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DE SUELOS RECIENTEMENTE DEGLACIADOS A TAPETES MICROBIANOS DE CIANOBACTERIAS: SUCESIÓN DE COMUNIDADES MICROBIANAS ANTÁRTICAS

FROM RECENTLY DEGLACIATED SOILS TO CYANOBACTERIAL-BASED MATS: SUCCESSION OF ANTARCTIC MICROBIAL COMMUNITIES

Tesis Doctoral / Doctoral Dissertation

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RESUMEN

Las cianobacterias contribuyen significativamente a la biomasa total del continente antártico. Son consideradas como los principales fotótrofos de la mayoría de los ecosistemas polares bentónicos de agua dulce, y como las arquitectas de los tapetes microbianos polares. Estos tapetes microbianos dominan muchos ecosistemas polares terrestres no marinos en la Antártida, y se espera que su presencia siga aumentando conforme aumente la disponibilidad de agua en el ecosistema terrestre como consecuencia del derretimiento de hielo y el aumento de las precipitaciones. Sin embargo, hasta la fecha desconocemos si existe un patrón en la composición de las comunidades de cianobacterias del suelo recientemente deglaciados que pueda relacionarse con factores abióticos o temporales, o si existen interacciones con el resto de la microbiota de los suelos. Tampoco sabemos cómo de similares son las comunidades de cianobacterias presentes en los suelos con aquellas que conforman los tapetes microbianos. Por otro lado, se desconocen cómo son las relaciones tróficas de su comunidad, o cuáles son los factores que las condicionan.

El objetivo general de la presente tesis doctoral es estudiar y entender con mayor detalle el microecosistema de los tapetes microbianos de cianobacterias, desde las primeras etapas de sucesión de las cianobacterias en los suelos recientemente deglaciados, hasta los cambios en las comunidades que conforman los tapetes microbianos frente a distintas condiciones ambientales.

ABSTRACT

Cyanobacteria contribute significantly to the total biomass of the Antarctic continent. They are considered as the main phototrophs of most polar benthic freshwater ecosystems, and as the architects of polar microbial mats. These microbial mats

dominate many non-marine terrestrial polar ecosystems in Antarctica, and its presence is expected to increase as the availability of water in the terrestrial ecosystem increases as a result of melting ice and precipitation. However, to date we do not know if there is a pattern in the composition of the cyanobacterial communities in glacier retreat areas that can be related to abiotic or temporal factors, or if there are interactions with the soil microbiota. We also do not know how similar the cyanobacterial communities from soils are to those that constitute the microbial mats, neither how the trophic relationships of mats community take place, or which are the factors that condition these interactions.

The scope of this doctoral thesis is to explore the microecosystem of cyanobacterial microbial mats, from the presence of pioneer cyanobacteria in recently deglaciated soil, to the trophic interactions of the communities that constitute the microbial mats, also considering the dynamics in their populations due to different environmental conditions.

A mis padres, por todo

A Bea

“— ¿Cuál es la razón por la que deseabais estar en una expedición que asumía grandes riesgos y afrontaba graves peligros?

—Majestad, el deseo de saber y el espíritu de aventura. Una expedición como aquella podía acabar en desastre, pero también descubrir cosas extraordinarias. Participar en esa expedición me ha permitido escribir un libro donde cuento las cosas, dignas de ser reflejadas, que han ocurrido durante los tres años de esa expedición. Para mi sería un honor que la conocierais. Algunos datos son de mucho interés para su majestad.”

José Calvo Poyato, *La travesía final*.

