BACTERIA FROM AQUATIC ECOSYSTEMS

3.1 INTRODUCTION

The process of cell death in bacteria is an intriguing and ill-understood event, especially in bacteria from aquatic ecosystems. Classically, bacterial death is a process of morphologic and functional loss of cell integrity, including cell lysis and destruction of genetic material (Servais et al., 1985). However, in recent years, a more complex process of cell death termed programmed cell death (PCD), has been considered potentially ubiquitous in prokaryotes organisms (Reviewed in Nedelcu et al., 2011).

The 'programmed' component refers to a genetically encoded, energy-dependent death phenotype and comprises several regulated mechanisms (Kroemer et al., 2005; Galluzzi et al., 2007; Kroemer et al., 2009; Galluzzi et al., 2012; Galluzzi et al., 2015). In eukaryotes, the most known type of PCD is the process of apoptosis, which is characterized by a series of defined biochemical and morphological events that predispose, precede, and accompany death (Danial and Korsmeyer, 2004). Apoptosis occurs as part of a developmental program or to preserve tissue homeostasis, that is, it is a "physiological cell death", important and necessary during the life of multicellular organisms (Kerr et al., 1972; Wyllie et al., 1980).

There is increasing evidence for the occurrence of apoptosis in bacteria, mainly in genera of medical interest. For example, *Escherichia, Myxococcus, Streptococcus, Staphylococcus, Pseudomonas,* and *Escherichia* can activate genetic modules when faced with cell death-triggering stresses, such as antibiotics, oxidative stress, virus infection and nutrient depletion. This activation can lead to cell death programs that are similar to those involved in eukaryotic cell apoptosis. Biochemical signs of apoptosis found in these bacteria include phosphatidylserine (PS) exposure, chromosome condensation, DNA fragmentation and/or proteases activity (Hazan et al., 2004; Mai-Prochnow et al., 2006; Regev-Yochay et al., 2007; Rice et al., 2007; Kolodkin-Gal and Engelberg-Kulka, 2008; Søgaard-Andersen and Yang, 2008; Hakansson et al., 2011; Pang et al., 2011; Dwyer et al., 2012).

In bacteria from aquatic ecosystems, apoptosis has been documented only in some species of photosynthetic bacteria (Cyanobacteria) during stress conditions

(reviewed in Bidle, 2016). In response to oxidative stress (Berman-Frank, 2004; Berman-Frank et al., 2007) and allelochemical compounds (He et al., 2016) cyanobacteria show typical apoptosis hallmarks, such as very high caspase activity, controlled degradation of internal components (i.e., DNA, thylakoids, carboxysomes, and gas vesicles), and increased vacuolization, with no evidence of plasma membrane rupture. However, it is still unknown if heterotrophic bacteria from aquatic ecosystems use a programmed mechanism to die. Morphological and biochemical manifestations of PCD in these microorganisms remain to be explored.

In the present work, we sought to determine whether physiological and ultrastructural hallmarks of apoptosis could be observed in heterotrophic bacteria from aquatic ecosystems under different stress situations. By studying bacteria exposed to increased temperature or virus infection, stimuli that may trigger bacterial death (Fuhrman and Noble, 1995; Pomeroy and Wiebe, 2001; Berggren et al., 2010), we detected, for the first time, biochemical and ultrastructural features of apoptosis, thus identifying apoptosis as a potential mechanism that underlies bacterial death in aquatic ecosystems.

2.2 MATERIAL AND METHODS

3.2.1 Sampling

Water samples were collected directly from the subsurface (0.5 m; 28◦C) of three tropical ecosystems Negro River, Solimões River and Chapéu D'uvas reservoir. No specific permissions were required for the water samples collected from these ecosystems. Our field studies did not involve endangered or protected species.

3.2.2 Bacterial Cultures

Cultures of bacteria were established from freshwater ecosystems. Bacterial isolates and stock cultures in LB broth media at 28◦C were established as previously work (Gamalier et al., 2017). Prior to experiments, bacterial isolates were thawed and streaked onto a plate containing LB agar. Solid cultures were grown at 28◦ C until observation of visible growth. After, colonies were extracted from plates and grown in LB broth media overnight at 28◦C with 225 rpm shaking.

3.2.3 Temperature Treatment

For temperature treatment, the bacterial cultures were re-suspended in 50 mL of LB medium at an initial concentration of 10^6 cells/mL and held at 32ºC. Control cultures were cultivated at same conditions of the stock (28ºC). All experiments were performed in triplicates. Aliquots of control and temperature treated cultures were collected at beginning of the experiment (0 h) and after 0,5h, 1h and 2h for following analyses.

3.2.4 VLP Inocula and Treatment

Because most viruses in aquatic ecossytems are bacteriophages (Bergh et al., 1989), we consider here the generical term “viral like particles” (VLPs) to describe viral components of these ecosystems. Inocula with VLPs were obtained from water samples from Chapéu D’uvas Reservoir. First, VLPs and bacteria were counted in water samples as previous study (Barros et al., 2010). Briefly, samples were fixed with glutaraldehyde solution (2% final concentration; pre-filtered on a 0.02 µM-pore-size filter) and bacteria and viruses were stained with SYBR green I (Molecular Probes, Eugene, OR,USA), on a 0.02 µm-pore-size Anodisc membrane filter (Whatman aluminum oxide) with a 0.45 µm-pore-size backing membrane filter. After being dried, the filter was placed on a glass slide and mounted with an antifade mounting solution (Patel et al., 2007). Analyses were performed under 1,000x magnification with an epifluorescence microscope (BX-51; Olympus, Melville, NY, USA) using light filters for blue excitation (450–490nm). For each filter, more than 200 viruses and 100 bacteria were directly counted in 20 random fields. Next, virus-bacteria ratio (VBR) was established.

Second, to obtain VLP inocula, water samples were sequentially filtrated in decrease pore sizes (0.8 µm, 0.4 µm, 0.2 µm and 0.04 µm) and added to bacterial cultures. The initial concentration of VLP added to bacterial cultures was based on the proportion of VBR found in water samples. Control cultures did not receive VLP inocula. These experiments were done in triplicates. Aliquots were collected each hour and analyzed as following described. Moreover, to ensure VLPs persistency along the experiment, treated cultures were stained with SYBR Green I and bacteria/VLPs were counted as described above. VLPs were observed by

fluorescence microscopy in all treated groups. Quantitative analyses showed that VLP numbers and VBR from inocula did not vary significantly in the VLP-treated groups during different time points (Supplementary Figure 1). This means that VLP persistency was constant along the experiment ensuring VLP infection.

3.2.5 Bacterial Density

For cell quantification, bacteria were stained with 4,6-diamidino-2-phenylindole (DAPI) (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 4%. After, samples (1 mL) plus DAPI (0.01 µg mL−1 final concentration) were placed in megafunnels (Shandon Mega funnel, Thermo, UK) for immediate centrifugation in a cytocentrifuge (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. Cytocentrifugation 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.

3.2.6 Bacterial Viability

Cell membrane viability was investigated using the LIVE/DEAD® BacLightTM kit (Molecular Probes Eugene, inc, ThermoFisher Scientific, OR, USA, catalog number L7012). 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 citocentrifuge (Shandon Cytospin 4, Thermo Electron Corporation, Madison, WI, USA) as previously described (Silva et al., 2014; Gamalier et al., 2017). 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 1,000 x magnification. The average percentage of live/dead bacteria was established for each slide sampled.

3.2.7 Apoptosis Probes

3.2.7.1 DNA fragmentation assay (TUNEL)

DNA fragmentation assessment was performed by terminal deoxynuclieotidyl transferase-mediated deoxyuridine triphosphate biotin nick end-labeling (TUNEL). Labeling of DNA fragments was performed with APO-BrdU™ TUNEL Assay Kit (Molecular Probes, Eugene, USA, catalog number A232110) that detects the DNA fragmentation of cells, once the DNA breaks expose a large number of 3´hydroxyl ends.

Samples containing at least $1x10^7$ bacteria/mL were fixed in filtered paraformaldehyde 4% for 1 h, washed twice in PBS (0.1M, 7.4 pH), re-suspended in 70% ethanol and stocked at -20 overnight (Dwyer et al., 2012). Next, samples were centrifuged, and cell pellets washed twice in the kit wash buffer. Cells were resuspended in a reagent solution (reaction buffer, FITC-dUTP, and the enzyme deoxynucleotidyl transferase was dissolved in sterile distilled water) and incubated at 37 ° C for 1 h. The aliquots were gently mixed every 15 min. After the incubation period, 1mL of the stop buffer (kit rinse buffer) was added to each sample and centrifuged. The cells were then re-suspended in 100 µl of kit alexa fluoride solution for 30 min and washed in PBS (0.1 M, 7.4 pH).

Analyzes were performed by fluorescence microscopy and flow cytometry, as described below. Negative controls for flow cytometry were performed following the same procedure, but without the incorporation of the TdT enzyme.

3.2.7.2 Annexin V assay

The asymmetric distribution of phospholipids in plasma membranes is a fundamental feature of all cell types. PS belongs to a class of acidic phospholipids normally found on the internal leaflet of the plasma membrane (Naito et al., 1997).

PS externalization has been reported to be a distinct event during PCD in many organisms including bacteria (Gautam and Sharma, 2002a; Raju et al., 2006; Sahoo et al., 2006; Elmore, 2007; Dwyer et al., 2012; Wadhawan et al., 2013, 2014). It is a downstream event and occurs after caspase activation. PS externalization was evaluated by labeling with FITC-conjugated annexin V (Invitrogen, Carlsbad, CA, USA catalog number A13201) (Akuthota et al., 2016). Samples ($1x10^7$ cells/mL) were collected, washed in PBS (0.1M, pH 7.4) and stained with annexin V (20μM) (DWYER et al., 2012). After incubation for 20 min, samples were analyzed by fluorescence microscopy and flow cytometry, as described below.

Analyzes were performed by fluorescence microscopy and flow cytometry, as described below. Negative controls for flow cytometry were performed following the same procedure, but without any staining.

3.2.7.3 Caspases-3 e -7 assay

Caspases are proteases that initiate targeted protein degradation and the execution of apoptotic programmed cell death (PCD) pathways in eukaryotic cells (Elmore, 2007; McIlwain et al., 2013). In prokaryotes, caspase-analogs (i.e. caspase-like) active proteins that are able to cleavage cell structures during PCD have been described (Berman-Frank, 2004; Hakansson et al., 2011; Dwyer et al., 2012). For detection of bacterial capase proteins, we used a fluorogenic substrate for activated caspases (CellEvent™ Caspase-3/7 Green Detection Reagent kit, Molecular Probes, catalog number C10423). The reagent consists of a four amino acid peptide (DEVD) conjugated to a nucleic acid binding dye. This cell-permeant substrate is intrinsically non-fluorescent, because the DEVD peptide inhibits the ability of the dye to bind to DNA. After activation of caspase-3 or caspase-7 in apoptotic cells, the DEVD peptide is cleaved, enabling the dye to bind to DNA and produce a bright, fluorogenic response. The substrate was added (20 μM) directly in culture medium during experiments for at least 30 min. After, samples were pelleted, fixed in paraformaldehyde 4% and re-suspended in buffer solution. The slides were prepared and analyzed, as described below.

3.2.8 Sample Preparation for Apoptosis Analyses

For DNA fragmentation, PS externalization and caspases analyses, samples were prepared by citocentrifugation at high acceleration (452 g) for 10 min (Shandon Cytospin 4, Thermo Electron Corporation, Madison, WI, USA), as previously described (Silva et al., 2014). Slides were mounted with Prolong gold antifade reagent (Molecular Probes) and kept in 4ºC. Analyses were performed under fluorescence microscopy (BX-60, Olympus, Melville, NY, USA) at 450–480 nm excitation wavelengths (Alexa Flour488/FITC).

Tunel and annexin positivity were done by direct visualization. Caspase-positive cells were counted in 10 random fields at 1000 x magnification using an ocular graticule grid and the percentage of positive cells were determined in each experimental group considering the total bacterial count (bacterial density).

3.2.9 Flow Cytometry

For detection and quantification of DNA fragmentation (TUNEL assay) and PS externalization (Annexin assay), samples were collected with a FACS Aria II flow cytometer (Becton Dickinson) equipped with a 488 nm argon laser for excitation, and 515 ± 15nm (with 495 nm long-pass mirror for FITC fluorescence) emission filter, and used with a 70 mm nozzle at 70 PSI. The following PMT voltages were used: 353 (FSC), 271 (SSC) and 675 (FITC) or 488 (alexa fluor). At least 100,000 cells were collected for each sample. The percent of positive cells was determined with FlowJo software and reflect the number of annexin V-positive or TUNEL-positive cells exceeding the fluorescence of 95% of untreated cells.

3.2.10 Cell Preparation for TEM

Water samples (filtered through a 30-µm membrane mesh) and treated and controls cultures 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 (Silva et al., 2014;

Gamalier et al., 2017), so that uniformly distributed specimens could be processed as easily handled blocks of cells. They then were re-suspended in molten 2% agar (Merck, Darmstad, 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.

3.2.11 Quantitative TEM Analyses

To study morphological aspects of the bacterial cells, electron micrographs were randomly taken at magnifications of 30,000–100,000 x and a total of 152 electron micrographs from bacteria were analyzed (n = 48 cells from natural ecosystems; n = 38 cells from temperature-control group; n = 76 cells from temperature-treated group; n = 38 from VLP-control group; and n = 96 from VLP-treated group). Morphometric analyses were determined using the software Image J 1.41 (National Institutes of Health, Bethesda, MD, USA).

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.

3.2.12 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. Statistical analyses and graphs were performed using the software Prism 6.0.1 (GraphPad software, San Diego, CA).

3.3 RESULTS

3.3.1 Bacteria from Tropical Freshwater Ecosystems *in Situ* Exhibit Morphological Features of Apoptosis

Apoptosis is characterized by a stereotypical set of ultrastructural features that are well documented in eukaryotic cells by TEM (Kerr et al., 1972; D'Avila et al., 2008; Ribeiro et al., 2013). By analyzing the ultrastructure of freshwater bacteria in water samples collected from three different aquatic ecosystems, we noticed similar characteristics indicative of apoptosis in these cells (Fig. 1). In all samples, we found both intact bacteria with typical morphology (Fig. 1A) and damaged cells with morphological signs of apoptosis such as cytoplasmic condensation (Fig. 1B), cell retraction (Fig. 1C), and peripheral clustering of amorphous, electron-dense material (Fig. 1D). Bacteria with an apoptotic-like profile had not-disrupted cell envelopes (Fig. 1B-E) and evidence of cell compartmentalization (Fig. 1E), apoptosis-related features as seen in eukaryotic cells (Kerr et al., 1972). TEM quantitative analyses revealed that 22.91% of all freshwater bacteria evaluated by TEM exhibited an apoptotic cell profile (Fig. 1F).

