Metagenomic analysis of *Raphidiopsis raciborskii* microbiome: beyond the individual

Paula Vico‡, Andrés Iriarte§, Sylvia Bonilla, Claudia Piccini‡

‡ Instituto de Investigaciones Biológicas Clemente Estable, Montevideo, Uruguay  
§ Instituto de Higiene, Facultad de Medicina, UDELAR, Montevideo, Uruguay  
| Sección Limnología, Facultad de Ciencias, UDELAR, Montevideo, Uruguay

Corresponding author: Claudia Piccini (cpiccini@iibce.edu.uy)

Academic editor: Chloe Robinson

Received: 02 Aug 2021 | Accepted: 05 Oct 2021 | Published: 21 Oct 2021

Citation: Vico P, Iriarte A, Bonilla S, Piccini C (2021) Metagenomic analysis of *Raphidiopsis raciborskii* microbiome: beyond the individual. Biodiversity Data Journal 9: e72514. https://doi.org/10.3897/BDJ.9.e72514

Abstract

*Raphidiopsis raciborskii* is a toxic, invasive bacteria with a defined biogeographic pattern attributed to the generation of ecotypes subjected to local environmental filters and to phenotypic plasticity. The interactions taking place between the cyanobacterium and the other bacteria inhabiting the external polysaccharide-rich matrix surrounding the cells, or phycosphere, may be ecotype-specific and would have different influence on the carbon and nutrient cycling in the ecosystem. Here, we describe the bacterial community or microbiome (assessed by 16S rRNA metagenomics) associated to two *R. raciborskii* strains that have been described as different ecotypes: the saxitoxin-producer MVCC19 and the non-toxic LB2897. Our results showed that both ecotypes share 50% of their microbiomes and differ in their dominant taxa. The taxon having the highest abundance in the microbiome of MVCC19 was *Neorhizobium* (22.5% relative abundance), while the dominant taxon in LB2897 was the Planctomycetes *SM1A02* (26.2% relative abundance). These groups exhibit different metabolic capabilities regarding nitrogen acquisition (symbiotic nitrogen-fixing in *Neorhizobium* vs. anammox in *SM1A02*), suggesting the existence of ecotype-specific microbiomes that play a relevant role in cyanobacterial niche-adaptation. In addition, as saxitoxin and analogues are nitrogen-rich (7 atoms per molecule), we hypothesise that saxitoxin-producing *R. raciborskii* benefits from external sources of nitrogen provided by the microbiome bacteria. Based on these findings, we
propose that the mechanisms involved in the assembly of the cyanobacterial microbiome community are ecotype-dependent.

Keywords

Raphidiopsis raciborskii, microbiome, 16S rRNA metagenomics, phycosphere

Introduction

Cyanobacteria have a polysaccharide-rich microzone outside the cell wall that surrounds the cells, filaments or colonies, which are colonised with heterotrophic bacteria. Despite the recognised role of heterotrophic bacteria in carbon and nutrient recycling of aquatic ecosystems, little is known about the composition and the interaction of these bacteria with the bacteria. In this microzone, called the phycosphere (Bell and Mitchel 1972), the metabolites are readily exchanged before their diffusion to the water and represent a central meeting place for bacteria and bacteria (Seymour et al. 2017). The interactions occurring between individual organisms within this phycosphere have an ecosystem-level effect on several processes, for example, carbon and nutrient cycling, toxin biosynthesis etc. The incorporation of bacteria into the phycosphere likely occurs through chemotaxis, random contacts and vertical transmission (Seymour et al. 2017).

The close association between bacteria and bacteria that occurs in the phycosphere may be a strategy to achieve a higher number of genes and functions to cope and thrive through a range of environmental conditions (Humbert et al. 2013). These interactions translate into a selective advantage for both partners, which widen the spectrum of "goods" that they can obtain. The concept of the "common good" proposes that natural selection would keep those functions available in the whole community (Morris et al. 2011). By sharing these metabolites that are extracellularly available, the ability to synthesise them by themselves would no longer be necessary (Pande and Kost 2017). Thus, the bacterial species that are able to co-evolve in synergy and interaction would constitute the microbiome of a community or the "interactome" (Cook et al. 2019). This has been addressed in the cyanobacterium Microcystis sp., where a metagneomics-based study of blooms from twelve lakes showed that their microbiomes share a large number of functional genes despite the fact that bacteria were taxonomically distinct at the 16S rRNA level (Jankowiak and Gobler 2020). As a consequence, changes at the taxonomic level would be functionally equivalent and guarantee the permanence of essential metabolic functions.

The synthesis of the external polysaccharide-rich (EPS) matrix that surrounds the cyanobacterial cells is thought to be a physiological response to fluctuations in environmental conditions, allowing bacteria to maintain their fitness and also the associated microbiota (Rossi and De Philippis 2015). The chemical characteristics of EPS and its abundance depend on the species and strain of bacteria and the culture or environmental conditions. Under oligotrophic conditions, EPS is the source of available
organic carbon for heterotrophs, while during blooms, the relative abundance of bacteria living in the EPS varies depending on the biomass of the bacteria and its EPS composition (Woodhouse et al. 2018). It is then possible that bacteria having different metabolic functions are selected and that structural and functional differences in the community are influenced by the available organic matter and nutrients in the EPS (Louati et al. 2015).

In some toxic bacteria, such as *Microcystis* spp., the bacteria present in the phycosphere were shown to differ markedly from free-living planktonic ones (Wu et al. 2019) and allow the bacteria the access to specific compounds, such as vitamins and some components of the outer membrane lipopolysaccharide, while providing bacteria with highly bioavailable carbon. For example, *Microcystis* spp. do not have the ability to fix nitrogen and appeared to benefit from the nitrogen released from the *Rhizobiales* present in their microbiome and to the enrichment of N$_2$-fixation genes (Jankowiak and Gobler 2020).