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INTRODUCCION GENERAL

A. ANTÁRTIDA

A.1 El continente antártico

La Antártida es el continente más austral de la Tierra. Para los propósitos del Tratado Antártico, la Antártida queda definida como 'la región situada al sur de los 60° de latitud Sur, incluidas todas las barreras de hielo', mientras que para el Comité Científico de Investigaciones Antárticas (SCAR), la región antártica quedaría conformada por el continente antártico, sus islas costeras y el Océano Austral circundante. A excepción del extremo norte de la Península Antártica, la región antártica se encuentra por debajo del círculo polar antártico. El continente antártico cubre un área de aproximadamente 14 millones de km^2, convirtiéndose así en el cuarto continente más grande después de Asia, América y África. Del total de su superficie, solo alrededor del 0.18 % (21,745 km^2) queda libre de hielo en algún momento del año (Burton-Johnson *et al.,* 2016). El 99.82 % restante queda oculto por la cubierta de hielo o *indlandsis* antártico. Esta capa de hielo se constituyó primeramente como un campo de hielo a inicios del Oligoceno (33.9-23.3 Ma) con períodos de avance y retroceso. No fue hasta el Plioceno (5.3-2.6 Ma) cuando la Antártida se ubicó, por la deriva continental en el área polar meridional, y cuando los hielos cubrieron la totalidad de la superficie. Esta masa de hielo, con un espesor medio de 1829 m, y máximo de 4776 m, alberga el 91 % del volumen total de hielo del Planeta (Fox y Cooper 1994). El resto del hielo se concentra en el Ártico (7.5 % aprox.) y en los glaciares de montaña. En muchos puntos de la costa antártica, el manto de hielo se continúa con grandes masas de hielo parcialmente flotante llamadas barreras o plataformas de hielo. Estas plataformas de hielo flotantes suponen alrededor del 11 % de la cubierta de hielo antártico, y presentan un comportamiento muy dinámico,

variando su extensión a lo largo del año desde los 20 x 10^6 km^2 durante gran parte del año, a los 3 x 10^6 km^2 en otoño (Summerhayes *et al.,* 2009).