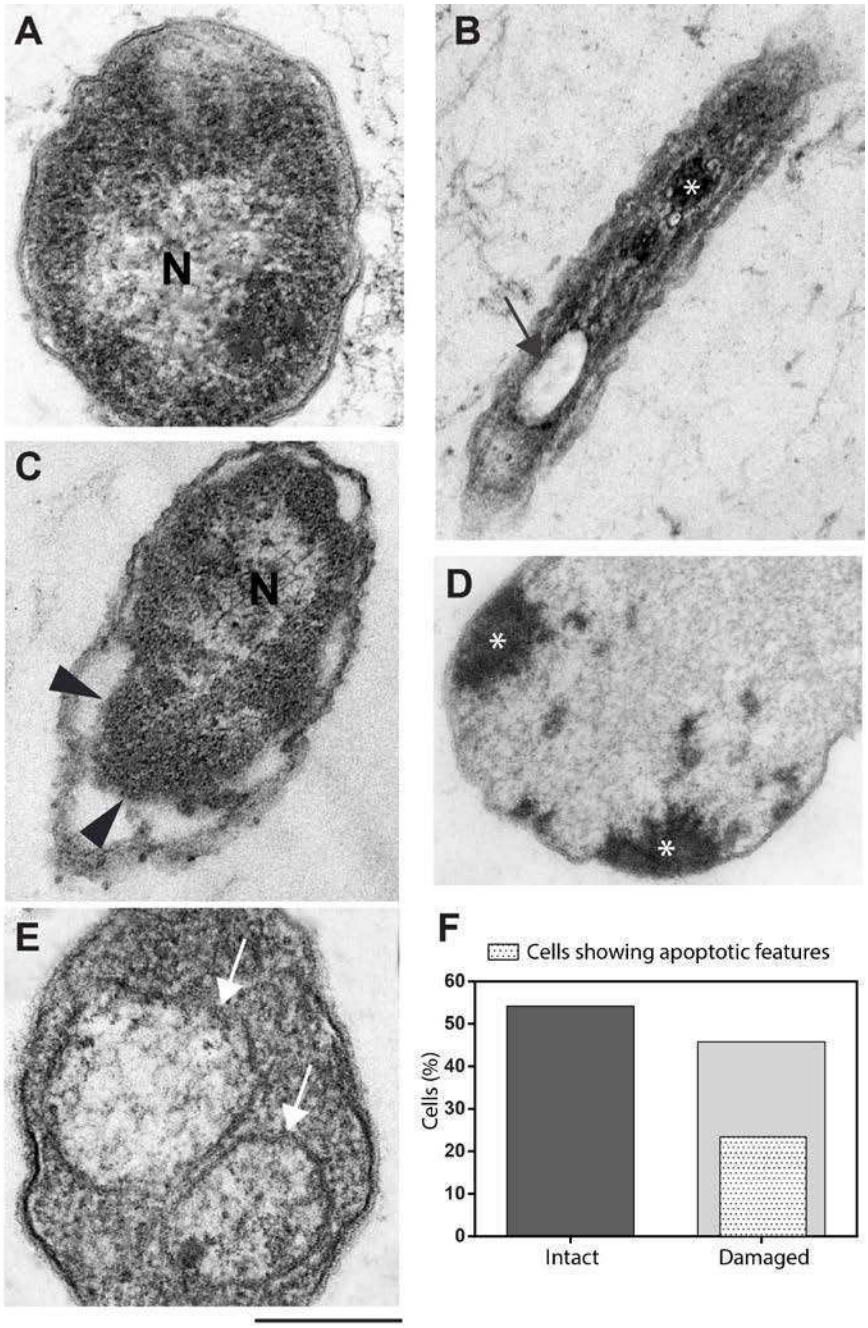

Figure 1: Freshwater bacteria show ultrastructural features of apoptosis. (A) An intact bacterium shows typical morphology with a well-characterized nucleoid (N). (B-C) Bacteria with cytoplasmic condensation (B, asterisk) and retraction (C, arrowheads). In (D), observe focal peripheral clusters of electron dense materials (asterisk), similar to eukaryotic chromatin margination. Arrows in (B) and (E) indicate intracytoplasmatic membrane-bound formations indicative of cell compartmentalization. Note that degenerating cells exhibit not-disrupted bacterial envelopes. (F) Quantitative TEM of intact and damaged bacteria. Water samples were collected from Negro River, Trombetas River and Solimões River, filtered, fixed and processed for TEM. Scale bar = 250 nm.

We then decided to investigate whether freshwater bacteria kept in cultures exhibited physiological and ultrastructural hallmarks of apoptosis in response to stressor agents that trigger bacterial death, such as increased temperature (Pomeroy and Wiebe, 2001; Berggren et al., 2010) and virus infection (Fuhrman and Noble, 1995; Barros et al., 2010).

3.3.2 Bacterial Viability is Affected by Temperature and VLP exposure

First, we investigated if the temperature and VLP treatments would induce changes in bacterial density and viability. Quantitative analyses by fluorescence microscopy after DAPI staining demonstrated that cell density did not change during temperature treatment (Fig. 2A), while significant decrease ($P < 0.05$) of cell numbers was detected after 1 h of VLP treatment (Fig. 2B). We next wondered if the cell density would be associated with bacterial death. By using a probe for membrane integrity (BacLight), which directly detects non-viable/dead cells (Fig. 2C and D), we identified higher percentage of dead cells during both treatments (Figs. 2 A-D) in comparison to untreated controls. This increase was significant at 1h and 2h of temperature treatment (Fig. 2A; $P = 0.05$) and at 1h of VLP treatment (Fig. 2B; $P = 0.001$). Our results show that both treatments induced cell death at specific time points and that cell density and cell viability are not necessarily associated.

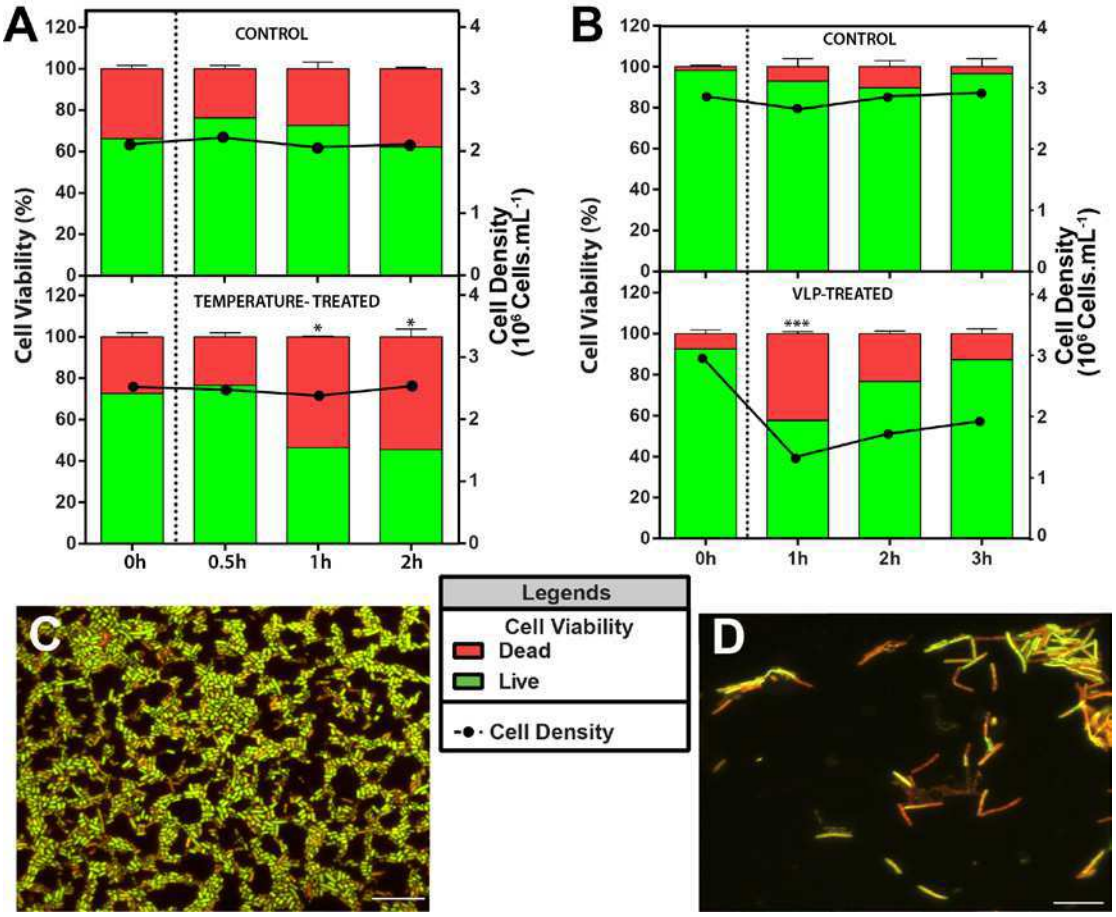

Figure 2: Bacterial cell density and viability after temperature or VLP-treatments. (A) Temperature-treated cultures showed an increase in bacterial death at 1 h and 2 h of treatment but not in cell density. (B) VLP-treated cultures showed a significant decrease of cell numbers after 1 h of VLP treatment paralleled by an increase of cell death in the same time point. (C, D). Representative images of control (0h) (C) and VLP-treated (1h) bacteria after staining for cell viability. Viable cells stain in green and dead cells in red. Bacteria were stained with DAPI or Baclight for evaluation of cell density and viability, respectively. * p≤ 0.05, ***p =0.001. Scale bar = 2 µm.

3.3.3 Temperature and VLP Treatments Induce DNA Fragmentation

To determine whether bacteria exposed to increased temperature and virus infection showed apoptosis-induced DNA fragmentation, we next performed TUNEL assay (Dwyer et al., 2012). Positive cells were analyzed by both fluorescence microscopy (Fig. 3A and B) and flow cytometry (Fig. 3C-E). Quantitative flow cytometric analyzes revealed increased number of TUNEL-positive cells at 2 h (41.60 ± 8.5 %; $p = 0.005$, Fig. 3C) of the temperature treatment compared to the control group at the same point (27.80 ± 6.2 %; $p = 0.30$ %, Fig. 3C) and to the control group at the beginning of the experiment (temperature at 0h = 23.20 ± 2.30 %; $p < 0.005$, Fig. 3C).

VLP treatment led to increased DNA fragmentation at 1 h of treatment (VLP-control 1h = 24.63 ± 2.5 %, VLP-treated 1h = 51.03 ± 6.25 %; $p <0.001$, Fig. 3D) and compared to the control group at the beginning of the experiment (VLP-treated 0h = 21.63 ± 2.7; $p<0.001$, Fig. 4 D). We also found a significant difference between the means of DNA fragmentation at 2h (Control 2h = 27.8 ± 4.6 %; VLP-treated 2h = 44.63 ± 10.85 %; $p=0.009$) and in comparison to the beginning of the experiment (temperature-treated 2h = 23.20 ± 2.30 %), Fig. 4 D). Representative cytograms from treated groups and controls are presented in Fig. 4 E.

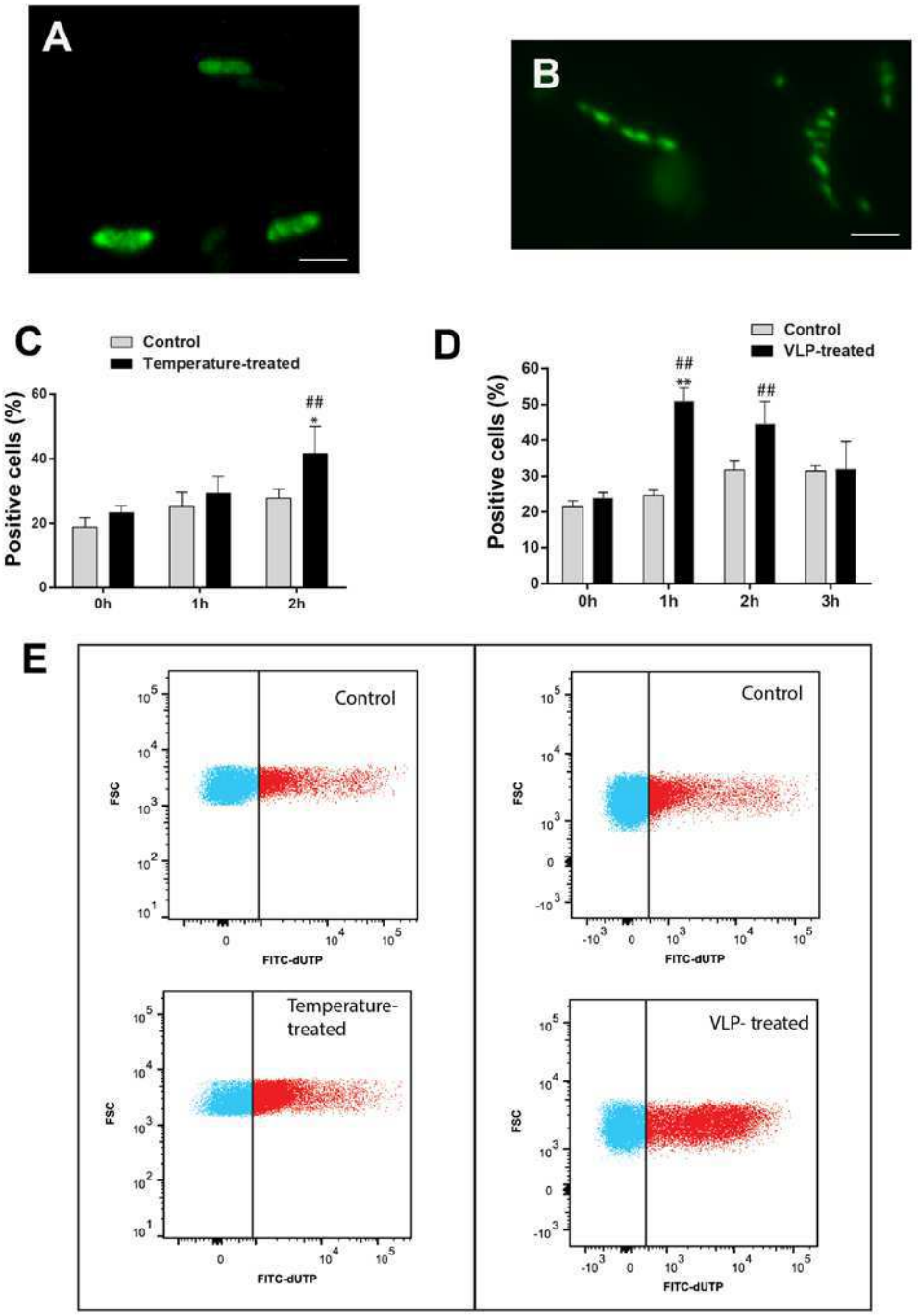

Figure 3: DNA fragmentation induced by environmental stressors. (A, B) Representative images of TUNEL-positive bacteria in temperature- (A) and VLP- (B) treated groups. (C, D) Quantitative analyses by flow cytometry showed higher DNA fragmentation at 1 h in temperature-treated (C) and 1-2 h in VLP-treated cultures (D). (E), Representative cytograms obtained in each experimental group. DNA fragmentation was performed with APO-BrdU™ TUNEL Assay Kit. Samples were analyzed by both fluorescence microscopy and flow cytometry. The location of the vertical threshold line between TUNEL negative cells (highlighted in red) and TUNEL positive cells (highlighted in blue) was determined based on untreated and unstained cells (negative controls). $^{\#\#}$significant difference to control at 0h, $p \leq 0.05$. **significant difference to control at the same time point, $p \leq 0.05$. Scale bar = 1 µm.

3.3.4 PS Externalization Increases in Temperature- and VLP-exposed Bacteria

It is recognized that PS is relocated to the outer membrane leaflets at sites of the cell surface when the cells undergo apoptosis (Danial and Korsmeyer, 2004). Thus, PS externalization is considered a general event for identification of apoptotic cells (Galluzzi et al., 2015). Here, PS externalization was investigated by an annexin-V assay (Dwyer et al., 2012) applied to bacteria submitted to temperature and VLP treatments. Positive cells were analyzed by both fluorescence microscopy (Fig. 4A) and flow cytometry (Fig. 4B). Quantitative flow cytometric analyzes showed an increase of PS externalization at 2 h of temperature treatment compared to control at the same point (28.48 ± 5.0 %; $p = 0.005$ %, Fig. 4C) and to the beginning of experiment (temperature at 0h = 29.00 ± 5.5 %; $p < 0.02$, Fig. 4C).

VLP-treatment showed increased PS externalization at 1 h of treatment compared to the control at the same time point (VLP-1h = 40.45 ± 5.9 %, VLP-control 1h = 21.5 ± 6.0 %; $p < 0.002$, Fig. 4D) and to the beginning of experiment (VLP-0h = 18.83 ± 2.4 %; $p < 0.05$, Fig. 4D). Representative cytograms from treated groups and controls are presented in Fig. 4E.