*Raphidiopsis* (*Cylindrospermopsis*) *raciborskii* (Order Nostocales) is a cyanobacterium that forms toxic blooms that has attracted worldwide interest due to its increasing expansion from warm latitudes to temperate zones. This species is capable of alternately producing saxitoxins (STX) or cylindrospermopsin (CYN) or being non-toxic. However, strains capable of synthesising both STX and CYN at the same time have not yet been described (Vico et al. 2020, Soares et al. 2012, Burford et al. 2018, Antunes et al. 2015, Neilan et al. 2002, Briand et al. 2004, Moreira et al. 2014, Wood et al. 2014, Dokulil 2015, Aguilera et al. 2019).

Although it is proposed that the success of this species in tolerating, colonising and adapting to different environmental conditions (temperature, light and nutrients) is due to a strategy combining phenotypic plasticity (Bonilla et al. 2016, Bonilla et al. 2012, Willis et al. 2018, Soares et al. 2012) and the existence of different ecotypes (Piccini et al. 2011), it is unknown how these environmental changes affect the structure of the microbiota. The increasing incidence of toxic blooms of saxitoxin-producing (SxP) *R. raciborskii* in South America raises concern, since this alkaloid has a very serious neurological effect in humans and animals. Therefore, knowing the composition of its microbiome is relevant, not only to understand its role in *R. raciborskii* growth and population dynamics, but also as a means of discovering bacterial taxa able to degrade saxitoxins to use as a water treatment strategy.

This is the first description of the heterotrophic bacterial community associated with *R. raciborskii* strains described as different ecotypes of the species (Vico et al. 2020, Piccini et al. 2011). We used 16S rRNA gene metagenomics to assess the bacterial microbiomes associated with the phycosphere of two *R. raciborskii* strains, one SxP isolated from South America (MVCC19) and one non-producer (NoP) (LB2897) isolated from North America. The dataset describes the amplicon sequence variants (ASVs) associated with each strain and their taxonomic affiliations.
Methods

Cyanobacterial strains, culturing conditions and samples

We analysed the microbiome of two strains of *R. raciborskii* from the Americas, one isolated from a lake located at the northernmost latitude where the species was detected and the other from a lake at the southernmost latitude where the species can be found. The LB2897 strain, originally isolated from Lemon Lake (USA, 39.2568, −86.3929) was obtained from the UTEX culture collection (see Yilmaz and Phlips 2011 for further description) and the MVCC19 strain was isolated in 2007 from Javier Lake (Uruguay, −34.8640, −56.0409) (Vidal and Kruk 2008, Piccini et al. 2011) (Table 1).

Static cultures of both strains were grown in a nitrogen-free BG11 medium (Stanier et al. 1971) at 26°C and PAR light intensity of 80 μmol photons m²s⁻¹ with a 16:8 h light:dark photoperiod. Under these conditions, the morphology of the two strains was analysed and compared, measuring 30 cells and 50 filaments under the microscope (400 x and 1000 x magnifications). The biovolume was calculated following Hillebrand et al. 1999. The genome of both strains and their phylogenetic and ecological characteristics have been previously published (Vico et al. 2020). The MVCC19 and LB2897 whole-genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession VIRO00000000 and VOIM00000000, respectively (Vico et al. 2020). After 7 days of incubation in the above-mentioned conditions, 5 ml of each strain culture were sampled and subjected to DNA extraction.
DNA extraction and 16S RNA gene sequencing

To harvest the cells, samples were filtered on to 2 µm pore size polycarbonate hydrophilic membranes. The DNA extraction was performed as described in Martínez de la Escalera et al. 2014 using the filters containing the biomass as starting material. Briefly, the lysis was achieved by incubating the filters in extraction buffer containing 1% CTAB, EDTA and proteinase K at 37°C during 30 min on a shaker. Then, SDS was added and the mix was incubated at 65°C for 2 h. The resulting lysate was separated from the proteinaceous phase by centrifugation using 24:1 chloroform:isoamylalcohol (three times). After collecting the aqueous upper phase, DNA was precipitated with 0.1 vol. sodium acetate, pH 5.2 and 0.6 vol. isopropanol for 1 h at room temperature. Precipitated DNA was pelleted by centrifugation at 12,000 x g, 45 min, washed with 70% (v/v) cold ethanol (same centrifugation procedure) and suspended in 1 x TE overnight at 4°C. After extraction, the concentration and purity of DNA were spectrophotometrically determined at 260 and 280 nm (NanoDrop).

The 16S rRNA gene was amplified (three pooled technical replicates) and sequencing at the Macrogen Sequencing Service (South Korea). Sequencing libraries were prepared according to the Illumina 16S Metagenomic Sequencing Library protocols to amplify the V3-V4. Primer sequences used for the first amplifications were as follows: 341F/805R(V3-V4, 341F: CCTACGGGNGGCWGCAG, 805R: GACTACHVGGGTATCTAATCC) (Herlemann et al. 2011). The final purified product was then quantified using qPCR according to the qPCR Quantification Protocol Guide (KAPA Library Quantification Kits for Illumina Sequencing platforms) and qualified using the TapeStation DNA screentape (Agilent Technologies, Waldbronn, Germany). Then, the paired-end (2 × 300 bp) sequencing was performed by the Macrogen Sequencing Service, using the MiSeq™ platform.
Data processing

Bioinformatic analyses of the microbiome were performed in R (version 4.1.0) using the DADA2 package (Callahan et al. 2016). Quality profiles of the forward and reverse reads were inspected by the recommended parameters of DADA2. Sequences were then quality filtered, denoised, merged and the chimera were removed using the DADA2 and amplicon sequence variants (ASVs) of the V3-V4 region of 16S rRNA gene were defined and taxonomically classified using version 132 of Silva Database as a reference (Quast et al. 2012, Glöckner et al. 2017). For multiple alignments, the Decipher package was used (Wright 2015) and the phylogeny tree was constructed with Phangorn (Schliep 2010) with the parameters recommended by Callahan et al. 2016 (see Suppl. material 1).