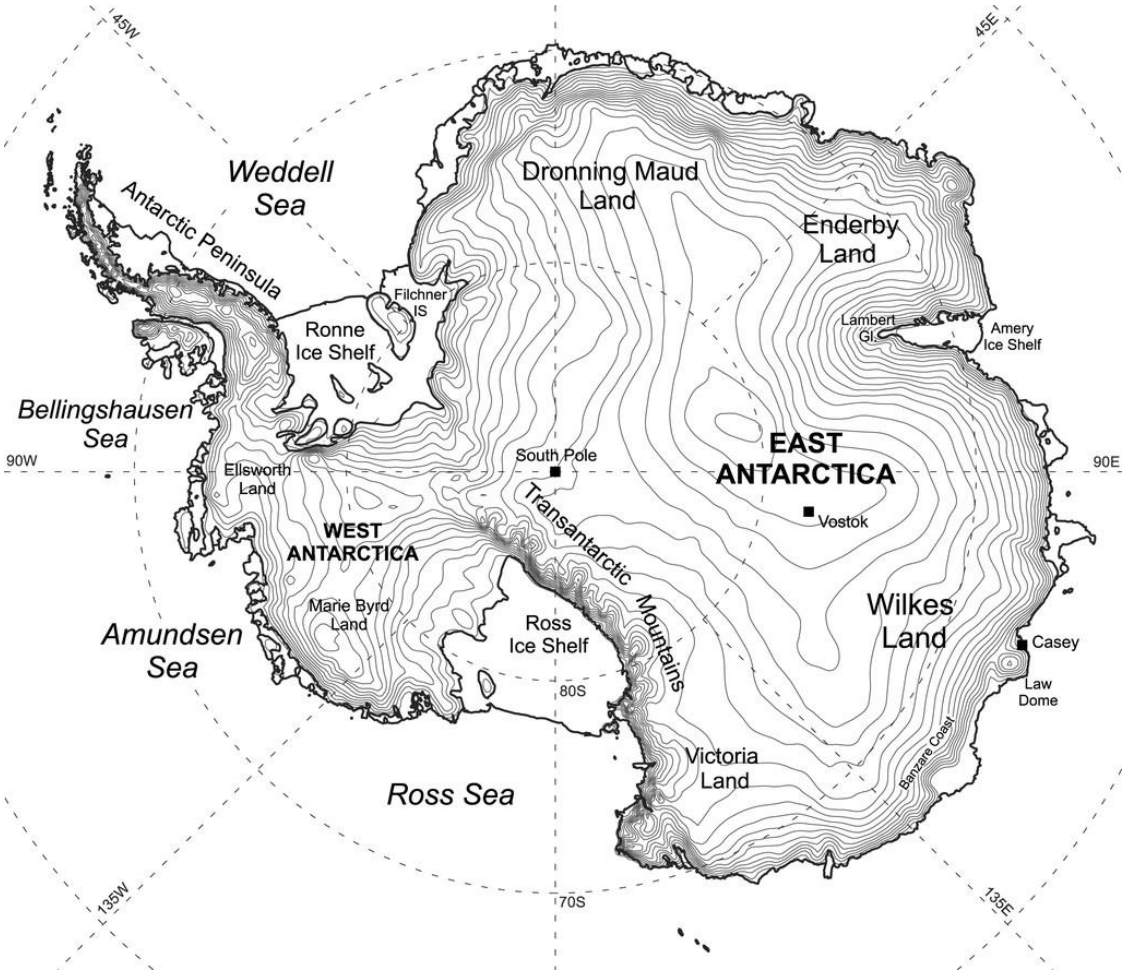


Figura 1. Mapa de la Antártida que muestra las tres zonas morfológicas en que se divide el continente (la Antártida oriental, la Antártida occidental y la Península Antártica), las principales plataformas de hielo flotantes (plataforma de hielo Filchner-Ronne y de Ross), así como los mares en que se divide el océano austral (mar de Ross, mar Amundsen, mar de Bellingshausen y el mar de Weddell). Los contornos de elevación representan intervalos de 200 m (topografía de Liu *et al.* 2001).

La cubierta de hielo de la Antártida se divide en tres zonas morfológicas: la Antártida oriental, la Antártida occidental y la Península Antártica (**Figura 1**). El este y el oeste de la Antártida están separados por las Montañas Transantárticas, una cadena de montañas que se extiende 3500 km desde Victoria Land hasta la plataforma de hielo

Ronne-Filchner. La mayor parte de la cubierta de hielo de la Antártida está compuesta por la capa oriental, que contiene el hielo más frío y que estaría en su mayoría congelado hasta el lecho. La capa de hielo de la Antártida occidental, por otro lado, es mucho más cálida tanto en la superficie como en el lecho. Tanto las capas de hielo de la Antártida oriental como occidental pierden masa principalmente a través del desprendimiento de icebergs y el derretimiento de las plataformas de hielo. La Península Antártica es una región montañosa estrecha que se proyecta hacia el norte desde la capa de hielo principal durante aproximadamente 1250 km (hasta 63°S). La capa de hielo de la Península Antártica difiere sustancialmente de la Antártida oriental y occidental, y consiste en capas de hielo más pequeñas y delgadas que están sujetas a un derretimiento significativo del hielo superficial (Summerhayes *et al.* 2009).

El clima antártico se caracteriza por tres aspectos claramente definidos: bajas temperaturas permanentes, escasa precipitación y fuertes e incesantes vientos. Aquí se ha registrado la temperatura del aire más baja: -89.2 °C, medida en la base de investigación Vostok el 21 de julio de 1983. Pero datos más recientes de satélite recopilados durante el periodo de 2004–2016 revelan una amplia región de la meseta antártica oriental que alcanza regularmente temperaturas de la superficie de la nieve por debajo de -90 °C (Scambos *et al.,* 2018). En la Antártida también se han registrado las rachas de viento más intensas de la superficie terrestre, llegándose a superar los 327 km/h, en julio de 1972, en la base de investigación Dumont d'Urville. La precipitación media anual se sitúa en torno a los 155-166 mm (van de Berg *et al.* 2006; Krinner *et al.* 2007). Sin embargo, hay que considerar que estos parámetros climáticos varían ampliamente entre la zona continental y la marítima. Así, la Península Antártica se diferencia de las otras dos regiones morfológicas del continente, mostrando una

precipitación media anual que varía entre 200 a 1000 mm (Balks *et al.,* 2013), una humedad media superior al 80 %, y unas temperaturas que durante el verano austral pueden oscilar entre los 0 y 5 °C. Es por ello que las costas de la Península Antártica occidental e islas vecinas concentran la mayor biodiversidad del continente, siendo también la región más sensible a los efectos del cambio climático reciente, ya que el riesgo de cambios irreversibles, y en cascada, debido al incremento de unas temperaturas que se sitúan cercanas al punto de congelación, es mayor.