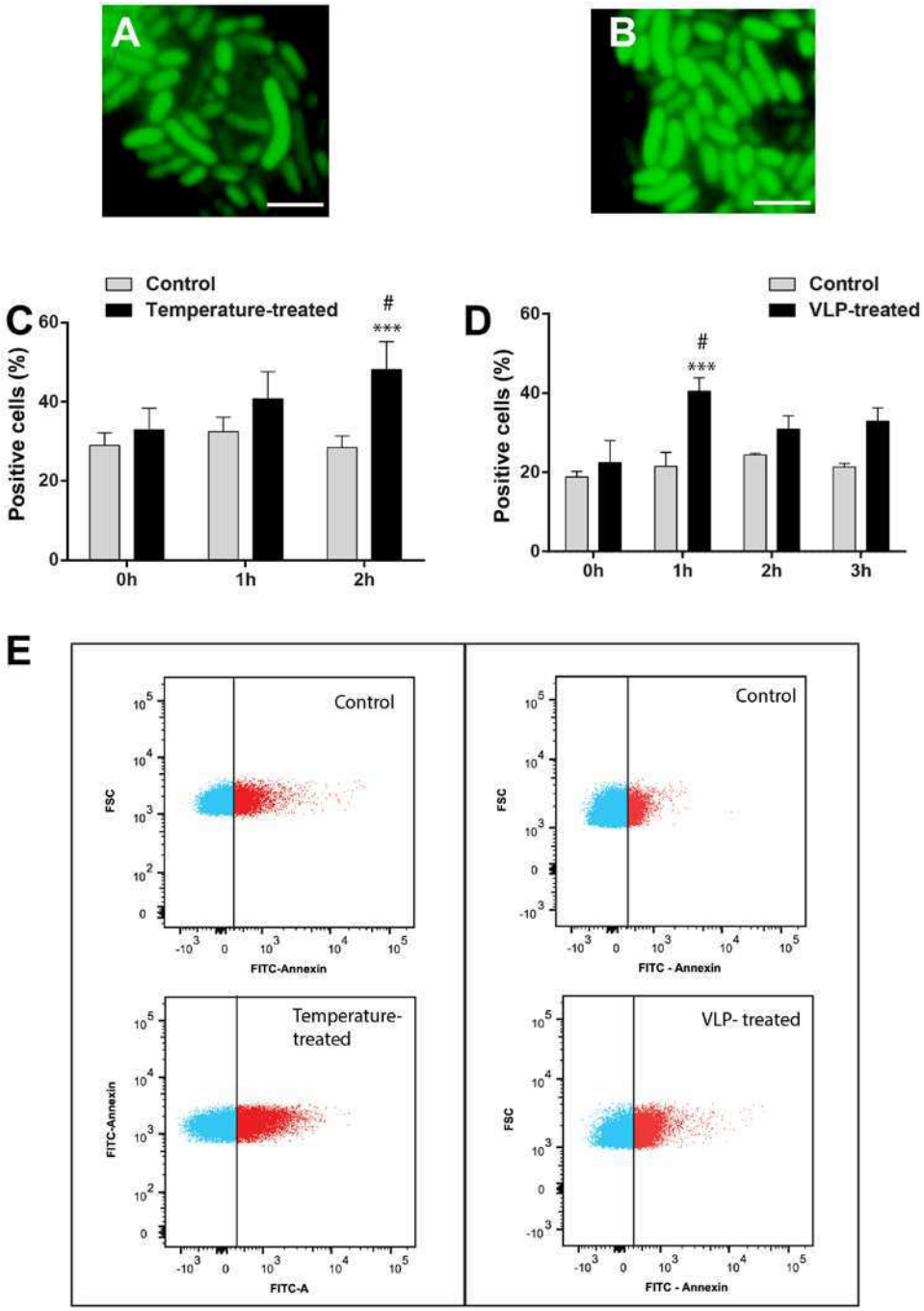

Figure 4: Temperature and VLP treatments induced phosphatidilserine (PS) exposition in freshwater cultured bacteria. (A, B) Representative images of annexin-V positive cells in temperature- (A) and VLP- (B) treated groups, indicating PS translocation. (C, D) Quantitative analyses by flow cytometry showed significant increase in PS externalization after temperature (C) or VLP-treatment (D). (E) Representative cytograms obtained in each experimental group. Bacteria were stained with FITC-conjugated annexin V. Samples were analyzed by both fluorescence microscopy and flow cytometry. The location of the vertical threshold line between annexin-V negative cells (highlighted in red) and annexin-V positive cells (highlighted in blue) was determined based on untreated and unstained cells (negative controls). ##significant difference to control at 0h, $p \leq 0.05$. ** significant difference to control at the same time point, $p \leq 0.05$. Scale bar = 1 µm.

3.3.5 Caspase Activity Increases In Response to Bacterial Exposure to Temperature and Virus

To detect active caspases, treated and non-treated bacteria were stained with a caspase-3/7 probe and analyzed by fluorescence microscopy (Fig. 5). Quantitative analyses showed increased caspase levels after 2 h of temperature exposure (temperature control 2 h = 12,0 ± 3,19 %, temperature treated 2 h = 22,75 ± 3,16%; p = 0,005; Fig. 5A). VLP-treatment induced increase of active caspases at 1 h and 2 h of the treatment (Control-VLP 1h = 22.00 ± 1.2 %, VLP-treated = 35.50 ± 2.5 %; p=0.04, Fig. 5B).

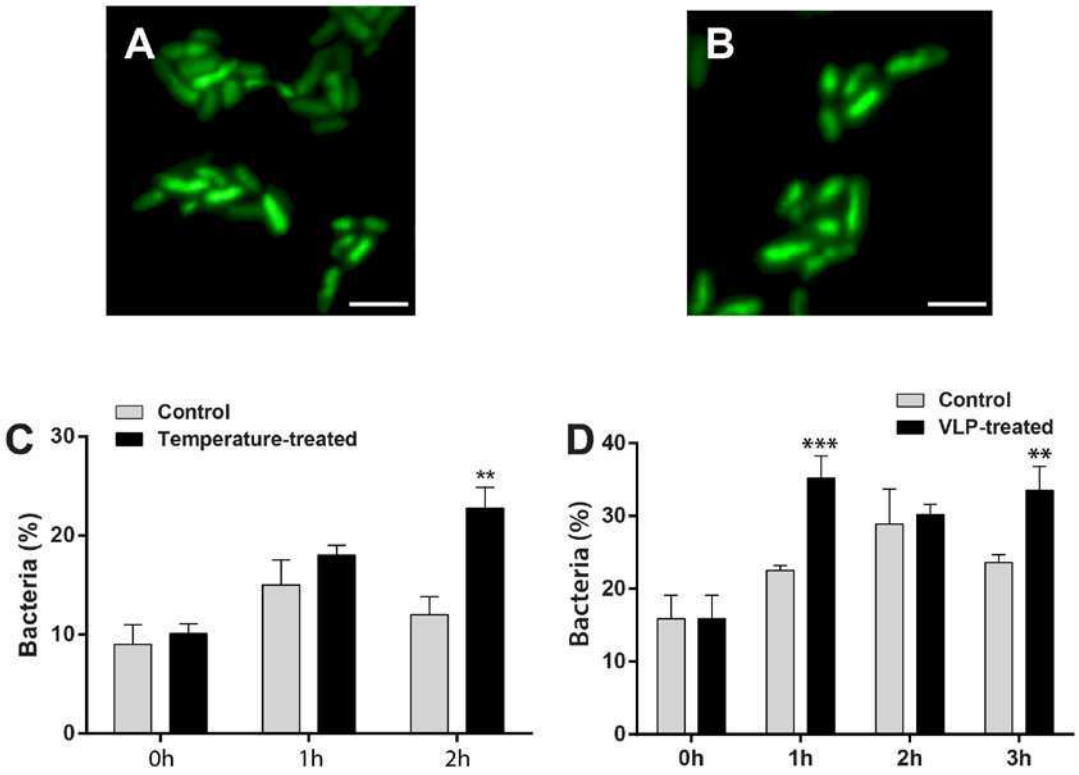

Figure 5: Temperature and VLP treatments induced increased caspase activation in freshwater cultured bacteria. (A, B) Representative images of caspase-positive cells in temperature- (A) and VLP- (B) treated groups. (C, D), Quantitative analyses showed significant increase in caspase activation at 2 h after of temperature treatment (C) or at 1 and 3 h in VLP-treated cultures (D). Bacterial samples were stained with a caspase-3/7 probe, and prepared by citocentrifugation for enumeration of unstained and stained cells under a fluorescence microscope. *** $p \leq 0.001$; ** $p \leq 0.005$, significant difference to control at the same time point. Scale bar = 1 µm.

3.3.7 Temperature and VLP Treatments Induce Ultrastructural Changes Indicative of Apoptosis in Cultured Freshwater Bacteria

To investigate ultrastructural alterations underlying cell death, cultured temperature-treated and VLP-treated bacteria were then prepared for TEM. TEM analyses showed clear ultrastructural changes induced by both treatments. While in the control group there was predominance of intact cells with typical morphology and well-preserved cell envelope (Fig. 6), treated groups exhibited damaged cells with several ultrastructural changes such as cytoplasmic condensation (Fig. 7 A and B), loss of cell shape (Fig. 7B-D), cell envelope disarrangement with cytoplasmic shedding (Fig. 7C and D), and cell elongation (Fig. 7D, 8A). VLPs, identified as small-enveloped dots with electron-dense cores (Fig. 8A) or small pin-shaped forms (Fig. 8B), were clearly observed within bacteria in the VLP-treated group, thus confirming the effectiveness of the viral infection.

Having identified general ultrastructural alterations in treated bacteria, we next performed a detailed qualitative and quantitative evaluation of all damaged bacteria. First, we found morphological signs similar to those that occur before completely cell demise during apoptosis events in eukaryotic cells (Pianetti et al., 2009; He et al., 2016). We observed a range of progressive alterations since different degrees of cytoplasmic condensation and retraction (Fig. 7A,B and Fig. 9 A-C) to lack of internal recognizable cytoplasmic structures (i.e. empty cells) (Fig. 9D). Interestingly, most damaged bacteria presented intact, not-disrupted cell envelopes (Fig. 9, Fig. 10 A-E) a typical apoptotic feature described in eukaryotic cells, which undergo morphological changes but preserve their plasma membranes (Kerr et al., 1972).

Other signs of apoptosis were observed in treated bacteria, such as focal peripheral clustering of amorphous material (Fig. 10A and B) which is similar to eukaryotic chromatin margination that occurs in apoptosis (Pianetti et al., 2009). Interestingly, we observe cell compartmentalization with clear formation of membrane-bound structures within the cytoplasm (Fig. 10C-E, asterisks) and isolated, round and compacted bodies (Fig. 10F). These observations of cell compartmentalization and packaging resemble those that can occur at the late stages of apoptosis in eukaryotic cells, such as formation of apoptotic bodies (refs).

Next, to get more insights into the occurrence of apoptosis in cultured freshwater bacteria, cells were quantitated as intact and damaged as before

(Heissenberger et al., 1996) and the number of damaged bacteria with a apoptosis-like profile was established (Fig. 10F). TEM quantitative analyses revealed increase in the frequency of damaged cells after both treatments, with highest proportions of apoptotic-like bacteria found at 2h of temperature-treatment (13.1% of all bacteria and 34,3% of damaged cells) and at 1 h of VLP-treatment (25.0% of all bacteria and 49.0% of damaged cells).

Taken together, our data demonstrate that apoptosis is a potential mechanism that underlies bacterial death in aquatic ecosystems.

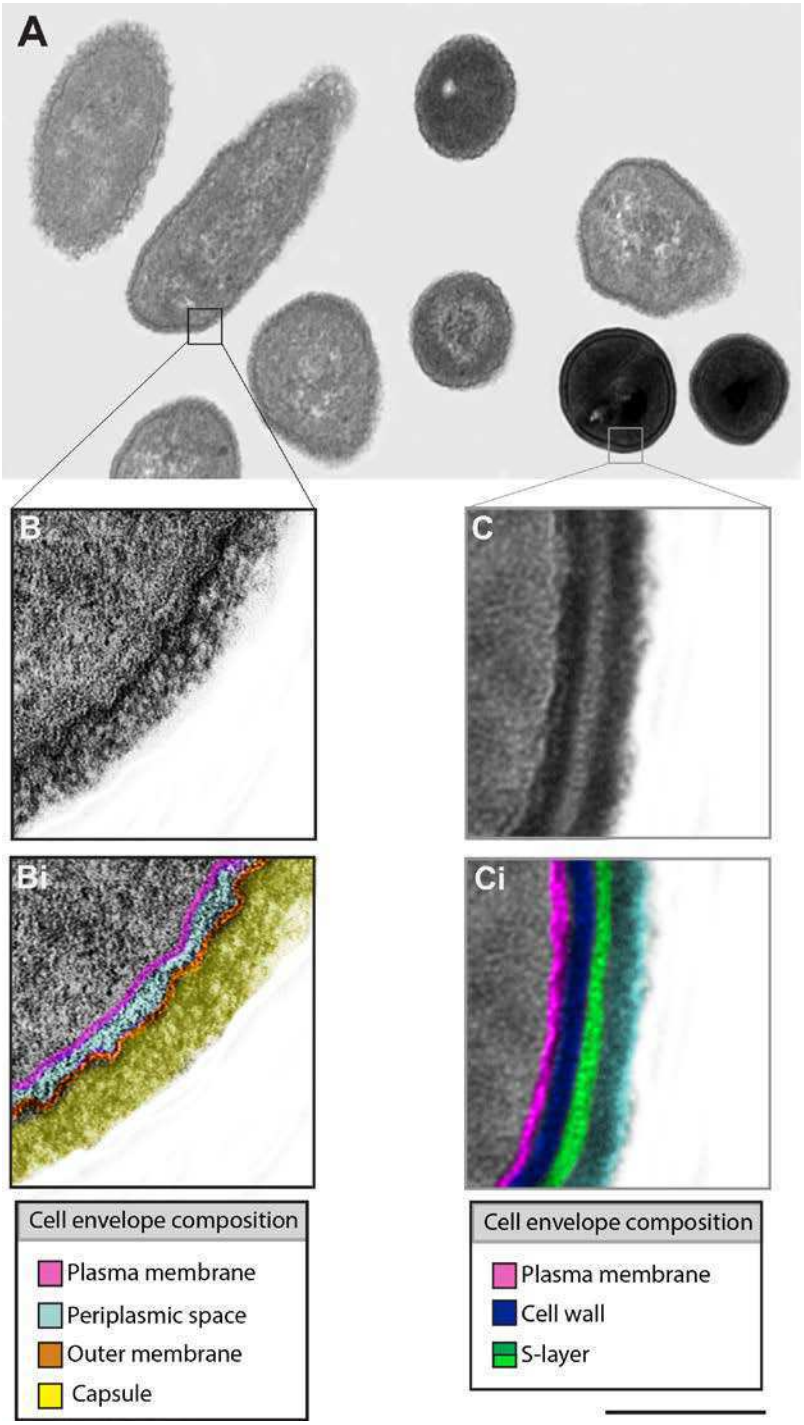

Figure 6: Ultrastructure of freshwater bacteria in control cultures. (A) A panoramic view of intact bacteria shows morphological aspects of the cytoplasm and cell envelope. (B, C) The cell envelopes of two morphologically distinct bacteria are seen in high magnification. The ultrastructural organization of them was highlighted in different colors in (Bi) and (Ci). Note that the differential cell envelope components: plasma membrane, outer membrane enclosing the periplasmic space and a external capsular structure (Bi); or plasma membrane, a thick cell wall and an extracellular structure termed S-layer (Ci). Cultured cells were fixed in a mixture of freshly prepared aldehydes and prepared for TEM. Scale bar = 1.0 µm (A); 80 nm (B, C).