The composition of bacterial microbiomes at different taxonomic levels was analysed with the Phyloseq package (McMurdie and Holmes 2013) and plots were generated using the package Ggplot2 (Gómez-Rubio 2017). The sample rarefaction analyses were conducted using the library Ranacapa (Kandlikar et al. 2018) (Suppl. material 2). Alpha-diversity was assessed as the Shannon Diversity Index and beta-diversity as the Bray–Curtis distance. ASVs abundances were normalised by proportion (counts in each sample/column were scaled by the sample/column's sum).

Biodiversity scope

This study was focused on the microbiome of the bloom-forming cyanobacterium Raphidiopsis raciborskii, analysing the differences between the microbiome community composition of a saxitoxin-producing strain and a non-toxic one.

Target

16S ribosomal ARN gene.

Taxonomic range

Bacterial domain.

Data Resources

Sequence data from this study have been deposited to NCBI SRA database. Resource identifiers are PRJNA737279 for the taxa obtained from R. raciborskii MVCC19 microbiome and PRJNA737280 for those obtained from R. raciborskii LB2897.
Taxonomic composition of the microbiome

After filtering by quality, denoising, merging and removing the chimeras, a total of 50,753 and 35,908 reads were obtained for the microbiome of LB2897 and MVCC19, respectively. They were clustered into amplicon sequence variants (ASVs) with 100% sequence identity. Taxa richness was 31 and 22 and Shannon Diversity was 2.2 and 2.4 for LB2897 and MVCC19, respectively. Bray–Curtis Distance Index between both microbiomes was 0.26. The most represented phylum was Proteobacteria. Amongst these, the Alphaproteobacteria were dominant (Table 2, Fig. 1).

In both microbiomes, few bacterial genera accounted for more than 50% of the community. In the case of the SxP (*R. raciborskii* MVCC19), the most abundant bacteria belonged to the *Rhizobium–Allorhizobium–Agrobacterium* clade (or *Neorhizobium*) (Mousavi et al. 2014) (Rhizobiaceae family, Alphaproteobacteria class), with a relative abundance of 22.5%, followed by 13.8% SM1A02 (Phycisphaeraceae family, Planctomycetes), 12.3%
**Brevundimonas** (Caulobacteraceae family, Alphaproteobacteria class) and 9.8% **Emticicia** (family Cytophagaceae, phylum Bacteroidetes).

| Genus       | Relative abundances (%) | Closer relative (% identity ≥ 97) and GeneBank accession number | Environmental source                                                                 |
|-------------|--------------------------|------------------------------------------------------------------|-------------------------------------------------------------------------------------|
| SM1A02      | 26.17 13.79              | Uncultured bacterium partial 16S rRNA gene (99%) LR643748        | Wastewater treatment system                                                          |
| Hirschia    | 20.54 5.80              | Uncultured Hyphomonadaceae bacterium clone 1d_33690 (100%) MG805085 | Sewage sludge of the completely autotrophic nitrogen removal over nitrite process with a submerged aerated biological filter and the effect of inorganic carbon on nitrogen removal and microbial activity. |
| Labrys      | 13.35 0.00              | Uncultured alpha proteobacterium clone caf51349 (100%) MF438647.1 | Floodplain lake water                                                               |
| Cutibacterium | 12.36 0                  | More than 100 sequences having 100% identity, including C. acnes (MT242489) and environmental clones associated with P arameciun (MH566018) | Oral microbiome. Isolation and Characterization of Predominant Microorganisms during decomposition of Waste Materials in a Field-Scale Composter |
| Gemmobacter | 10.74 0                  | Gemmobacter aquaticus strain 05SS-25 (100%) MG780340            | Freshwater sediment                                                                  |
| Neorhizobium | 0.23 22.51             | Rhizobium sp. TH167 (100%) KT826347                              | Cyanobacterial aggregates                                                            |
| Brevundimonas | 0 12.30                | Brevundimonas lenta strain P4-2 (100%) M N181016, MH348813, MG642117 | Water. Constructed wetlands. Ice                                                     |
| Blastomonas | 0.24 9.64               | Blastomonas sp. strain MPSM-12 (100%) M G494710                 | Daphnia is a reservoir for mercury-tolerant bacteria in the environment             |
| Azospirillum | 2.03 2.86               | Uncultured bacterium clone SIP21-RS-6 (99%) FR774694            | Rice rhizospheric soil                                                              |
| Asinibacterium | 0.80 6.47            | Sediminibacterium sp. strain FW305-C-49 (99%) MK402932          | Groundwater                                                                         |