A.2 Cambio climático reciente en la Península Antártica

Allí donde hay hielo los efectos del calentamiento se ven amplificados, ya que estas regiones contribuyen a la reflexión de grandes cantidades de la energía solar recibida. De hecho, así está ocurriendo en el contexto actual de calentamiento global en todas las regiones geográficas de la Antártida. La capa de hielo de la Antártida occidental ha sufrido una intensa pérdida de masa desde al menos la década de 1990 (Paolo *et al.* 2015), impulsada principalmente por la aceleración de los glaciares de salida asociados con el adelgazamiento de las plataformas de hielo flotantes (Pritchard *et al.* 2012; Dutrieux *et al.* 2014). Se estima que en torno a 2720 ± 1390 mil millones de toneladas de hielo se perdieron entre 1992 y 2017, lo que corresponde a un aumento en el nivel medio del mar de 7.6 ± 3.9 mm (Shepherd *et al.* 2018). El aumento de las temperaturas en la Península Antártica durante la segunda mitad del siglo XX ha sido mayor que el de cualquier otro medio terrestre del hemisferio sur, similar en magnitud al Ártico y sustancialmente más rápido que la media global (Robinson *et al.,* 2020). Este incremento de las temperaturas se estima en ~2 °C (Vaughan *at al.,* 2001). Durante este período, la

tasa de pérdida de hielo de la Península Antártica ha aumentado de 7 ± 13 mil millones a 33 ± 16 mil millones de toneladas por año (Shepherd *et al.* 2018), debido en su mayoría al colapso de la plataforma de hielo.

Los efectos que el cambio climático actual tiene sobre el ecosistema Antártico son numerosos. Pero hay dos consecuencias de especial interés para las investigaciones llevadas a cabo en la presente tesis doctoral, como son el retroceso glaciar y el movimiento de poblaciones de macrofauna local.

A.2.1 Glaciares en retroceso y sucesión primaria

Los valores actuales de superficie total ocupada por glaciares en la Península Antártica son menores que en periodos anteriores. Para el periodo comprendido entre 2001 y 2015, el 60 % de los 1855 glaciares delimitados entre las latitudes 61° y 73°S retrocedió, mientras que el 9 % avanzó ligeramente. Esto ha supuesto una pérdida de 1340 km^2 de hielo (Silva *et al.,* 2020), y un aumento de las áreas libres de hielo, que actualmente ocupan el 1 % de esta región. La combinación de factores naturales y antropogénicos es considerada la causa principal de la pérdida global de masa de hielo (Marzeion *et al.* 2014). Sin embargo, estos cambios observados en los últimos años en zonas glaciares de todo el planeta son en realidad consecuencia de cambios climáticos ocurridos hace décadas (Field *et al.* 2014; Zemp *et al.* 2015), lo que sugiere procesos de retroceso glaciar mucho más acusados en el futuro cercano.

A medida que los glaciares retroceden, quedan expuestos nuevos hábitats que han permanecido cubiertos de hielo por miles de años. Estos sustratos de tipología heterogénea (p. ej. depósitos de arenas, materiales rocosos expuestos, superficies de

erosión, llanuras aluviales o incluso lodos, etcétera) (Bradley *et al.* 2014; Ciccazzo *et al.* 2015), son susceptibles de ser colonizados por distintas formas de vida, iniciándose entonces un proceso de sucesión primaria (Grubb 1986; Schulz *et al.* 2013; Rydgren *et al.* 2014). Tras una colonización inicial, llevada a cabo generalmente por microorganismos que pueden estar presentes previamente bajo la capa de hielo o han podido llegar a la zona arrastrados por el agua de deshielo o transportados por el aire (Fierer *et al.* 2010; Frey *et al.* 2013), o incluso a través de la lluvia o la niebla (Evans *et al.,* 2019), se inicia la sucesión, la cual conlleva variaciones en la estructura y composición de las comunidades (Bradley *et al.* 2014; Ciccazzo *et al.* 2016; Rime *et al.* 2015).

Desde el punto más próximo del límite glaciar hasta el más alejado al que llegó el glaciar en épocas pretéritas, se establece un eje espaciotemporal, donde los suelos más distantes representan los hábitats que llevan más tiempo expuestos, y por tanto albergan las comunidades más antiguas (Matthews, 1992). Por ello, estos sistemas naturales únicos son ideales para estudiar cómo cambian las poblaciones, las comunidades se ensamblan y los ecosistemas se desarrollan a lo largo del espacio y del tiempo en respuesta al calentamiento global (Caccianiga *et al.,* 2006; Pessi *et al.,* 2019; Losapio *et al.,* 2021).