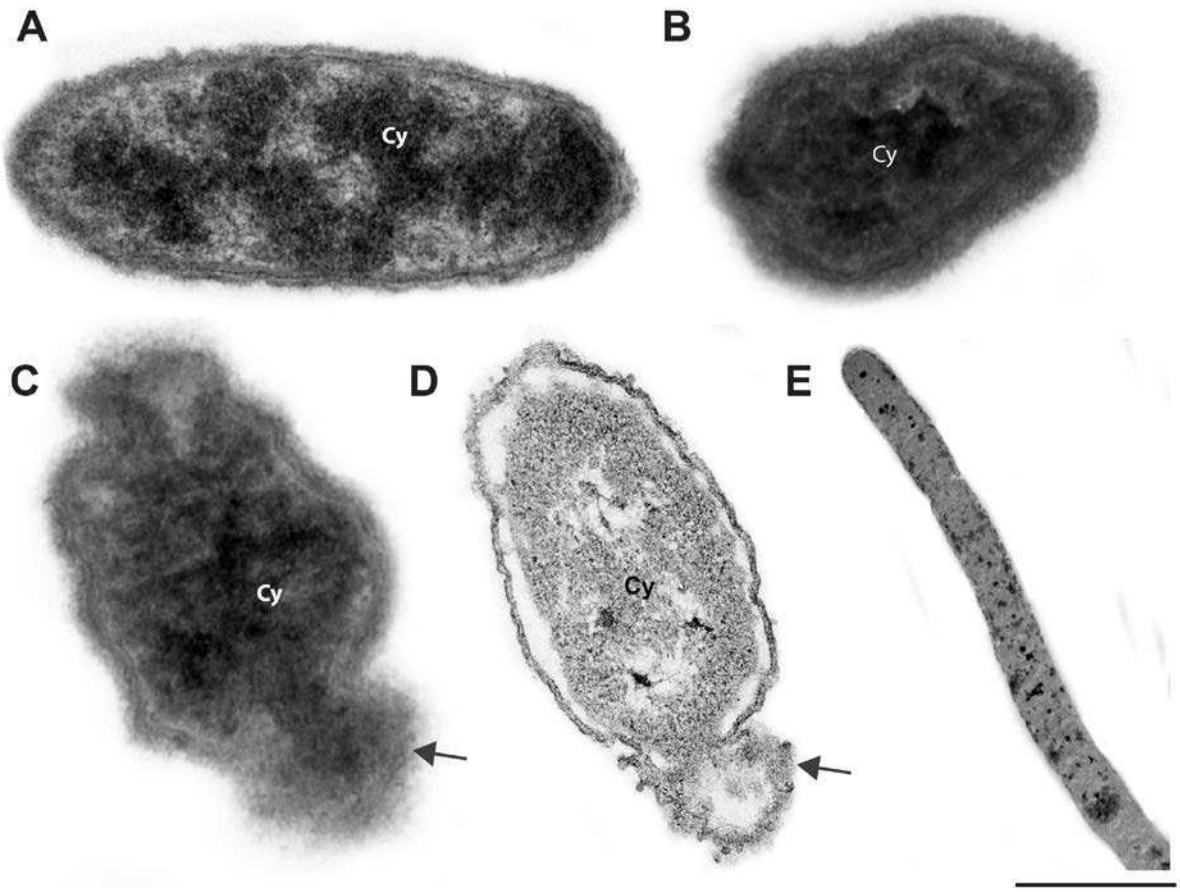

Figure 7: Cultured freshwater bacteria show ultrastrutural alterations after treatment with increased temperature or VLP infection.(A-C) Bacteria exhibiting cell condensation seen as electron-dense areas in the cytoplasm (Cy). (B-E) Damaged bacteria with partial loss of the cell shape, including cell elongation (E). Arrowheads in (C and D) indicate cell envelope disruptions with material shedding to the extracellular medium. Cultured bacteria were treated with increased temperature (B and C) or addition of VLP inocula (A, D and E), samples were collected after 1 h, fixed and processed for TEM. Scale bar = 120 nm (A, B, C and D) and 200 nm (E).

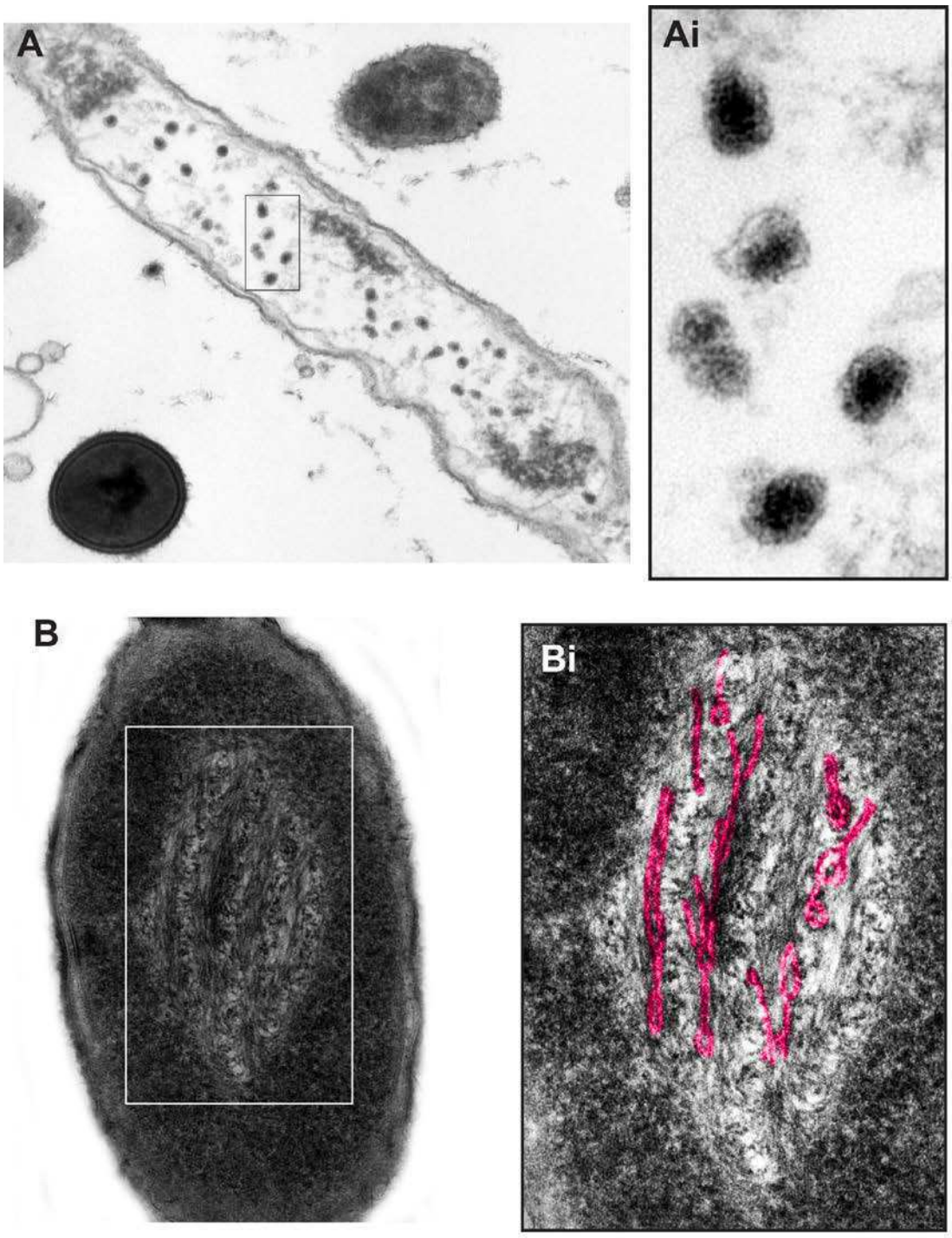

Figure 8: Bacteria treated with VLP inocula show viral forms in the cytoplasm. (A) An elongated bacterium with many viral particles in the cytoplasm. The boxed area in (A) is shown in (Ai). Note the typical virus morphology visualized as small enveloped dots with electron-dense cores. (B) An infected bacterium with pin-shaped viral forms (highlighted in pink in Bi) in the nucleiod region. Note in (A) that infected bacteria have intact cell envelope in conjunction with cytoplasmic morphological alterations. Cultured cells were fixed in a mixture of freshly prepared aldehydes and prepared for TEM. Scale bar = 300 nm (A); 50 nm (Ai); 90 nm (B) and 45 nm (Bi).

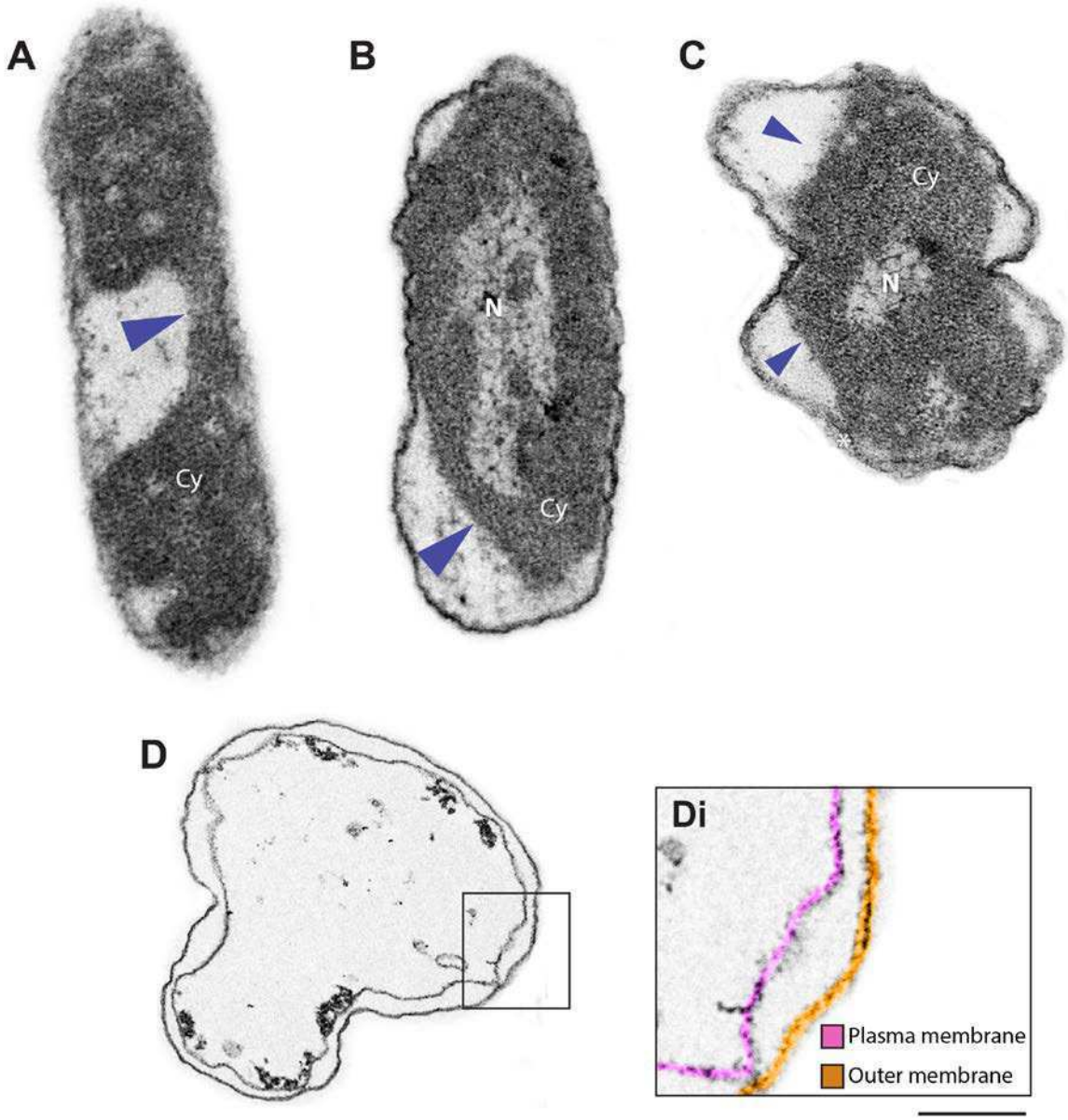

Figure 9: Ultrastructural features of apoptosis in freshwater bacteria.. (A-C) Arrowheads indicate a progressive retraction of the cytoplasm (Cy) in bacteria with intact cell envelopes. (D) A damaged, empty bacterium shows cytoplasm with absence of internal structures. The boxed area in (D) is shown in high magnification in (Di). Note that the cell envelope is not disrupted, although the cell is in late stage of degeneration. Samples were collected from cultures after 1 h and 3 h of VLP treatment and processed for TEM. Scale bar = 130 nm (A-D) and 45 nm (Di).

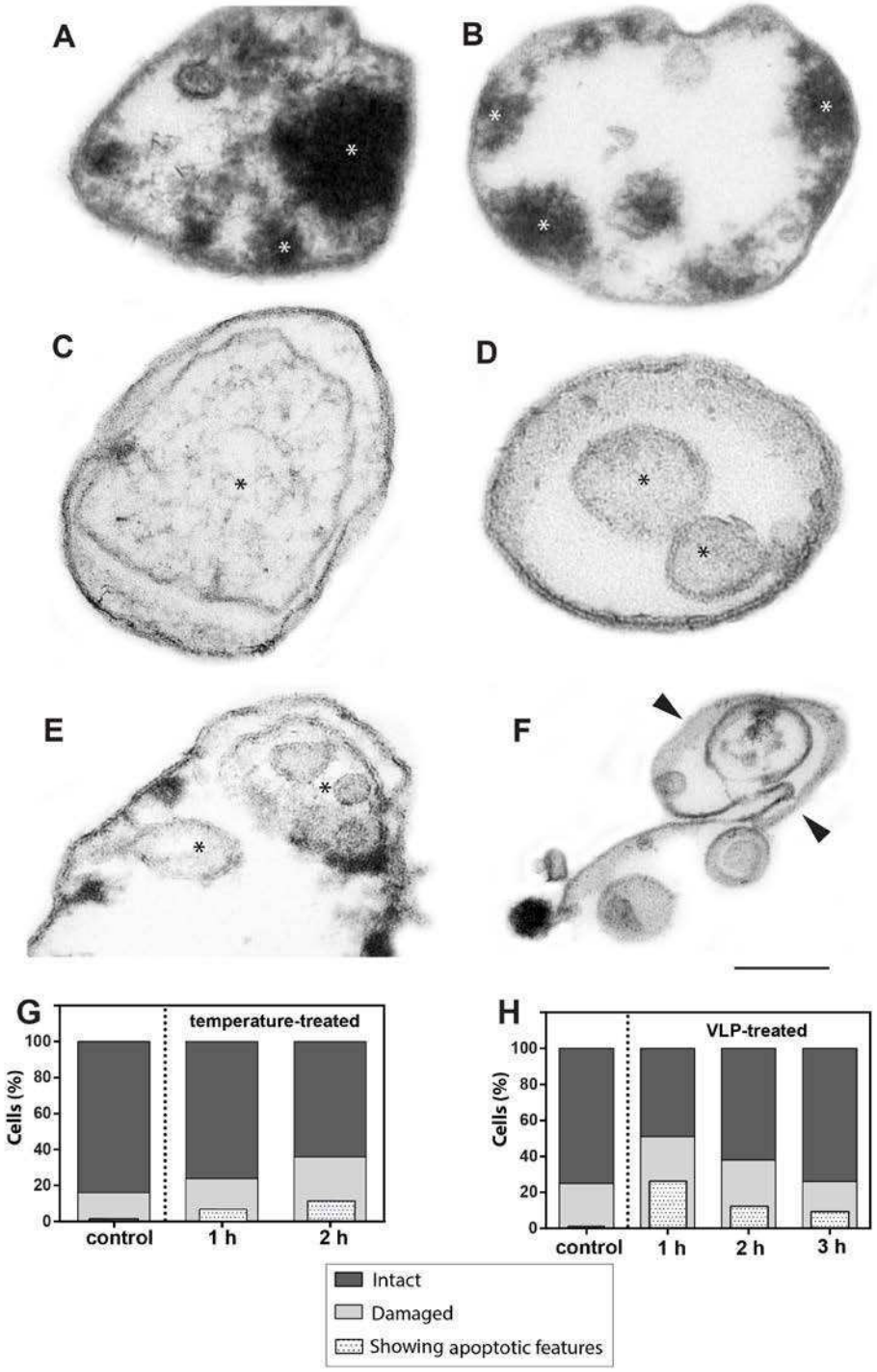

Figure 10: Bacteria at late stages of cell degradation show typical apoptotic morphological features. (A, B), Bacteria with amorphous condensed material (white asterisks) arranged in the peripheral cytoplasm. (C-E) Bacteria in process of compartimentalization, with membrane-bound vesicular structures (black asterisks) in the cytoplasm. Note the typical trilaminar aspect of the delimiting membranes. (E) Membrane-bound and compacted bodies (arrowheads) are seen at late stage of bacterium degeneration. (G, H) Quantitative TEM analyses of intact and damaged bacteria. Samples were collected from cultures after 1 h (A, B), 2 h (C) of VLP treatment or at 2 h (D) of temperature treated, fixed and processed for TEM. Scale bar = 90 nm (A-D); 70 nm (E) and 110 nm (F).