Table 2. GenBank retrieved sequences having more than 97% identity with the ASVs obtained in this study.
| Genus                      | Relative abundances (%) | Closer relative (% identity ≥ 97) and GeneBank accession number | Environmental source                                                                 |
|----------------------------|--------------------------|------------------------------------------------------------------|---------------------------------------------------------------------------------------|
| *CL500-29_marine_group*    | 2.36 0                   | Uncultured bacterium clone Wat111 (100%) KC189789               | Bacterial Community Structure on *Hydrilla verticillata* and *Vallisneria americana* in a Freshwater Spring |
| *Niveispirillum*           | 0.91 4.23                | LT628527.1                                                      | Eutrophic lake. associated with cyanobloom                                             |
| *Phenylobacterium*         | 0 5.68                   | Uncultured bacterium clone SPN0-300day-93 (99%) MF085152        | PAHs contaminated soil                                                                  |
| *Bryobacter*               | 1.81 0                   | Uncultured bacterium clone (100%) LC3362 49                    | Down-flow Hanging Sponge (DHS) reactor treating toluene gas as carbon source           |
| *Devosia*                  | 1.94 0                   | Uncultured bacterium clone (100%) LR6400 62                    | Wastewater treatment system                                                            |
| *Emticicia*                | 0.00 9.76                | Uncultured bacterium clone PiExp_89 (97%) KJ818846              | Impact of manufactured TiO nanoparticles on planktonic and sessile bacterial communities (Moselle river) |
| *Gemmatimonas*             | 1.60 0.25                | Uncultured *Aquabacterium* sp. clone bac21-T3-T2 (100%) KY606809 | Hot water biofilm after heat shock treatment                                             |
| *Roseomonas*               | 1.23 1.14                | *Roseomonas* sp. strain FW305-C-119 (100%) MK402959             | Groundwater                                                                            |
| *Phreatobacter*            | 1.12 0                   | Uncultured *Rhizobiales* bacterium clone 1d_92826 (100%) MG803495 | Sewage sludge of the completely autotrophic nitrogen removal over nitrite process with a submerged aerated biological filter and the effect of inorganic carbon on nitrogen removal and microbial activity. |
| *OLB12*                    | 0.75 0                   | Uncultured bacterium clone F5KQ4C046QQN (99%) GU911896         | Activated sludge                                                                      |
| *Muricoccus*               | 0 1.88                   | *Roseomonas* sp. strain FW305-C-119 (99%) MK402959             | Groundwater                                                                            |
| *Caulobacter*              | 0 1.82                   | Uncultured bacterium clone HK31-1-39-10 (100%) KX163332        | Basaltic subsurface ecosystems                                                         |
| Genus            | Relative abundances (%) | Closer relative (% identity ≥ 97) and GeneBank accession number | Environmental source |
|------------------|-------------------------|---------------------------------------------------------------|----------------------|
| Roseococcus      | 0.55 0.25               | Uncultured bacterium clone N3 (100%) HQ697534                 | Biologically activated carbon for drinking water treatment |
| IMCC26207        | 0.36 0.10               | Uncultured bacterium clone SZB6 (100%) A1M176889              | Mangrove sediment    |
| Neorhizobium     | 0 0.80                  | Rhizobium sp. strain A&R_E177 (97%) KX550303                  | Chimney Hills Pond, Tulsa |
| Aminobacter      | 0.18 0.00               | Uncultured bacterium (100%) LR654214                         | Wastewater treatment system |
| oc32             | 0.15 0.29               | Betaproteobacteria bacterium 5-B6 (98%) LC523959             | Root of aquatic plant |
| Bauldia          | 0.11 0                   | Uncultured bacterium clone MPB2-18 (99%) AB630694            | Microflorae of aquatic moss pillars in a freshwater lake, East Antarctica |
| Sphingomonas      | 0.09 0                  | Sphingomonas sp. strain SM1-b (100%) MT279454                 | Exopolysaccharide-producing bacteria from the Ghadikola lagoon water. |
| Pseudonocardia   | 0.07 0                  | Pseudonocardia sp. strain IB2014P10-1 (100%) MH978626        | Actinobacteria associated with deep-water endemic amphipods of Lake Baikal |
| Nocardioides     | 0.07 0                  | Nocardioides kribbensis strain P86 (100%) MT487642           | Spacecraft associated microbial organisms from the Mars odyssey and Pathfinder missions |
| Sphingopyxis     | 0.04 0.08               | Sphingopyxis sp. strain T93 9100% MT611302                    | Bacteria isolated from highland barley cultivation soil in Tibet |
| Lacibacter       | 0.06 0                  | Uncultured prokaryote clone OTU029 (99%) KF680692            | Drinking water biofilm |
| Leifsonia        | 0.05 0                  | Uncultured Microbacteriaceae bacterium clone UVmen1_31 (99%) JC701147 | Water from long-term experimental oligotrophic mesocosms in Cuatro Cienegas* |
| alphal_cluster   | 0.05 0                  | Uncultured bacterium clone Espejo_1_17_12_Water.240996 (98%) KM184952 | Water from Espejo lake, Argentina |
| Genus               | Relative abundances (%) | Closer relative (% identity ≥ 97) and GeneBank accession number | Environmental source                                      |
|--------------------|-------------------------|------------------------------------------------------------------|----------------------------------------------------------|
|                    | LB2897                  | MVCC19                                                           |                                                          |
| Paludibaculum      | 0.03 0.06               | Uncultured bacterium clone FL_03_167 (100%) KC666531            | Bacterial communities associated to Microcystis colonies  |
| Novospingobium     | 0 0.14                  | Uncultured bacterium clone LNH_9_9_11_Pumice.207741 (99%) KM124 853 | Water from Nahuel Huapi lake, Argentina                   |
| Stenotrophomonas   | 0 0.14                  | No match                                                         | -                                                        |
| Pseudoxanthobacter | 0.04 0                  | Uncultured bacterium clone EF_bacC09 (100%) JX564275             | Sediment from slow sand filtration columns (wastewater)  |

In the NoP (*R. raciborskii* LB2897), *SM1A02* was the most abundant genus (26.2% of the total), followed by 20.5% *Hirschia* (Hyphomonadaceae family, Alphaproteobacteria class), 13.3% *Labrys* (Xanthobacteraceae family, Alphaproteobacteria class) and 12.36% *Cutibacterium* (Propionibacteriaceae family, Actinobacteria class) (Fig. 2).