A.2.2 Cambios en la distribución de la fauna y flora nativa

El aumento de las temperaturas en la región antártica tiene consecuencias significativas en la fauna nativa. Para la megafauna asociada al ecosistema marino, se han observado en los últimos años cambios en la distribución de colonias y concentración de individuos

a lo largo de la Península Antártica. Estudios realizados en la última década muestran como el pingüino Papua (*Pygoscelis papua*) está aumentando en abundancia y expandiéndose hacia el sur, mientras que los pingüinos Adelia (*Pygoscelis adeliae*) y barbijo (*Pygoscelis antárctica*) están disminuyendo en casi todos los lugares de la Península Antártica donde se tienen registros (Lynch *et al.,* 2012; Clucas *et al.,* 2014). Un escenario más desafortunado se plantea para el pingüino emperador (*Aptenodytes forsteri*), estrechamente vinculado al hielo marino. Si se cumplen las condiciones más pesimistas, y a su vez improbables (Hausfather y Peters, 2020), planteadas en el Quinto Informe de Evaluación del IPCC (2014), sus poblaciones podrían reducirse en un 80% en 2100 (Jenouvrier *et al.,* 2020).

Las consecuencias para los grupos de mamíferos pinnípedos (Pinnipedia) que habitan en la Antártida serían distintas dependiendo del nicho ecológico que ocupa cada especie. Considerando los cambios registrados en las comunidades estudiadas a lo largo de las últimas décadas, las poblaciones de foca cangrejera (*Lobodon carcinophagus*) y de Weddell (*Leptonychotes weddellii*) se verían especialmente afectadas a largo plazo por los cambios anuales en la extensión, la persistencia y el tipo de hielo marino. Por el contrario, las poblaciones de foca de Ross (*Ommatophoca rossii*) y foca leopardo (*Hydrurga leptonyx*) serían menos vulnerables a los cambios en las características del hielo marino, aunque podrían resultar afectadas por la alteración antropogénica de las redes tróficas (Siniff *et al.,* 2008). Los cambios en las poblaciones de elefantes marinos (*Mirounga leonina*) todavía son desconocidos a gran escala, aunque estudios realizados en áreas locales del continente sugieren que esta especie podría verse favorecida como consecuencia de la reducción del hielo marino (van den Hoff *et al.,* 2014; Clausius *et al.,* 2017). Estas mismas tendencias se han sugerido para el lobo marino (*Arctophoca*

gazella), aunque de nuevo, los cambios en los recursos alimenticios debidos a factores distintos al clima podrían perjudicarla significativamente (Siniff *et al.,* 2008).

La fauna estrictamente terrestre consiste solo en dos especies nativas de insectos, *Parochlus steinenii* y *Belgica antarctica*, ambas pertenecientes a la familia de los quironómidos (Chironomidae). Actualmente estas especies se distribuyen en su mayoría en las islas Shetland del Sur. Sin embargo, si las temperaturas continúan aumentado, estas poblaciones tendrían el potencial de expandirse hacia áreas de las costas oeste y este de la Península Antártica, e incluso llegar a la Antártida continental (Contador *et al.,* 2020). Por lo que respecta a la meiofauna, y considerando estudios experimentales y observaciones a largo plazo, se han sugerido respuestas divergentes para los distintos grupos estudiados. Por ejemplo, la especie dominante de nematodo *Scottnema lindsayae* muestra una disminución, mientras que la distribución y abundancia de taxones menos abundantes de nematodos, rotíferos y tardígrados aumenta (Convey y Wynn-Williams, 2002; Andriuzzi *et al.,* 2018). También se ha sugerido una reducción en las poblaciones de microartrópodos (Bokhorst *et al.,* 2008) en respuesta a estas alteraciones del ecosistema antártico.