3.4 DISCUSSION

Bacterial cell death is an important biological process in aquatic ecosystems in which it is thought to occur as a passive event during bacterial life or as a consequence of bacterial infection with viruses (Servais et al., 1985; Fuhrman and Noble, 1995). Here, we indicated that an active and more regulated process of bacterial cell death may be operating in freshwater bacteria. We identified, for the first time, that bacteria from freshwater ecosystems are capable to die through a PCD mechanism comparable to apoptosis. By using apoptosis markers, and detailed ultrastructural analyses, we found several characteristics typically associated with apoptosis and propose that freshwater bacterial cell death share many similarities with eukaryotic cells.

Apoptosis has been documented in cultured pathogenic bacteria of medical interest (Pianetti et al., 2009; Dwyer et al., 2012; Lee and Lee, 2014) but remains to be defined in heterotrophic bacteria from aquatic ecosystems. To understand the occurrence and potential significance of the apoptosis in these ecosystems, studies on the physiological, biochemical and structural aspects of PCD in aquatic bacteria are needed. Our ultrastructural examination of freshwater bacteria in situ found morphological features of apoptosis in cells from different aquatic environments (Fig. 1) similar to those classically described in eukaryotic cells (Kroemer et al., 2005).

To induce bacterial stress and death in cultures, we chose both a physical (temperature) and a biological (virus) stressor, which are of common occurrence in aquatic ecosystems (Hall and Cotner, 2007; Barros et al., 2010; Almeida et al., 2015; Xiong et al., 2016). In spite of the recognized role of temperature and virus as regulators of biological process rates in bacterial populations in aquatic ecosystems (Pace and Cole, 1994; Shish and Ducklow, 1994; Fuhrman and Noble, 1995; Fuhrman, 1999; Castillo et al., 2003), little is known about how aquatic bacteria, in terms of their metabolism and physiology, respond individually to these stressors. Aquatic systems, especially freshwaters, are subject to large seasonal and spatial temperature variations, that is a key bottom–up factor controlling bacterial dynamics and composition (Pomeroy and Wiebe, 2001; Farjalla et al., 2002; Farjalla et al., 2006; Amado et al., 2013). On the other hand, viruses can account for up to 40% of bacterial mortality in surface waters and their action is an important top–down mechanism of bacterial regulation in aquatic systems (Fuhrman and Noble, 1995).

Generally, increases in temperature are associated with an overall increase in bacterial metabolism with a positive effect (Hall et al., 2008). However, even a small increase in water temperature in tropical ecosystems is expected to drive organisms beyond optimal growing conditions (Deutsch et al., 2008), increasing energetic requirements for cell maintenance as a consequence of changes in the membrane fluidity or in the functioning of the cellular enzymatic machinery (Nedwell, 1999; Pomeroy and Wiebe, 2001). Thus, the temperature manipulations in our experiment are in the upper temperature limit found in inland waters, and are inserted in the regular temperature range found in natural ecosystems (Deutsch et al., 2008). Otherwise, viral infection directly impacts bacterial populations and indirectly acts on bacterial density and diversity (Maranger and Bird, 1995). In our experiments, VLP numbers and VBR from inocula did not vary significantly in the VLP-treated groups during different time points (Fig 1 C and D) ensuring that VLP infection was constant along the experiment.

First, we investigated physiological dynamics of bacteria submitted to both stressors (increased temperature and viral infection) through density and viability analyses (Fig. 2). By using a fluorescent marker for quantification of bacterial cell viability, we provided direct evidence that both stressors led to bacterial death. On the other hand, our results showed that cell viability is not necessarily associated with cell density (Fig. 2 A and B). This may explained by the fact that bacterial abundance is an indirect evaluation of cell death, based just on the presence of intact cells, while the viability assay directly detects dead bacteria, even if these cells are still intact (Joux and Lebaron, 2000). Thus, bacteria with functionally altered plasma membranes, which are considered non-viable/dead cells can be observed with viability probes, providing a better picture of the bacterial responses to environmental changes. Our data also highlight the fact that a considerable fraction of bacteria die before complete lysis.

We also observed that both bacterial numbers and viability are recovered 2h after significant reduction in VLP-treated groups. Adaptive mechanisms might be acting to explain this recovery. It is recognized that virus can trigger adaptive cell mechanisms may reflected on microbial population dynamics (Andersson and Banfield, 2008; Tyson and Banfield, 2008). Bacterial genetic and molecular devices can be executed to combat or prevent viral infection (reviewed in Barrangou and Marraffini, 2014).

Second, we investigated the occurrence of biochemical hallmarks of apoptosis in bacteria from aquatic bacteria. Our findings demonstrate, for the first time, that both stressors induced DNA fragmentation (Fig. 3), PS exposure (Fig.4) and increase of caspase activation (Fig. 5), which are typical apoptotic biochemical signatures commonly found in eukaryotic cells. DNA fragmentation is a major event that occurs during apoptosis in eukaryotic organisms (Galluzzi et al., 2012). Protease activation is likewise commonly required for the unpacking of DNA and for endonuclease activation during eukaryote death (Taylor et al., 2008). Proteases of the caspase family, especially -3 and -7 can be triggered at the apoptosis beginning and act as death effectors on cleavage of cell structures, including DNA (Galluzzi et al., 2012). On the other hand, the translocation of PS in eukaryotes indicates the late stages of apoptosis process and acts as signal for phagocytic cells (TAYLOR et al., 2008). Thus, our data indicate that bacterial apoptosis induced by environmental stressors may be occuruing through activation of caspase pathways, which potentially cleave the cellular components during the process of cell death. However, while the meaning of PS flip-flop in prokaryotic cells is not completely clarified, the exposition of PS during PCD in bacteria from freshwater open perspectives of how unicellular organisms respond in microbial community. Our results reinforce evidences that prokaryotes possess the requisite biochemical machinery to facilitate their own termination once cell death has been triggered by functionally distinct stresses (Dwyer et al., 2012).

TEM is considered an essential technique to characterize the process of apoptosis. TEM is the only technique with sufficient resolution to reveal morphological aspects indicative of cell viability and physiology enabling the detection of cell alterations that occur even before cell lysis (Silva et al., 2017).. Ultrastructural changes such as cytoplasm condensation and retraction, cell compartmentalization and maintenance of an intact plasma membrane are hallmarks of PCD/apoptosis in prokaryotes (Berman-Frank, 2004; Pianetti et al., 2009; Lee and Lee, 2014). Our TEM data demonstrated that damaged bacteria in the treated groups exhibited different morphological features with cell profiles highly compatible with apoptosis (Fig. 8-10). These non-lytic, dying bacteria may represent apoptotic cells in correlation with similar morphological features of apoptosis from eukaryotic cells in which the plasma membrane remains intact until late stages of the death process (Silva et al., 2017). Likewise, condensed amorphous material into bacterial cytoplasm

of damaged bacteria arranging in the cell periphery founded in present study (Fig. 9) is quite similar to the DNA condensation that occurs in cell nucleus of eukaryotic organisms during apoptotic events (Golstein, 2017). Moreover, we observe cell vacuolization and packaging accompanying bacterial death (Fig. 10), both evidences of an apoptotic-like profile eukaryotic cells at the late stages of apoptotic events (Kroemer et al., 2009). In fact, eukaryotic cells follow a compartmentalization process and their cytoplasmic and organellar remains are packaged into membrane-bound structures (i. e. apoptotic bodies) (Kroemer et al., 2009). Thus, according our TEM analyses, structural changes observed before completely cell degradation might represent progressive morphological alterations indicative of PCD mechanisms.

The present work also raises discussion on an important point of microbial ecology: are PCD events in bacteria from freshwater ecosystems critical for the population survival in stress situations in natural environments? For a long time bacterial mortality in aquatic ecosystems was viewed in terms of predation, nutrient depletion, sinking to the deep water and viral lysis [45]. However, the mode of bacterial death particularly PCD potentially might impact the microbial loop and play a role in the cycling of resources between trophic levels and the organisms occupying them. It has demonstrated that PCD in aquatic eukaryote unicellular organisms can increase the fitness of others in the group and could potentially increase the complexity of the microbial system (Orellana et al., 2013). Based on relative survivability of bacterial pathogenic populations, with and without PCD, it is hypothesized that PCD genotype can provide a selective advantage in environmental situations, since interactions between individuals are forged and in some instances a new unit of selection (that of the group) is created (Durand et al., 2016). What emerges here is that PCD is an active event with implications for the evolution of complexity and higher levels of organization beyond the bacterial cell.

Altogether, our data support the occurrence of an apoptotic process of death occurring in bacteria from freshwater ecosystems in response to environmental simuli. Although the meaning of PCD in the different ecological scenarios awaits further investigations, our findings show that bacterial death is not merely a feature of bacterial population dynamics where individuals exhaust their resources, starve and die or are predated upon, making way for new offspring. Bacterial death may be a much more complex with potential impact on the bacterial community regulation.

Moreover, the discovery of apoptosis-like events in freshwater bacteria highlights the fact that these organisms posses a conserved PCD pathway.

3.5 ACKNOWLEDGMENTS

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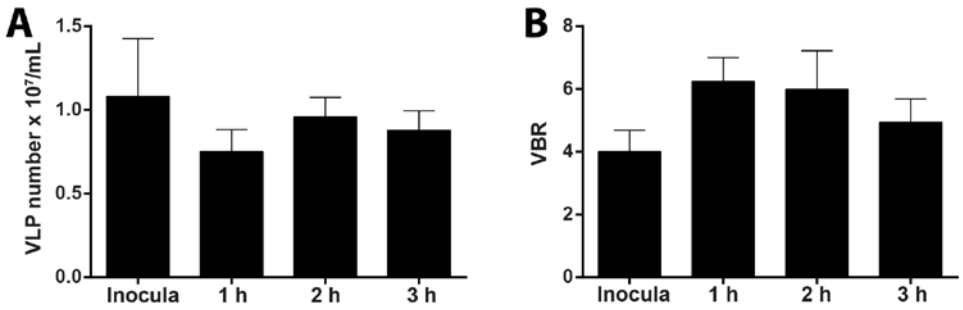
3.6 SUPPLEMENTAL MATERIAL



Supplementary Figure 1: Virus like particles (VLP) number and virus bacteria relation (VBR) in inocula and cultures. Observe that VLP numbers and VBR from inocula did not vary significantly in the bacteria treated groups during different time points. Inocula from aquatic samples and VLP-treated bacterial cultures were stained with SYBR green I and quantified by fluorescence microscopy.

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4. CONCLUDING REMARKS AND PERSPECTIVES

The field of aquatic microbiology has traditionally encompassed studies focused on cell populations or community levels. Information on how cells respond to their environment has been obtained mostly by inference from population level data. However, the cell is the ultimate, irreducible unit of biological integration. Populations of bacteria often present heterogeneous phenotypes and better understanding of bacterial functional capabilities and behavior in microbial food webs is largely dependent on individual cell responses.

Within the cell, information occurs and is regulated in multiple dimensions, including those of time and space. New appreciation for the existence and importance of cellular heterogeneity, coupled with recent advances in technology, create the need of the application of new tools to study individual microbial cells and their relations with ecological activities and functions at high levels of details. In this sense, TEM has proven to be a powerful technique to study aquatic microorganisms, allowing a better understanding of the bacterial structure and cell processes at high resolution. Our present data from environmental and culture-based TEM studies revealed an ultrastructurally diverse population of bacteria in freshwater ecosystems, characterized by distinct and complex cell structures. The recognition that these microorganisms have an ultrastructural diversity represented by internal and external structures is important to the understanding of their functional capabilities in freshwater ecosystems. In fact, application of single-cell imaging at both light and electron microscopy levels has helped to identify different aspects of the bacterial life such as cell viability, cell secretion, cell-cell interaction, and cell communication. Moreover, the ability of freshwater bacteria to respond to environmental stressors can be consistently detected at ultrastructural level

Our single-cell analyses provided, for the first time, structural evidence for the occurrence of apoptosis in freshwater bacteria from aquatic environments. Furthermore, we indentified the occurrence of biochemical and morphological features of apoptosis in these microorganisms in cultures exposed to environmental stressors, such as increased temperature and virus-

infection. Thus, our data helped to understand how freshwater bacterial die and provided insights into a programmed mechanism that possibly govern this event in bacterial communities from aquatic ecosystems.

PCD such as apoptosis is a considered a basic phenomenon that regulates tissue kinetics in eukaryotic cells and is responsible for focal elimination of cells during normal development of eukaryotic organisms. In other words, PCD/apoptosis is “natural death”, which is necessary for tissue growing, differentiation and functioning. In aquatic ecosystems, the identification of apoptotic-like bacteria raises many questions regarding the role of this mechanism of death in these ecosystems. Is PCD/apoptosis a basic mechanism involved in the regulation and maintenance of bacterial communities? How do bacterial communities benefit from PCD mechanisms? What environmental signs and conditions dictate the PCD/apoptosis pathway in aquatic bacteria? Since individual bacteria death can result in the export of resources to the group level, bacterial apoptosis potently may impact ecological process that occurs in freshwater ecosystems. However, the functional significance of PCD in bacteria and other microorganisms from aquatic ecosystems remain to be understood and future studies are need to get more insights into the contribution of this process of cell death to ecological processes.

5 RELATED PUBLICATION

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Potential effects of UV radiation on photosynthetic structures of the bloom-forming cyanobacterium *Cylindrospermopsis raciborskii* CYRF-01

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Cyanobacteria are aquatic photosynthetic microorganisms. While of enormous ecological importance, they have also been linked to human and animal illnesses around the world as a consequence of toxin production by some species. *Cylindrospermopsis raciborskii*, a filamentous nitrogen-fixing cyanobacterium, has attracted considerable attention due to its potential toxicity and ecophysiological adaptability. We investigated whether *C. raciborskii* could be affected by ultraviolet (UV) radiation. Non-axenic cultures of *C. raciborskii* were exposed to three UV treatments (UVA, UVB, or UVA + UVB) over a 6 h period, during which cell concentration, viability and ultrastructure were analyzed. UVA and UVA + UVB treatments showed significant negative effects on cell concentration (decreases of 56.4 and 64.3%, respectively). This decrease was directly associated with cell death as revealed by a cell viability fluorescent probe. Over 90% of UVA + UVB- and UVA-treated cells died. UVB did not alter cell concentration, but reduced cell viability in almost 50% of organisms. Transmission electron microscopy (TEM) revealed a drastic loss of thylakoids, membranes in which cyanobacteria photosystems are localized, after all treatments. Moreover, other photosynthetic- and metabolic-related structures, such as accessory pigments and polyphosphate granules, were damaged. Quantitative TEM analyses revealed a 95.8% reduction in cell area occupied by thylakoids after UVA treatment, and reduction of 77.6 and 81.3% after UVB and UVA + UVB treatments, respectively. Results demonstrated clear alterations in viability and photosynthetic structures of *C. raciborskii* induced by various UV radiation fractions. This study facilitates our understanding of the subcellular organization of this cyanobacterium species, identifies specific intracellular targets of UVA and UVB radiation and reinforces the importance of UV radiation as an environmental stressor.

Keywords: ultraviolet radiation, cyanobacteria, cell death, thylakoid membranes, transmission electron microscopy, cell viability

INTRODUCTION

Cyanobacteria are a widely distributed group of aquatic photosynthetic organisms. Over 2.4 billion years ago, oxygenic photosynthesis carried out by primitive cyanobacteria transformed early Earth's reducing atmosphere into an oxidizing one (Rasmussen et al., 2008). Their widespread ecological importance includes symbiotic interactions (Adams, 2002) and impacts on nutrient cycling (e.g., fixing atmospheric nitrogen) (Capone et al., 1999).

Cyanobacteria species frequently form massive, harmful blooms, which contribute to a reduction in water quality as dissolved oxygen in the water is depleted. In turn, secondary problems arise such as fish mortality and the discharge of toxic substances (Carmichael, 2001). As a consequence, the presence of toxic cyanobacterial blooms in natural waters used for drinking or recreational purposes represents a serious risk to human health (Funari and Testai, 2008; Zanchett and Oliveira-Filho, 2013; Gehringer and Wannicke, 2014).