**Discussion**

The data, presented in this work, show that the composition of the bacterial community inhabiting the EPS of two strains of *R. raciborskii* is different, mainly due to the dominant genera. In the case of the NoP ecotype, the 16S rRNA sequences of the most abundant taxa share high identity with bacteria from wastewater treatments and sediment (Table 2). Wastewater bacteria are characterised by being very efficient in nutrient removal (Villaverde 2004). This seems to be the case of the *SM1A02* genus, dominant in NoP microbiome, but also abundant in the phycosphere of SxP. It has been proposed as a novel anammox (anaerobic ammonia oxidiser) bacterium (Tian et al. 2017) found in many activated sludges with good nitrifying performance (Chen et al. 2019). It has been also found in the phycosphere of the marine microalgae *Gambierdiscus* from different locations (Pacific Ocean, Atlantic Ocean, Caribbean Sea), suggesting that it may be broadly associated with this dinoflagellate (Rambo et al. 2019). The anaerobic oxidation of ammonium to nitrogen gas appears to be a metabolic pathway present in virtually any anoxic environment where fixed nitrogen (ammonium, nitrate, nitrite) is found. Therefore, in order to thrive in the EPS layer of *R. raciborskii*, the *SM1A02* anammox bacteria should be located in the anaerobic niches generated by degradation of the mucilage.

In the SxP ecotype, a genus affiliated to *Neorhizobium* (absent in NoP), was the most abundant taxon (Table 2). Bacteria from the Rhizobiaceae family are well known as symbiotic, nitrogen-fixing organisms that live in close association with plants. This nitrogen-fixing redundancy found in the toxic strain could reflect the fact that the saxitoxin molecule is nitrogen-rich, probably requiring high concentration of this nutrient to be produced under the nitrogen-deprived conditions of the BG11 medium. Due to the extreme oxygen...
sensitivity of nitrogenase, the environmental oxygen partial pressure regulates the nitrogen fixation activity. Thus, it is a process that requires anaerobic or microaerobic conditions. In the case of rhizobia, a finely-tuned symbiosis with a plant is the most common strategy, which leads to a root nodule that avoids the high oxygen concentrations generated during photosynthesis (Ledermann et al. 2021). However, as our data do not provide functional information, more work involving, for example, RNA sequencing should be performed in order to determine the spatial location of active *Neorhizobium* inside the EPS of a cyanobacterium, as well as the actual role of this highly abundant microorganism in *R. raciborskii* EPS.

Other abundant bacterial groups associated to the SxP strain have been found in environments contaminated with polycyclic aromatic hydrocarbons (Yang et al. 2018) or nanoparticles (Jomini et al. 2015). The presence of highly abundant bacteria (from 5 to 9%) with the ability to cope with complex compounds could be associated with the use of saxitoxin and analogues as carbon and nutrient source. In addition to organisms related to the nitrogen cycle, we also found taxa commonly found in water or sediment, cyanobacterial blooms and associated to eukaryotic microorganisms, these latter having a symbiotic lifestyle (Table 2).

Hence, the microbiome community, inhabiting the phycosphere of *R. raciborskii* grown under nitrogen-free conditions, showed a combination of functional groups involved in nitrogen cycling, degradation of complex organic compounds and the presence of symbiotic organisms. This functional coupling taking place in the phycosphere of *R. raciborskii* could be related to its small genome content (Stucken et al. 2010). This genome reduction decreases the replication-related energy costs and can be overcome by obtaining metabolic products from the community (Cook et al. 2019). In this way, shifts in
the microbiome may help the bacteria to cope with changing environmental conditions faster than by mutation and selection, implying that the whole community represents the unit of natural selection (Rosenberg et al. 2007).

Moreover, some of the taxa, accounting for the higher bacterial richness observed in the NoP microbiome, showed relative abundances ranging from 0.1 to 12.4% and were affiliated to bacteria described as symbionts of eukaryotic organisms (aquatic plants and mosses, ciliates, amphipods) (Table 2). The absence of these bacteria in the microbiome of the SxP ecotype probably reflects not only the differences in toxin production, but also the different environmental characteristics of the lakes from where they were isolated (Table 1) and suggests a tighter coupling between the non-toxic cyanobacterium and its heterotrophic partners. Since bacterial ecotypes are characterised by having different specific niches and responses to the environment (Cohan 2019), our results suggest that the mechanisms involved in the assembly of the microbiome community are ecotype-dependent. This implies that, to understand and predict the appearance and dominance of bacteria in different environmental conditions, it would be necessary to study them as a community of organisms.

Conclusions

We found that the dominant bacterial genus in SxP and NoP microbiomes is involved in nitrogen metabolism. Interestingly, while the SM1A02 genus reached its highest abundance in NoP, it also showed a high relative abundance in SxP. On the other hand,
the most abundant genus in SxP (*Neorhizobium*) was in extremely low abundance in NoP. *SM1A02* has been described as anaerobic ammonia oxidisers (anammox) that convert ammonium and nitrite to nitrogen gas and *Neorhizobium* (22.5%) is a nitrogen-fixing Alphaproteobacteria. As saxitoxin and analogues are nitrogen-rich molecules (7 atoms per molecule), toxic ecotypes would need more nitrogen supply than non-toxics to maintain fitness. We hypothesise that saxitoxin-producing *R. raciborskii* benefits from external sources of nitrogen provided by the anammox and nitrogen-fixers partners.

**Caveats and Limitations**

The cultures of both strains were performed without added nitrogen, which implies that nitrogen fixation is the main way of obtaining the needed reduced nitrogen to growth. Further studies involving different nitrogen concentrations in the culture medium should be performed in order to determine if the dominant members of the microbiome are still those related to nitrogen cycling.

**Usage Rights**

Sequence data from this study will be publicly available at NCBI.

**Acknowledgements**

This work was supported by PEDECIBA and CSIC-Universidad de la República and was carried out in partial fulfilment of the requirements of P.V. for the Doctoral degree from PEDECIBA. P.V. was the recipient of a fellowship from ANII (Agencia Nacional de Investigación e Innovación, Uruguay).

**Author contributions**

Paula Vico - laboratory work, bioinformatic analysis, writing.

Andrés Iriarte - bioinformatic analysis, supervision, writing, revision.

Sylvia Bonilla - laboratory work (supervision of strains growth), revision.

Claudia Piccini - laboratory work, supervision, writing, revision.