Las diferentes comunidades vegetales presentes en la Península Antártica también han mostrado distintas respuestas al cambio climático reciente. Las comunidades de las dos especies de plantas vasculares presentes, *Colobanthus quitensis* y *Deschampsia antárctica*, han respondido al incremento de las temperaturas en la Península Antártica durante las últimas cuatro décadas, con aumentos en el número y tamaño de sus poblaciones, así como en su capacidad de reproducirse sexualmente (Fowbert y Smith, 1994). En contraste con las plantas vasculares, el aumento de las temperaturas se ha

relacionado con una disminución en el crecimiento de los musgos a lo largo de la Península Antártica (Day *et al.,* 2009). Por lo que respecta a los líquenes, los datos publicados muestran diferentes sensibilidades al calentamiento. Se ha reportado un aumento en poblaciones de *Usnea antarctica* tras un período de 11 años con temperaturas estivales menos frías (+0.42 °C en comparación con las temperaturas anuales de verano durante los 34 años anteriores) (Sancho *et al.,* 2017), frente a una reducción de hasta el 71 % de cobertura en parcelas experimentales sometidas a un incremento de la temperatura durante un periodo de 10 años (Bokhorst *et al.,* 2016).

Las respuestas de las distintas especies nativas de fauna y flora de la Antártida ante los cambios ambientales actuales son diversas. Esto es debido, en parte, a la distinta capacidad de adaptación de dichas especies, pero también a que el cambio climático es un proceso complejo (Helmuth *et al.,* 2005) que involucra cambios en múltiples condiciones ambientales.

B. LAS CIANOBACTERIAS

B.1 Cianobacterias en el ecosistema

Las cianobacterias son el grupo más grande, diverso y ampliamente distribuido de procariotas fotosintéticos, además de ser uno de los grupos procariotas más antiguos presentes en el planeta. En términos generales, pueden definirse como organismos que albergan, dentro de una célula procariota de carácter bacteriano, un aparato fotosintético muy similar en aspectos funcionales, estructurales y moleculares al cloroplasto presente en las células eucariotas.

Estas bacterias han tenido y tienen una enorme relevancia en la evolución de nuestro planeta y la vida que tiene lugar en él. Por el registro fósil, sabemos que las cianobacterias han estado presentes al menos desde el Proterozoico, y probablemente ya existieron en los períodos fríos anteriores (Schopf, 2000). Su papel clave en la acumulación de oxígeno en la atmósfera hace 2500 millones de años es incuestionable, como resultado de la fotosíntesis oxigénica. Este proceso de 'inyección de oxígeno', provocó un cambio trascendental y sin precedentes en las condiciones ecológicas para la vida en la Tierra, promoviendo una adaptación hacia condiciones aerobias mediante el desarrollo de un metabolismo aerobio, de mayor eficiencia metabólica. Esto permitió, entre otras cosas, el desarrollo de la célula eucariótica y posteriormente los organismos pluricelulares, considerándose además el ancestro evolutivo de los plástidos fotosintetizadores presentes en algas y plantas superiores. Por tanto, las cianobacterias habrían sido responsables de dos grandes cambios geológicos y evolutivos, como son el Gran Evento de Oxigenación y la Endosimbiosis. Además, algunas presentan la capacidad de fijar nitrógeno atmosférico (N_2), contribuyendo significativamente a la cantidad total de nitrógeno fijada y disponible para la red trófica de ciertos ecosistemas.

Las cianobacterias son un grupo de organismos ubicuo (Pandey *et al.,* 2004), es decir, con distribución mundial y presentes en toda clase de hábitats. Estas bacterias pueden encontrarse fácilmente en la atmósfera, ecosistemas del mediterráneo, las zonas polares, el desierto de Atacama, el Sáhara, y a varios metros de profundidad en la tierra (Vincent, 2000; Wierzchos *et al.,* 2018; Puente-Sánchez *et al.,* 2018; Almela *et al.,* 2019a; Galbán *et al.,* 2021; Mehda *et al.,* 2021). Gracias a su gran diversidad morfológica, estructural y fisiológica, son capaces de adaptarse a variaciones lumínicas, de temperatura, disponibilidad de nutrientes, humedad y radiación, entre otros factores.

La enorme adaptabilidad de estos microorganismos les dota de unas características y una versatilidad decisivas para soportar los factores de estrés ambiental, adquiriendo especial relevancia en aquellos ambientes considerados extremos. Las cianobacterias ejercen un papel clave como reguladores centrales del ciclo del carbono y del nitrógeno en entornos empobrecidos. Las contribuciones de este filo bacteriano clave al ciclo biogeoquímico, y en particular al carbono (C) y el nitrógeno (N), se ejemplifican en diversos ecosistemas desérticos fríos, incluidos los suelos de la meseta tibetana (Wong *et al.* 2010) y el permafrost ártico (Hultman *et al.* 2015), donde las comunidades microbianas del suelo median los procesos funcionales centrales relacionados con la renovación de nutrientes del suelo (Barrow 1992; Delgado-Baquerizo *et al.* 2018).