The bloom-forming cyanobacterium, *C. raciborskii*, has attracted considerable attention due to its broad distribution and recent geographic expansion, as well as its ability to produce potent toxins such as hepatotoxins and neurotoxins (Molica et al., 2002; Carneiro et al., 2013). Although it is considered a tropical/subtropical species, the prevalence of *C. raciborskii* in temperate climatic zones has rapidly increased over the past two decades (Hamilton et al., 2005; Figueredo and Giani, 2009; Everson et al., 2011; Sinha et al., 2012). Overall, the geographical expansion of *C. raciborskii* has been partially attributed to a number of factors, which include increasing temperatures and eutrophication (reviewed in Sinha et al., 2012). While the effects of increasing temperatures and other aspects, such as nutrient availability, on *C. raciborskii* have been extensively investigated (reviewed in Sinha et al., 2012), the effect of ultraviolet (UV) radiation, which is an important source of ionization energy in the biosphere, on this species has not.

Although very small proportions of solar UV radiation contribute to the total irradiance of the Earth's surface (UVC: 0%, 100–290 nm; UVB: <1%, 280–315 nm; and UVA: <7%, 315–400 nm), this portion of the solar spectrum is highly energetic (Kirk, 1994). Anthropogenic activities over the last three decades have contributed to the depletion of the ozone layer and, therefore, the consequent increase in solar UV radiation reaching the Earth's surface has become an important issue (Crutzen, 1992; Madronich, 1992; Kerr and McElroy, 1993). Despite international efforts made to reverse such negative processes, levels have not returned to those seen prior to the 1980s; the timing of a return to pre-1980 UV levels cannot be precisely predicted (McKenzie et al., 2011).

Several processes are affected by UV irradiance. For instance, UVB induces a reduction in the metabolism of heterotrophic bacteria (e.g., Amado et al., 2015), as well as aquatic primary producers, such as cyanobacteria, mainly due to DNA damage (Buma et al., 2001; Helbling et al., 2001; Rastogi et al., 2010). Furthermore, detrimental effects by UVA on phytoplankton have been observed on primary production, pigment degradation and changes in nitrogen metabolism (Kim and Watanabe, 1994; Döhler and Buchmann, 1995; Palffy and Voros, 2006).

In the present work, we investigated the potential effects of different wavelength bands of UV radiation on the concentration, viability and ultrastructure of a *C. raciborskii* strain. Our data demonstrate that UV radiation, mainly UVA, induces drastic damage to the cyanobacterium's cytoplasmic thylakoid membranes and their associated pigments, leading to cell death.

MATERIALS AND METHODS

Cyanobacteria Strain and Culture

The cyanobacterium *C. raciborskii* (CYRF-01) was obtained from the culture collection of the *Laboratório de Ecofisiologia e Toxicologia de Cianobactérias (IBCCF –UFRJ*, Brazil). This *C. raciborskii* strain is able to produce saxitoxins and gonyautoxins (Ferrão-Filho et al., 2009). A non-axenic cyanobacterial stock culture was maintained in sterile ASM-1 growth medium in 300-mL Erlenmeyer flasks placed in a climate-controlled room at 25°C, 35 μmol photons m^{-2} s^{-1}, with a light–dark cycle of 12:12 h (Gorham et al., 1964).

UV Irradiation Exposure

In order to evaluate the effects of different UV wavebands, we administered the following treatments: UVA + UVB (280–400 nm), UVA (315–400 nm), UVB (280–315 nm) and control (Photosynthetically Active Radiation [PAR]; 400–700 nm). For each group, samples of *C. raciborskii* from the same stock culture 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. Subsequently, groups 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. The UV intensities used in experiments were 11.8 Wm^{-2} (UVA) and 0.54 Wm^{-2} (UVB). These values were based on natural solar radiation measurements taken during May, 2009 in Juiz de Fora City (21°45′51″ S) in southeast Brazil. UVA + UVB and UVB treatments were performed in borosilicate glass Erlenmeyers (40 mL) incubated under UVA and/or UVB lamps. UVA treatment was performed in quartz flasks (40 mL) incubated under UVA lamps, while the control treatment was performed in quartz flasks (40 mL) under PAR radiation using the same conditions as the stock culture. According to our measurements, borosilicate glass decreased UVB intensity by 50% and UVA by 10% while quartz glass has a transmittance of ∼90% for UVA, UVB, and PAR radiations (Six et al., 2007). The distance between the UV lamps and flasks (∼30 cm) was calculated using a radiometer (IL 1400A; International Light Technologies, Peabody, MA, USA) in order to ensure the radiation intensity used was in accordance with previous descriptions. Treatments were performed during 6 h at room temperature (RT; 20 ± 1°C) and all samples were carefully homogenized prior to analysis. All experiments were performed in triplicate.

Cell Concentration

Cyanobacteria samples were taken from each experimental group for cell concentration evaluations as previously described (Sipaúba-Tavares and Rocha, 2003). Briefly, samples were fixed with Lugol solution and cells counted in a light microscope (BX 41; Olympus, Tokyo, Japan) at 400 × magnification using an improved Neubauer hemocytometer (Sipaúba-Tavares and Rocha, 2003). Analyses were performed at different time points of UV exposure (0, 2, 4, and 6 h).

Cell Viability

Cell membrane integrity was investigated using a fluorescent probe (*Baclight*) as an indicator of cell viability (Boulos et al., 1999). Samples were collected from each group before UV exposure (0 h) and at the end of 6 h. The percentage of live/viable and dead/non-viable cells was determined using a LIVE/DEAD BacLight Viability kit (Molecular Probes, Inc, ThermoFisher Scientific, Eugene, OR, USA), which contains a mixture of fluorescent dyes, SYTO® 9 and propidium iodide. These probes differ both in their spectral characteristics and in 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 each stain (total volume of 0.9 μL) was added to 300 μL 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 Corporation, Madison, WI, USA), as previously described (Silva et al., 2014), at 28 × g for 5 min at medium acceleration and then evaluated under a fluorescence microscope (BX-60, Olympus, Tokyo, Japan) at 450–480 nm excitation wavelengths, which enable simultaneous fluorescence visualization of live and dead cells. This fluorescence did not cross talk with chlorophyll autofluorescence emitted by cyanobacteria using green (510–550 nm excitation wavelengths) and UV (330–385 nm excitation wavelengths) filters (Supplementary Figure S1). For each group, 30 cyanobacterial filaments were counted and the percentage of live/dead cells determined. Images were taken using an Evolution VF (Media Cybernetics, Rockville, MD, USA) digital camera and Image Pro-Plus 5.0 software (Media Cybernetics).

Transmission Electron Microscopy (TEM)

Samples collected for each group at the 6 h time point 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, washed twice in the same buffer at 1500 × g for 10 min, and stored at 4°C for subsequent use. After fixation, agar embedding was performed as previously described (Barros et al., 2010; Silva et al., 2014), so that uniformly distributed specimens could be processed as easily handled blocks of cells. Briefly, samples were centrifuged at 1,500 × *g* for 1 min. They were then re-suspended in molten 2% agar (Merck, Darmstad, Germany) for further processing. 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 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, USA) (Melo et al., 2006). 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). Cyanobacteria were examined using a transmission electron microscope (Tecnai Spirit G12; FEI Company, Eindhoven, The Netherlands) at 80 kV.

Quantitative Ultrastructural Studies

To study the ultrastructural alterations potentially induced by UV radiation exposure, a total of 64 electron micrographs were randomly taken at magnifications of 9,300× to 30,000×. The following data were quantitated: (i) total cytoplasmic area; (ii) cytoplasmic area occupied by thylakoids; (iii) total number and area of polyphosphate granules; and (iv) total number of polyhedral bodies (carboxysomes). Quantitative analyses were performed using software Image J 1.41 (National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis

Cell concentration, the percentage of viable and non-viable cells and ultrastructural morphometric data were compared by one-way ANOVA, followed by Tukey's comparison test using Prism 6.01 software (GraphPad software, San Diego, CA, USA).

RESULTS

UVA and UVA + UVB Affect Cell Concentration

Both UVA and UVA + UVB treatments led to significant decreases in *C. raciborskii* cell concentrations of 56.44 and 64.39%, respectively, after 6 h of treatment compared to controls ($P < 0.001$; **Figure 1**). In contrast, UVB treatment did not induce any significant change in cell concentration ($P = 0.4894$; **Figure 1**).

UV Radiation Induces *C. raciborskii* Death

To evaluate if the observed decreases in cell concentrations at 6 h were due to cell death, we next investigated cell viability (at 0 and 6 h) using a marker for plasma membrane integrity (Decamp and Rajendran, 1998). Live and dead cyanobacteria were observed in both untreated and treated groups (**Figures 2A,B**). However, all UV treatments induced a significant increase in the percentage of dead cells after 6 h of exposure (**Figure 2B**) compared to the control group ($P < 0.0001$). Radiation induced cell death in 91.65, 99.28, and 56.63% of cells for UVA + UVB, UVA, and UVB treatments, respectively. We also evaluated the viability of each single cell in each filament. Viable and non-viable cells were observed within the same cyanobacterial filament (**Figure 2C**).

Ultrastructure of Untreated *C. raciborskii*

Because this work was, to our knowledge, the first to investigate in extensive detail the ultrastructure of *C. raciborskii*,

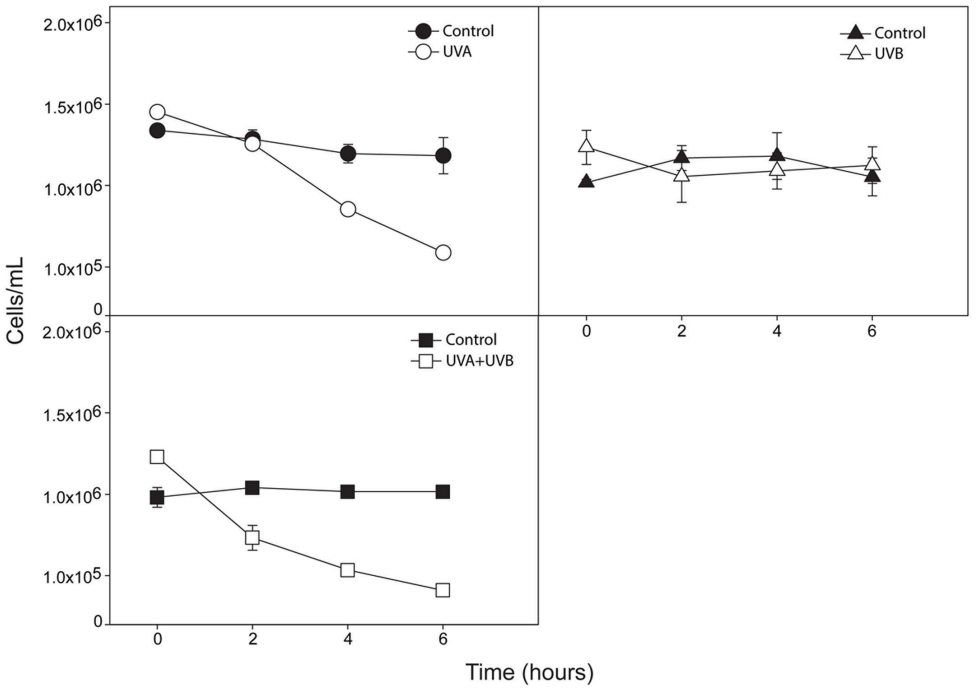

FIGURE 1 | Cell concentration evaluation after 6 h of exposure to ultraviolet radiation (UVA, UVB, and UVA + UVB). For each time point, the concentrations of UV-treated cultures (UVA, $n = 3$; UVB, $n = 3$ and UVA + UVB, $n = 3$) and their respective controls (total $n = 9$) were analyzed after fixation and cells counted by light microscopy using a hemocytometer. All cultures were in the exponential phase of growth. Data are expressed as mean ± SD ($n = 3$). Open symbols represent treatments whereas filled symbols represent control groups.

untreated organisms (controls) were firstly carefully analyzed (**Figures 3A–E**). Similar to other filamentous cyanobacteria, this species was characterized by an envelope composed of three layers: an inner membrane (plasma membrane), an intermediate (cell wall) and an outer membrane with a mucilaginous sheath on its extracellular surface (**Figure 3B**). Longitudinal and oblique sections clearly showed filament organization, with cells closely interrelated by this envelope (**Figures 4A,Aii)**.

The cytoplasm of *C. raciborskii* was characterized by a large number of thylakoid membranes and thylakoid-associated round structures (phycobilisomes), the sites of accessory photosynthetic pigments (phycocyanin and/or phycoerythrin; **Figures 3F** and **4,Ai)**. Other structures frequently identified in the cytoplasm were: gas vesicles (**Figure 3C**), lipid bodies (**Figure 3C**), polyhedral bodies, also termed carboxysomes (**Figure 3D**) and polyphosphate granules (**Figure 3E**). Gas vesicles, structures involved with body buoyancy, appeared as vacuoles of different sizes and shapes, and contained an electron-lucent content (**Figure 3C**). Polyhedral bodies, structures involved in the process of carbon fixation during photosynthesis (reviewed in Cannon et al., 2001), were observed isolated, or in groups, in association with thylakoid membranes (**Figures 3D**). TEM quantitative analysis revealed 3.41 ± 1.06 carboxysomes per cell section (mean ± SEM, $n = 20$ cell sections). Polyphosphate granules were seen as roughly rounded structures, characterized by aggregates of electron-dense phosphates, with an irregular surface and a diameter of about 0.3 μm (**Figure 3E**).

Cyanobacteria Photosynthetic Structures are Drastically Damaged by UV Radiation

To investigate ultrastructural alterations underlying cell death in *C. raciborskii*, samples of cyanobacteria were prepared for TEM after 6 h of UV treatment. All three UV treatments induced marked alterations of the cyanobacteria ultrastructure when compared to the control. The most evident changes were related to photosynthetic structures. Thylakoids were drastically damaged by UV radiation, showing significant reductions in cytoplasmic area occupied by these membranes of 95.8, 77.6, and 81.3% for UVA, UVB, and UVA + UVB treatments, respectively, compared to the untreated control group ($P < 0.05$; **Figure 5**).

Phycobilisomes and polyphosphate granules were also altered. In control cells phycobilisomes could be clearly identified as individual electron-dense dots, uniformly distributed in association with thylakoid membranes (**Figures 4,Aii)**. However, in UV-treated cells, phycobilisomes were found disarranged and frequently formed amorphous aggregates. These were dispersed in the cytoplasm, as mainly seen in the UVA-treated group (**Figure 6**).

Polyphosphate granules also appeared as disarranged structures (**Figure 7**). In control cells, such granules generally appeared having round forms (**Figures 7A,Ai)**, but after radiation exposure, most had lost their morphological characteristics, and presented as amorphous structures (**Figures 7B,C**). Quantitative

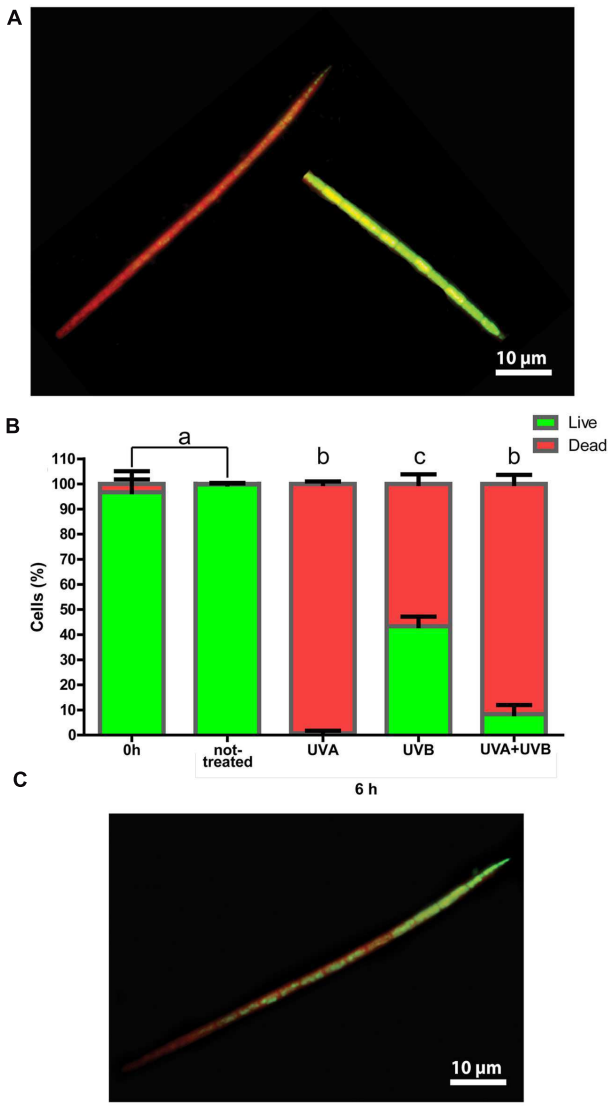

FIGURE 2 | Cell viability of *Cylindrospermopsis raciborskii* exposed to UV radiation. In **(A)**, live (green) and dead (red) cyanobacteria are observed. **(B)** After 6 h, all treatments induced significant cell death compared to untreated and 0 h groups ($P < 0.0001$ for all). Letters indicate a significant difference ($P < 0.0001$). In **(C)**, both live and dead cells are seen on the same filament (after UVB treatment). Samples were stained with Syto 9 and propidium iodide (BacLight). Live and dead cells were viewed simultaneously, and 30 filaments/group were counted by fluorescence microscopy.