**Conflicts of interest**

The authors declare no conflicts of interest
References

- Aguilera A, Gómez EB, Kaštovský J, Echenique R, Salerno G (2019) The polyphasic analysis of two native *Raphidiopsis* isolates supports the unification of the genera *Raphidiopsis* and *Cylindrospermopsis* (Nostocales, Cyanobacteria). Phycologia 57 (2): 130-146. https://doi.org/10.2216/17-2.1
- Antunes J, Leão P, Vasconcelos V (2015) *Cylindrospermopsis raciborskii*: review of the distribution, phylogeography, and ecophysiology of a global invasive species. Frontiers in Microbiology 6 https://doi.org/10.3389/fmicb.2015.00473
- Bell W, Mitchel R (1972) Chemotactic and growth responses of marine bacteria to algal extracellular products. The Biological Bulletin 143 (2): 265-277. https://doi.org/10.2307/1540052
- Bonilla S, Aubriot L, Soares M, González-Piana M, Fabre A, Huszar VM, Lürling M, Antoniades D, Padisák J, Kruk C (2012) What drives the distribution of the bloom-forming bacteria *Planktothrix agardhii* and *Cylindrospermopsis raciborskii*? FEMS Microbiology Ecology 79 (3): 594-607. https://doi.org/10.1111/j.1574-6941.2011.01242.x
- Bonilla S, González-Piana M, Soares MS, Huszar VM, Becker V, Somma A, Marinho M, Kokociński M, Dokulil M, Antoniades D, Aubriot L (2016) The success of the cyanobacterium *Cylindrospermopsis raciborskii* in freshwaters is enhanced by the combined effects of light intensity and temperature. Journal of Limnology https://doi.org/10.4081/jlimnol.2016.1479
- Briand J, Leboulanger C, Humbert J, Bernard C, Dufour P (2004) *Cylindrospermopsis raciborskii* (bacteria) invasion at mid-latitudes: selection, wide physiological tolerance, or global warming? Journal of Phycology 40 (2): 231-238. https://doi.org/10.1111/j.1529-8817.2004.03118.x
- Burford M, Willis A, Chuang A, Man X, Orr P (2018) Recent insights into physiological responses to nutrients by the cylindrospermopsin producing cyanobacterium, *Cylindrospermopsis raciborskii*. Journal of Oceanology and Limnology 36 (4): 1032-1039. https://doi.org/10.1007/s00343-018-7179-5
- Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP (2016) DADA2: High-resolution sample inference from Illumina amplicon data. Nature Methods 13 (7): 581-583. https://doi.org/10.1038/nmeth.3869
- Chen Y, Wang C, Dong S, Jiang L, Shi Y, Li X, Zou W, Tan Z (2019) Microbial community assembly in detergent wastewater treatment bioreactors: Influential rather than inoculum source plays a more important role. Bioresource Technology 287 https://doi.org/10.1016/j.biortech.2019.121467
- Clark M, Jones W (2006,2011,2013) Lake Lemon annual monitoring report, prepared for the Lake Lemon Conservancy District, Unionville, IN. School of Public and Environmental Affairs, Indiana University, Bloomington, Indiana.
- Cohan F (2019) Transmission in the Origins of Bacterial Diversity, From Ecotypes to Phyla. Microbial Transmission 311-343. https://doi.org/10.1128/9781555819743.ch18
- Cook KV, Li C, Cai H, Krumholz LR, Hambright KD, Paepl HW, Steffen MM, Wilson AE, Burford MA, Grossart H, Hamilton DP, Jiang H, Sukenik A, Latour D, Meyer El, Padisák J, Qin B, Zamor RM, Zhu G (2019) The global Microcystis interactome. Limnology and Oceanography 65 (Suppl 1): S194-S207. https://doi.org/10.1002/lno.11361
• Dokulil M (2015) Vegetative survival of *Cylindrospermopsis raciborskii* (Cyanobacteria) at low temperature and low light. Hydrobiologia 764 (1): 241-247. https://doi.org/10.1007/s10750-015-2228-y

• Glöckner FO, Yilmaz P, Quast C, Gerken J, Beccati A, Ciuprina A, Bruns G, Yarza P, Peplies J, Westram R, Ludwig W (2017) 25 years of serving the community with ribosomal RNA gene reference databases and tools. Journal of Biotechnology 261: 169-176. https://doi.org/10.1016/j.jbiotec.2017.06.1198

• Gómez-Rubio V (2017) ggplot2 - Elegant graphics for data analysis (2nd Edition). Journal of Statistical Software 77 https://doi.org/10.18637/jss.v077.b02

• Herlemann DP, Labrenz M, Jürgens K, Bertilsson S, Waniek JJ, Andersson AF (2011) Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. The ISME Journal 5 (10): 1571-1579. https://doi.org/10.1038/ismej.2011.41

• Hillebrand H, Dürselen C, Kirschelt D, Pollingher U, Zohary T (1999) Biovolume calculation for pelagic and benthic microalgae. Journal of Phycology 35 (2): 403-424. https://doi.org/10.1046/j.1529-8817.1999.3520403.x

• Humbert J, Barbe V, Latifi A, Gugger M, Calteau A, Coursin T, Lajus A, Castelli V, Oztas S, Samson G, Longin C, Medigue C, de Marsac NT (2013) A tribute to disorder in the genome of the bloom-forming freshwater cyanobacterium *Microcystis aeruginosa*. PLOS One 8 (8). https://doi.org/10.1371/journal.pone.0070747

• Jankowiak J, Gobler C (2020) The composition and function of microbiomes within *Microcystis* colonies are significantly different than native bacterial assemblages in two North American lakes. Frontiers in Microbiology 11 https://doi.org/10.3389/fmicb.2020.01016