TEM analysis revealed that cells exposed to UVA or UVB showed a significant reduction in the number of polyphosphate granules per cell section compared to the control group ($P = 0.0008$; **Figure 7D**); this reduction corresponded to 66.87 and 63.76% for UVA- and UVB-treated groups, respectively. However, the number of granules did not significantly vary between control and UVA + UVB groups (**Figure 7D**). The total number of polyhedral bodies and gas vesicles also did not vary between groups ($P > 0.3366$ and $P > 0.810$, respectively).

DISCUSSION

The current study shows that UV radiation induces death of the cyanobacterium *C. raciborskii,* and that this process occurs through directly damaging cell structures. It appears that the entire photosynthetic cell apparatus of *C. raciborskii* is a target of this radiation, in conjunction with the cell envelope and structures related to cellular metabolism (polyphosphate granules).

The effect of UV radiation, especially UVB, has been evaluated in different species of cyanobacteria (reviewed in Singh et al., 2010), but not in *C. raciborskii*. For example, growth of the cyanobacteria, *Oscillatoria priestleyi* and *Phormidium murrayi,* was suppressed by 100 and 62%, respectively, following exposure to UVB radiation (Quesada and Vincent, 1997). Similarly, Han et al. (2003) reported the inhibition of growth of a rice-field cyanobacterium *Anabaena sp.* during exposure to UVA + UVB radiation, while solar UV radiation inhibited *Anabaena sp.* PCC 7120 by up to 40% (Gao et al., 2007). Other work with two cyanobacteria (*Nostoc muscorum* and *Phormidium foveolarum*) found that while a UVB dose of 1.0 µmol m^{-2} s^{-1} induced a 14–21% growth decrease, treatment using a lower UVB dose (0.1 µmol m^{-2} s^{-1}) did not influence growth (Singh et al., 2012).

A study with several strains of cyanobacteria demonstrated a growth decrease of 48% after 30 min of exposure to UVB alone (14.4 ± 1 Wm^{-2}), without the addition of PAR (Kumar et al., 2003); moreover, all cell strains died after 90 min of exposure (Kumar et al., 2003). Using a lower intensity of UVB (3 Wm^{-2}) and another cyanobacterium strain (*Artrospira platensis)*, other groups have observed 28 and 40% growth decreases after 30 and 60 min of exposure, respectively (Ganapathy et al., 2015).

Here, we used two approaches to investigate the effect of UV radiation on *C. raciborskii* growth/death: (i) a classical cell concentration evaluation, which demonstrated a significant reduction in cell numbers in response to UVA + UVB and UVA treatments (**Figure 1**), and (ii) a cell viability test, which identified that all treatments, including UVB, were able to trigger significant cyanobacterial death (**Figure 2**). This means that while UVA and UVA + UVB led to *C. raciborskii* lysis, as indirectly shown by the cell concentration analysis, all treatments were able to elicit cellular changes indicative of cell death, i.e., loss of plasma membrane integrity. Therefore, while UVB radiation did not induce cell rupture, it still caused a loss of cell viability, although to a lesser extent compared with UVA and UVA + UVB treatments (**Figure 2B**). Thus, cell viability data were important in allowing the detection of cell death-related changes prior to cell lysis (Pearl, 2000; Agusti et al., 2006).

The use of markers for cell viability also enabled us to demonstrate the presence of viable and non-viable cells within the same cyanobacterial filament (**Figure 2C**). This likely represents the initial response of individual cells to UV radiation. It is possible that higher UV doses and/or longer times to UV exposure lead to death or inactivation of all cells within the filament.

Ultrastructural studies of cyanobacteria are still scarce, although such studies would be of great importance in better understanding the biology and diversity of these organisms,

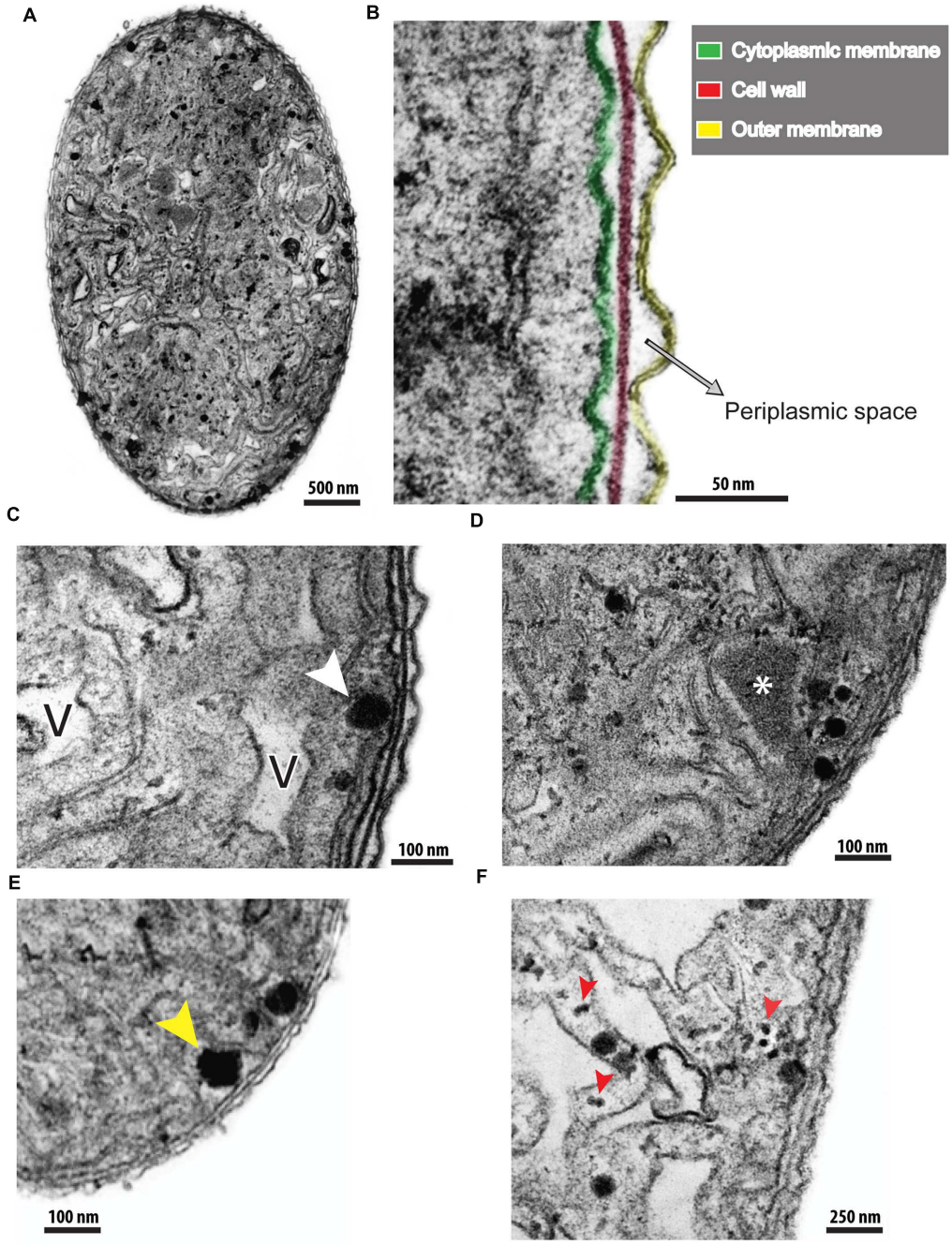

FIGURE 3 | Different views of untreated *C. raciborskii* cells observed by transmission electron microscopy (TEM). In **(A)**, a cross-section of a cell shows a general view of the cytoplasm, cell envelope and their organization. **(B)** At higher magnification, the structure of the cellular envelope is more clearly revealed. Note the cytoplasmic membrane (green), cell wall (red) and outer membrane (yellow). **(C–E)** Cytoplasmic structures such as air vesicles (V), lipid bodies (white arrowhead), polyhedral bodies (*), and polyphosphate granules (yellow arrowhead) are observed. In **(F)**, the thylakoid membrane structure is seen at high magnification. Phycobilisomes (red arrowheads) are viewed as small electron-dense dots in association with thylakoid membranes.

and in providing insights into their ecological responses. The ultrastructure of *C. raciborskii* was analyzed here in detail by TEM. We used a method of pre-inclusion in agar with the aims of maintaining the optimal preservation of cells and of reducing artifacts caused by mechanic damage and the loss of specimens during sample manipulation (Melo et al., 2007; Silva et al., 2014). Untreated cells exhibited subcellular details such as an elaborate three-layered envelope, and a cytoplasm containing

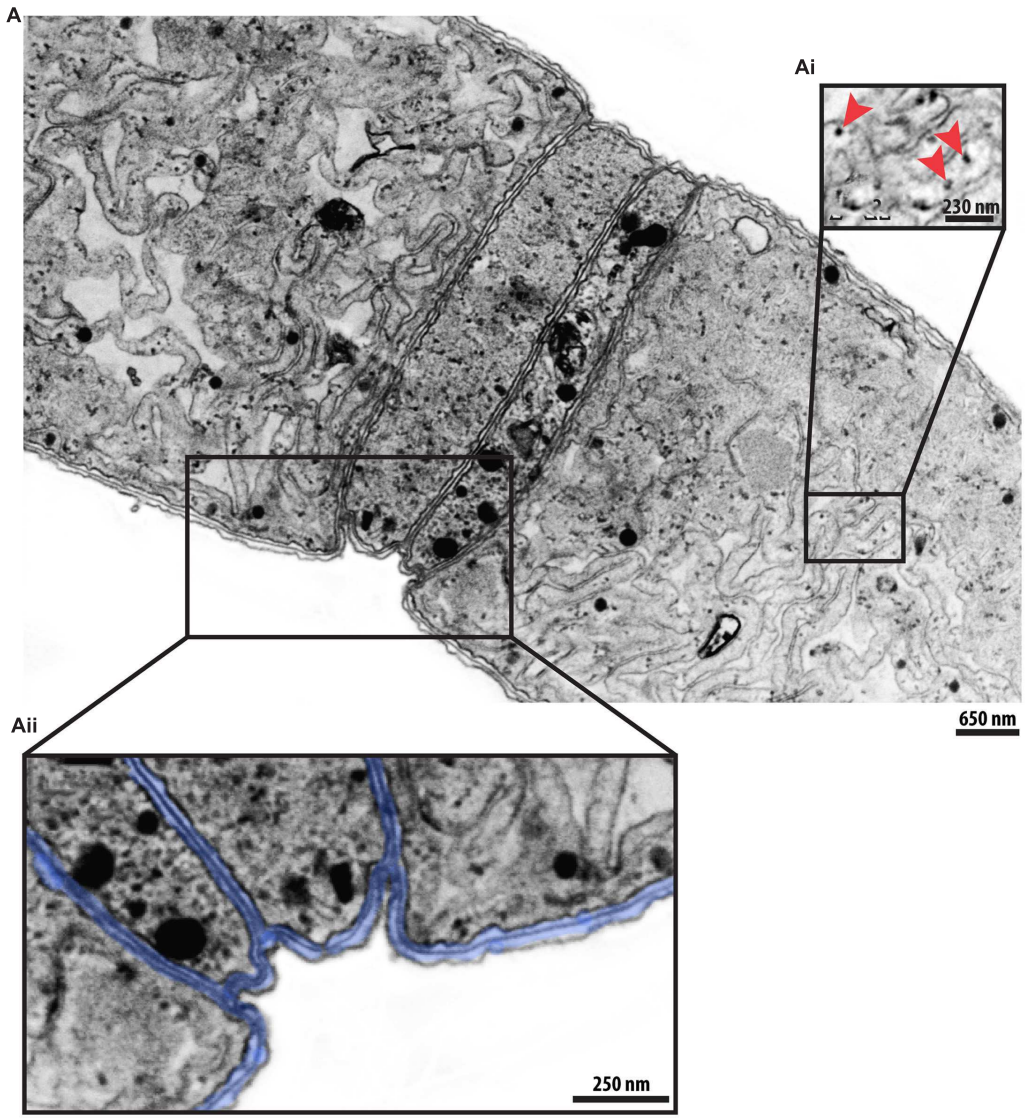

FIGURE 4 | A representative *C. raciborskii* filament seen by transmission electron microscopy (TEM). (A,Aii) A longitudinal section shows several cells tightly associated with each other by their envelopes (highlighted in blue in **Aii**). Thylakoid membranes with phycobilisomes (indicated by arrowheads in **Ai**) are clearly visible.

an intricate membrane system (thylakoids), with associated phycobilisomes and other structures such as polyphosphate granules and carboxysomes (**Figures 3** and **4**). Cells were tightly arranged within each filament (**Figure 4**).

The thylakoid membranes of cyanobacteria are the major sites of respiratory electron transport, as well as photosynthetic light reactions (reviewed in Mullineaux, 2014). Knowledge of the arrangement and number of thylakoids is taxonomically important (Whitton, 1972; Lang and Whiton, 1973) because these are considered to be stable cyanobacterium features (Komárek and Vaslavská, 1991). For instance, in contrast to other cyanobacteria, such as *Annamia toxica gen. et sp. nov.,* which display concentric thylakoids (Nguyen et al., 2013), these structures are irregularly arranged in *C. raciborskii* (**Figures 3F** and **4,Ai)**.

Ultrastructural analyses identified subcellular structural targets of UV radiation. The main finding was the detection of accentuated damage to thylakoid membranes (**Figure 5**). These data are in agreement with previous studies that showed that treatment with UVB for 2 h affected thylakoid membranes in cyanobacteria of the genus *Synechococcus sp.* (Chauhan et al., 1998). Structural alterations and subsequent reductions of these specialized structures were also observed in eukaryotic phytoplankton affected by UVB (Meindl and Lütz, 1996; Lütz et al., 1997).