• Jomini S, Clivot H, Bauda P, Pagnout C (2015) Impact of manufactured TiO₂ nanoparticles on planktonic and sessile bacterial communities. Environmental Pollution 202: 196-204. https://doi.org/10.1016/j.envpol.2015.03.022

• Kandlikar G, Gold Z, Cowen M, Meyer R, Freise A, Kraft NB, Moberg-Parker J, Sprague J, Kushner D, Curd E (2018) ranacapa: An R package and shiny web app to explore environmental DNA data with exploratory statistics and interactive visualizations. F1000Research 7 https://doi.org/10.12688/f1000research.16680.1

• Ledermann R, Schulte CM, Poole P (2021) How *Rhizobia* adapt to the nodule environment. Journal of Bacteriology 203 (12). https://doi.org/10.1128/jb.00539-20

• Louati I, Pascault N, Pascualt M, Meyer R, Freise A, Kraft NB, Moberg-Parker J, Sprague J, Kushner D, Curd E (2018) ranacapa: An R package and shiny web app to explore environmental DNA data with exploratory statistics and interactive visualizations. F1000Research 7 https://doi.org/10.12688/f1000research.16680.1

• Ledermann R, Schulte CM, Poole P (2021) How *Rhizobia* adapt to the nodule environment. Journal of Bacteriology 203 (12). https://doi.org/10.1128/jb.00539-20

• Louati I, Pascault N, Pascualt M, Meyer R, Freise A, Kraft NB, Moberg-Parker J, Sprague J, Kushner D, Curd E (2018) ranacapa: An R package and shiny web app to explore environmental DNA data with exploratory statistics and interactive visualizations. F1000Research 7 https://doi.org/10.12688/f1000research.16680.1

• Louati I, Pascault N, Pascualt M, Meyer R, Freise A, Kraft NB, Moberg-Parker J, Sprague J, Kushner D, Curd E (2018) ranacapa: An R package and shiny web app to explore environmental DNA data with exploratory statistics and interactive visualizations. F1000Research 7 https://doi.org/10.12688/f1000research.16680.1

• Louati I, Pascault N, Pascualt M, Meyer R, Freise A, Kraft NB, Moberg-Parker J, Sprague J, Kushner D, Curd E (2018) ranacapa: An R package and shiny web app to explore environmental DNA data with exploratory statistics and interactive visualizations. F1000Research 7 https://doi.org/10.12688/f1000research.16680.1

• Louati I, Pascault N, Pascualt M, Meyer R, Freise A, Kraft NB, Moberg-Parker J, Sprague J, Kushner D, Curd E (2018) ranacapa: An R package and shiny web app to explore environmental DNA data with exploratory statistics and interactive visualizations. F1000Research 7 https://doi.org/10.12688/f1000research.16680.1

• Louati I, Pascault N, Pascualt M, Meyer R, Freise A, Kraft NB, Moberg-Parker J, Sprague J, Kushner D, Curd E (2018) ranacapa: An R package and shiny web app to explore environmental DNA data with exploratory statistics and interactive visualizations. F1000Research 7 https://doi.org/10.12688/f1000research.16680.1

• Louati I, Pascault N, Pascualt M, Meyer R, Freise A, Kraft NB, Moberg-Parker J, Sprague J, Kushner D, Curd E (2018) ranacapa: An R package and shiny web app to explore environmental DNA data with exploratory statistics and interactive visualizations. F1000Research 7 https://doi.org/10.12688/f1000research.16680.1

• Martínez de la Escalera G, Antoniades D, Bonilla S, Piccini C (2014) Application of ancient DNA to the reconstruction of past microbial assemblages and for the detection of toxic bacteria in subtropical freshwater ecosystems. Molecular Ecology 23 (23): 5791-802. https://doi.org/10.1111/mec.12979

• McMurdie P, Holmes S (2013) Phylseq: An R package for reproducible interactive analysis and graphics of microbiome census data. PLOS One 8 (4). https://doi.org/10.1371/journal.pone.0061217