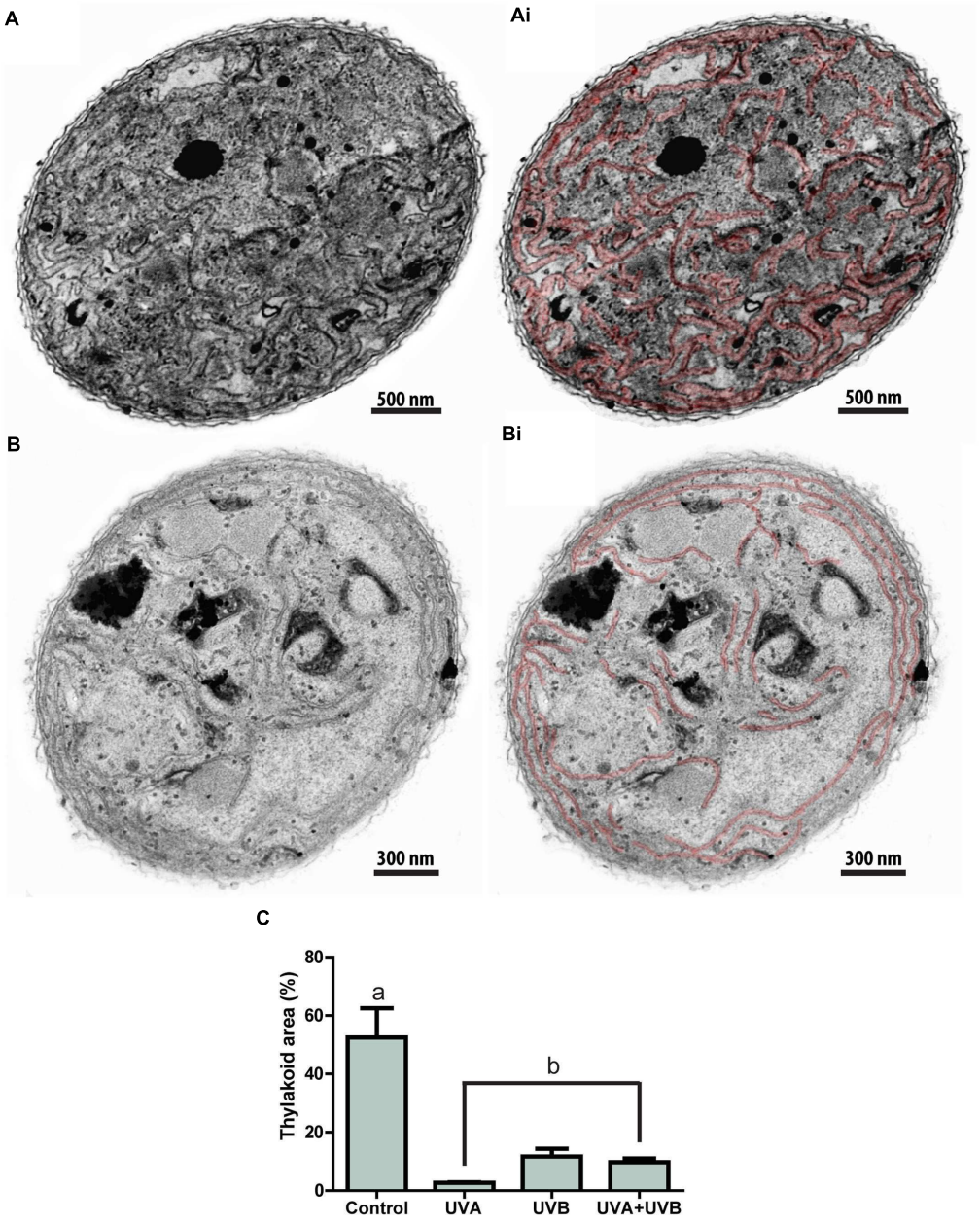

FIGURE 5 | Cyanobacterium thylakoid membranes are drastically affected by UV radiation. While the cytoplasm of control **(A)**, untreated *Cylindrospermopsis raciborskii* shows a high amount of thylakoid membranes, these structures were greatly reduced after exposure to UV radiation **(B)**. Membranes are highlighted in red in **(Ai)** and **(Bi)**. In **(C)**, quantitative analyses show a significant reduction of thylakoid areas induced by all UV treatments. Letters indicate a significant difference ($P < 0.05$). Cells were fixed in a mixture of glutaraldehyde and paraformaldehyde and prepared for TEM. A total of 64 electron micrographs were evaluated and thylakoid areas measured using software Image J 1.41.

In parallel to the thylakoid loss, we observed structural disarrangement of photosynthetic pigments (phycobilisomes; **Figure 6**). A study using molecular markers also reported the dissociation of phycobilisomes from thylakoid membranes when *Synechococcus sp.* were exposed to UVA + UVB + PAR (UVA: 4.3 Wm^{-2} and UVB 0.86 Wm^{-2}) (Six et al., 2007). Previous studies using only UV radiation (without PAR addition) suggested that phycobilisomes and photopigments on thylakoid membranes served as specific targets for UV radiation, particularly UVB (Rajagopal et al., 1998; Gupta et al., 2008; Ganapathy et al., 2015). It is believed that UV radiation affects the photosynthetic apparatus in some way (directly or indirectly through reactive oxygen species [ROS]) by, for instance, acting on proteins of thylakoid membranes, or on photosynthetic pigments, and/or

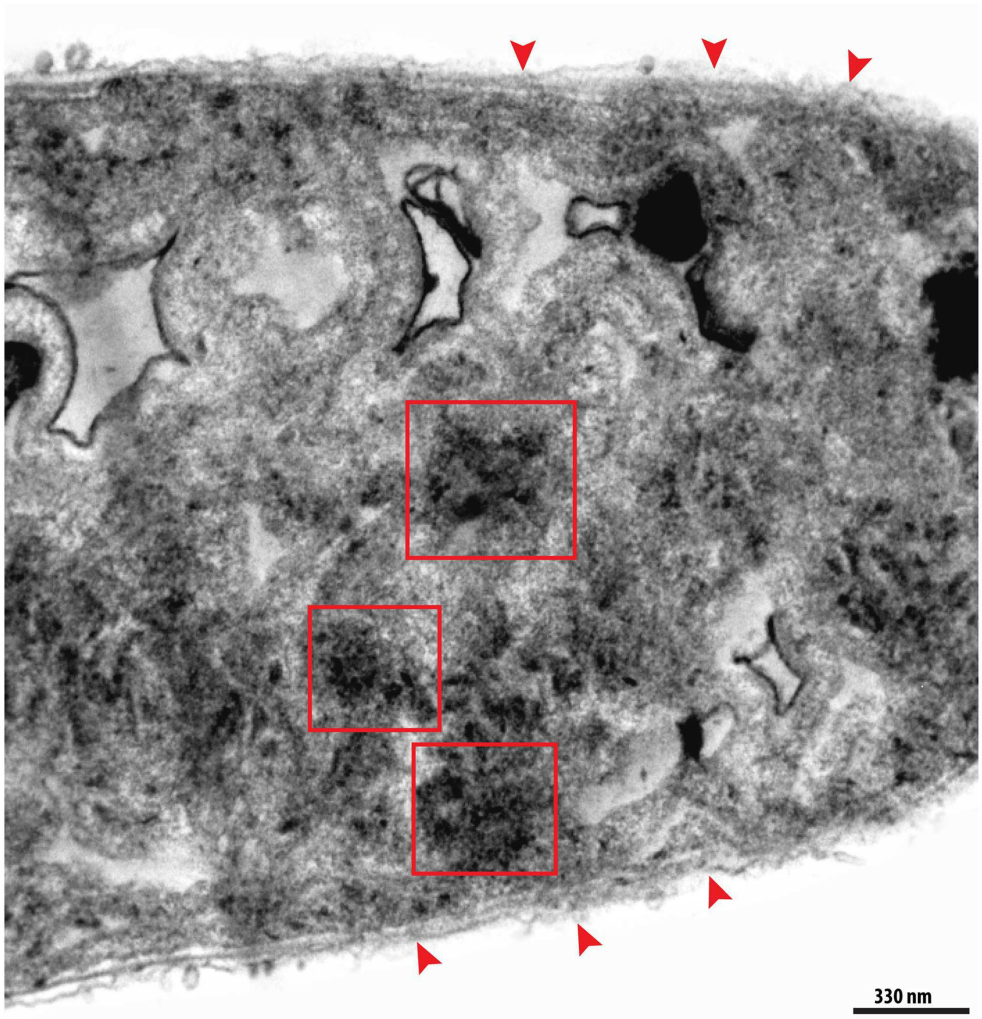

FIGURE 6 | Electron micrograph of a representative degenerating cyanobacterium. In addition to a clear loss of thylakoid membranes, aggregates of dissolving phycobilisomes (red boxes) were observed in the cytoplasm. Note the loss of the integrity of the cell envelope (arrowheads).

on proteins that link these pigments to thylakoid membranes. For example, under UVB stress, the cyanobacterium, *Spirulina platensis,* showed both structural (membrane distortions) and molecular (pigment protein complexes) alterations in thylakoid membranes (Rajagopal et al., 1998; Gupta et al., 2008). In *Arthrospira platensis,* levels of photosynthetic pigments, total chlorophyll, total carotenoids and *c*-phycocyanin decreased after long exposure times (9 h) to 3.0 Wm^{-2} UVB radiation (Ganapathy et al., 2015).

UVB radiation is the most energetic region of the UV spectrum that reaches ground level, affecting mainly cyanobacterial DNA (Kumar et al., 2004). In this regard, we therefore expected greater damaging effects with such treatment. However, our results demonstrated that UVB exposure was less deleterious in comparison to UVA and UVA + UVB treatments. This fact might be due to the UVB intensity used. While most studies used higher UVB doses compared to those of our study (Quesada and Vincent, 1997; Sinha et al., 1997; Kumar et al., 2003; Fijalkowska and Surosz, 2005), the intensities of UV radiation used in the current experiments corresponded well with UVA and UVB intensities on a sunny day in the Brazilian southeast region during autumn (UVA: 11.8 W m^{-2} and UVB: 0.54 W m^{-2}). Therefore, the UV intensities adopted here 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). We speculate that UVB at the intensity used in the present study may have had a potential photoprotective effect (Xu and Gao, 2010). Indeed, mechanisms of protection are triggered by light since high light intensities are usually accompanied by high intensities of biologically active UVB radiation in natural environments (Ding et al., 2013). However, cyanobacteria contain amino acids that provide UV protection (Sinha and Häder, 2008), and under only UV radiation are able to produce

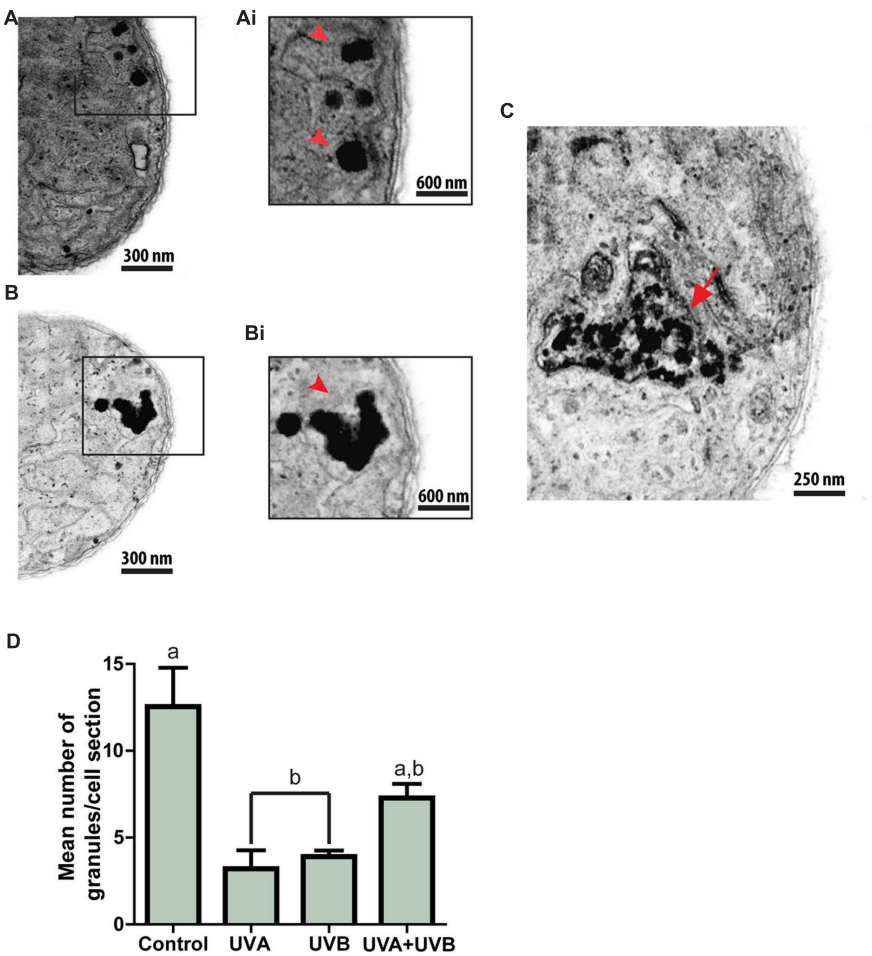

FIGURE 7 | Ultraviolet radiation induces disarrangement of *C. raciborskii* polyphosphate granules. Polyphosphate granules (boxed area in **(A)** and **(B)** and arrowheads in **Ai** and **Bi**) are seen as intact structures in control cells **(A,Ai)**, and as disrupted structures in UV-treated cells **(B,Bi)**. A polyphosphate granule is seen at an advanced stage of disintegration [**(C)**, arrow]. A graph of the number of polyphosphate granules per cell for each group is shown in **(D)**. A total of 64 electron micrographs were evaluated and the number of granules per section quantitated. A total of 273 granules were counted. Data are expressed as mean ± SD. Letters indicate significant differences between means ($P < 0.05$).

antioxidant enzymes (Ganapathy et al., 2015) and increase membrane lipid unsaturation (Gupta et al., 2008). These have been suggested to be protective mechanisms that keep cells alive under stress.

On the other hand, it is known that UVB effects are dependent on the wavelength incidence into this spectrum (Mitchell and Karentz, 1993). In fact, Meindl and Lütz (1996) observed that damage to the morphology and development of *Micrasterias denticulata* (Chlorophyceae) by UVB occurred within a short time frame when cells were treated with a wavelength shorter than 275 nm. Such results may explain the lower effect UVB had compared to UVA in our experiments, since the lamp used had an emission peak at a wavelength of 312 nm, which corresponds to an area of greater length and less energy in the UVB radiation spectrum. Other possibilities to be considered are differences in the sensitivity of cyanobacteria to various UV wavelength bands. Dissimilarities in UVB responses suggest a species-specific effect, which can be an important driver of community structure and the succession of species (Wangberg et al., 1996; Quesada and Vincent, 1997). Moreover, it has been demonstrated that the production of specific toxins by cyanobacteria may prevent cellular damage under moderate UV intensities. For example, a toxic strain of cyanobacteria *Microcystis aeruginosa* (a microcystin producer) increases bacterial fitness in high light intensities and shows a protective effect against UVB radiation (Zilliges et al., 2011; Ding et al., 2013).

In our experiments, UVA induced the most drastic alterations in *C. raciborskii* ultrastructure, both in thylakoid membranes and in photosynthetic pigments. In general, exposure to UVA was reported to cause the photo-inhibition of a natural population of cyanobacteria (Kim and Watanabe, 1993). However, the effects of UVA on other aspects of cyanobacteria, including ultrastructure, are poorly understood and their significance awaits further investigation.

Using TEM, we also found UV radiation affected other structures related to cellular metabolism, such as polyphosphate granules. The changes noted in these granules were another structural indication that the cell was in the process of dying in response to UV radiation. These cytoplasmic granules exhibited disarrangement, dissolution and a decrease in their number, mainly in response to UVA and UVB treatments (**Figure 7**). Polyphosphate granules have important functions in phosphate storage and energy metabolism, since phosphate is involved in the biosynthesis of nucleic acids, phospholipids and ATP (reviewed in Allen, 1984; Achbergerova and Nahalka, 2011). Moreover, *C. raciborskii* demonstrates a large capacity to stock phosphorous (as phosphate), which gives a competitive advantage to these cyanobacteria (Padisák, 1997). In accordance with the viability results, we also identified UV-induced ultrastructural changes of the cyanobacterial cell envelope (**Figure 6**), likely due to the absorption of UV radiation by membrane proteins, eventually resulting in cell death (Sinha et al., 1996).

Altogether, we demonstrated clear alterations in the viability and photosynthetic structures of *C. raciborskii* induced by UV radiation fractions. This study facilitates our understanding of the subcellular organization of this interesting and ecologically important cyanobacterium species, and identifies specific intracellular targets of UVA and UVB radiation. Moreover, our study opens new perspectives to investigate at high resolution possible signals of cell damage induced by UV radiation in *in situ* organisms based on TEM and also to evaluate the critical levels of UV to cell physiology and morphology that may leads to cell death in natural ecosystems.

In summary, our results reinforce and expand the importance of UV radiation as an environmental stressor and recognize subcellular structures involved in UV radiation-induced-cell damage, such as thylakoid membranes, phycobilisomes and polyphosphate granules. The knowledge of physiological responses at cellular level in cyanobacterial populations affected by UV radiation is important to understand the connection of environmental changes and persistence of species in aquatic ecosystems. Future investigations may link the individual physiology and the environmental conditions.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01202

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