• Moreira C, Fathalli A, Vasconcelos V, Antunes A (2014) Phylogeny and biogeography of the invasive cyanobacterium *Cylindrospermopsis raciborskii*. Archives of Microbiology 197 (1): 47-52. https://doi.org/10.1007/s00203-014-1052-5
- Morris JJ, Johnson Z, Szul M, Keller M, Zinser E (2011) Dependence of the cyanobacterium Prochlorococcus on hydrogen peroxide scavenging microbes for growth at the ocean’s surface. PLOS One 6 (2). https://doi.org/10.1371/journal.pone.0016805
- Mousavi SA, Österman J, Wahlberg N, Nesme X, Lavire C, Vial L, Paulin L, de Lajudie P, Lindström K (2014) Phylogeny of the Rhizobium–Allorhizobium–Agrobacterium clade supports the delineation of Neorhizobium gen. nov. Systematic and Applied Microbiology 37 (3): 208-215. https://doi.org/10.1016/j.syapm.2013.12.007
- Neilan BA, Saker ML, Fastner J, Törökné A, Burns BP (2002) Phylogeography of the invasive cyanobacterium Cylindrospermopsis raciborskii. Molecular Ecology 12 (1): 133-140. https://doi.org/10.1046/j.1365-294x.2003.01709.x
- Pande S, Kost C (2017) Bacterial unculturability and the formation of intercellular metabolic networks. Trends in Microbiology 25 (5): 349-361. https://doi.org/10.1016/j.tim.2017.02.015
- Piccini C, Aubriot L, Fabre A, Amaral V, González-Piana M, Giani A, Figueredo C, Vidal L, Kruk C, Bonilla S (2011) Genetic and eco-physiological differences of South American Cylindrospermopsis raciborskii isolates support the hypothesis of multiple ecotypes. Harmful Algae 10 (6): 644-653. https://doi.org/10.1016/j.hal.2011.04.016
- Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO (2012) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Research 41 https://doi.org/10.1093/nar/gks1219
- Rambo I, Dombrowski N, Constant L, Erdner D, Baker B (2019) Metabolic relationships of uncultured bacteria associated with the microalgae Gambierdiscus. Environmental Microbiology 22 (5): 1764-1783. https://doi.org/10.1111/1462-2920.14878
- Rosenberg E, Koren O, Reshef L, Efrony R, Zilber-Rosenberg I (2007) The role of microorganisms in coral health, disease and evolution. Nature Reviews Microbiology 5 (5): 355-362. https://doi.org/10.1038/nrmicro1635
- Rossi F, De Philippis R (2015) Role of cyanobacterial exopolysaccharides in phototrophic biofilms and in complex microbial mats. Life 5 (2): 1218-1238. https://doi.org/10.3390/life5021218
- Schliep KP (2010) Phangorn: phylogenetic analysis in R. Bioinformatics 27 (4): 592-593. https://doi.org/10.1093/bioinformatics/btq706
- Seymour J, Amin S, Raina J, Stocker R (2017) Zooming in on the phycosphere: the ecological interface for phytoplankton–bacteria relationships. Nature Microbiology 2 (7). https://doi.org/10.1038/nmicrobiol.2017.65
- Soares M, Lürling M, Huszar VM (2012) Growth and temperature-related phenotypic plasticity in the cyanobacterium Cylindrospermopsis raciborskii. Phycological Research 61 (1): 61-67. https://doi.org/10.1111/pre.12001
- Stanier RY, Kunisawa R, Mandel M, Cohen-Bazire G (1971) Purification and properties of unicellular blue-green algae (order Chroococcales). Bacteriological Reviews 35 (2): 171-205. https://doi.org/10.1128/br.35.2.171-205.1971
- Stucken K, John U, Cembella A, Murillo AA, Soto-Liebe K, Fuentes-Valdés JJ, Friedel M, Plominsky AM, Vásquez M, Glöckner G (2010) The smallest known genomes of multicellular and toxic bacteria: comparison, minimal gene sets for linked traits and the evolutionary implications. PIOS One 5 (2): e9235. https://doi.org/10.1371/journal.pone.0009235
• Tian S, Tian Z, Yang H, Yang M, Zhang Y (2017) Detection of viable bacteria during sludge ozonation by the combination of ATP assay with PMA-Miseq sequencing. Water 9 (3). https://doi.org/10.3390/w9030166

• Vico P, Bonilla S, Cremella B, Aubriot L, Iriarte A, Piccini C (2020) Biogeography of the cyanobacterium Raphidiopsis (Cylindropermopsis) raciborskii: Integrating genomics, phylogenetic and toxicity data. Molecular Phylogenetics and Evolution 148 https://doi.org/10.1016/j.ympev.2020.106824

• Vidal L, Kruk C (2008) *Cylindrospermopsis raciborskii* (Cyanobacteria) extends its distribution to Latitude 34°53'S: taxonomical and ecological features in Uruguayan eutrophic lakes. Pan-American Journal of Aquatic Sciences.

• Villaverde S (2004) Recent developments on biological nutrient removal processes for wastewater treatment. Reviews in Environmental Science and Bio/Technology 3 (2): 171-183. https://doi.org/10.1007/s11157-004-4565-6

• Willis A, Woodhouse J, Ongley S, Jex A, Burford M, Neilan B (2018) Genome variation in nine co-occurring toxic *Cylindrospermopsis raciborskii* strains. Harmful Algae 73: 157-166. https://doi.org/10.1016/j.hal.2018.03.001

• Woodhouse J, Ziegler J, Grossart H, Neilan B (2018) Cyanobacterial community composition and bacteria–bacteria interactions promote the stable occurrence of particle-associated bacteria. Frontiers in Microbiology 9 https://doi.org/10.3389/fmicb.2018.00777

• Wood S, Pochon X, Luttringer-Plu L, Vant B, Hamilton D (2014) Recent invader or indicator of environmental change? A phylogenetic and ecological study of *Cylindrospermopsis raciborskii* in New Zealand. Harmful Algae 39: 64-74. https://doi.org/10.1016/j.hal.2014.06.013

• Wright E (2015) Decipher: harnessing local sequence context to improve protein multiple sequence alignment. BMC Bioinformatics 16 (1). https://doi.org/10.1186/s12859-015-0749-z

• Wu Q, Zhang Y, Li Y, Li J, Zhang X, Li P (2019) Comparison of community composition between *Microcystis* colony-attached and free-living bacteria, and among bacteria attached with *Microcystis* colonies of various sizes in culture. Aquatic Ecology 53 (3): 465-481. https://doi.org/10.1007/s10452-019-09702-7

• Yang S, Gou Y, Song Y, Li P (2018) Enhanced anoxic biodegradation of polycyclic aromatic hydrocarbons (PAHs) in a highly contaminated aged soil using nitrate and soil microbes. Environmental Earth Sciences 77 (12). https://doi.org/10.1007/s12665-018-7629-6

• Yilmaz M, Philips E (2011) Diversity of and selection acting on cylindrospermopsin cyrB gene adenylation domain sequences in Florida. Applied and Environmental Microbiology 77 (7): 2502-2507. https://doi.org/10.1128/aem.02252-10
Supplementary materials

**Suppl. material 1: Sequence ASV table [doi]**

**Authors:** Paula Vico, Andrés Iriarte, Claudia Piccini  
**Data type:** Phylogenetic  
**Brief description:** The representative sequence of each identified ASV and their taxonomic identity according to SILVA database are shown.  
[Download file](48.00 kb)

**Suppl. material 2: Rarefaction curves [doi]**

**Authors:** Paula Vico, Andrés Iriarte, Sylvia Bonilla, Claudia Piccini  
**Data type:** image  
**Brief description:** Rarefaction curves obtained from the 16S rRNA gene sequencing for each *R. raciborskii* strain  
[Download file](604.37 kb)