B.2 Cianobacterias en las regiones polares

En la Antártida, la ausencia de plantas vasculares otorga más importancia a las cianobacterias en la configuración de la ecología del continente a escalas locales (Cary *et al.* 2010), y en las funciones propias mediadas por fotoautótrofos del suelo. Las cianobacterias albergan numerosas adaptaciones fisiológicas para hacer frente al estrés climático y son capaces de colonizar y crecer en amplios rangos ambientales en el espacio y el tiempo geográficos (Van Goethem y Cowan, 2019).

Considerando las temperaturas optimas de crecimiento, la mayoría de las cianobacterias aisladas de ecosistemas polares han sido clasificadas como psicrotolerantes, es decir, pueden crecer a temperaturas cercanas a 0 °C, pero sus óptimos metabólicos se sitúan por encima de los 15 °C (Tang *et al.,* 1997; Roos and Vincent, 1998; Singh and Elster, 2007; Vincent, 2007; Zakhia *et al.,* 2008). Solo unas pocas cianobacterias antárticas son

consideradas como verdaderamente psicrófilas, mostrando temperaturas de crecimiento optimas por debajo de 15 °C (Fritsen and Priscu, 1998; Nadeau and Castenholz, 2000; Vincent and Quesada, 2012; Chrismas *et al.,* 2015). Por ello, la posición de las cianobacterias como piezas clave de los hábitats bénticos polares no marinos reside en su capacidad de resistir las condiciones extremas de estas regiones. Esta flexibilidad y resistencia, que les permite dominar los ecosistemas someros de las zonas polares, se conoce como la 'estrategia del liquen' (Quesada y Vincent, 2012). Cuando encuentran las condiciones adecuadas para crecer, estos organismos procarióticos fundamentalmente filamentosos producen un mucílago orgánico (exopolisacáridos) que da lugar a estructuras cohesivas que ofrecen una base muy propicia para la creación de microhábitats, en los que pueden establecerse otros microorganismos con diferentes características ecológicas, constituyendo los tapetes microbianos.

B.3 Taxonomía de las cianobacterias

Las cianobacterias han sido históricamente estudiadas por botánicos debido a sus similitudes ecológicas e incluso morfológicas con las algas eucariotas. Como resultado, las cianobacterias se clasificaron inicialmente de acuerdo con el enfoque botánico, que se basa en las características morfológicas de muestras naturales y sigue las reglas del Código Internacional de Nomenclatura para algas, hongos y plantas (ICN; anteriormente conocido como el Código Internacional de Nomenclatura Botánica) (p. ej. Geitler 1932; Drouet 1981; Anagnostidis y Komárek 1985, 1988, 1990; Komárek y Anagnostidis 1986, 1989). En la década de 1970, surgió un nuevo paradigma de taxonomía de

cianobacterias, impulsado por el creciente reconocimiento de la naturaleza procariótica de las cianobacterias (Stanier *et al.* 1978). El enfoque bacteriológico, que sigue las reglas del Código Internacional de Nomenclatura de Procariotas (ICNP), también se basa principalmente en características morfológicas, pero difiere del enfoque botánico por la observación de cepas en cultivos axénicos en lugar de muestras naturales, y que también incorpora datos fisiológicos (p. ej. Rippka *et al.* 1979; Castenholz 2001).

Los análisis moleculares han proporcionado una contribución vital a nuestra comprensión actual de la taxonomía y la evolución de las cianobacterias. Esto puede incluir marcadores quimiotaxonómicos como la composición de lípidos, pigmentos y metabolitos secundarios, pero se basa principalmente en el análisis de secuencia del gen del ARNr 16S (Wilmotte, 1994). Con la integración de datos moleculares, quedó claro que ni la taxonomía tradicional botánica ni bacteriológica reflejaban la historia evolutiva de las cianobacterias. Por ello, el uso combinado de marcadores morfológicos, ecológicos, fisiológicos y moleculares (enfoque polifásico) es ahora el método de elección en la taxonomía de cianobacterias. La primera revisión taxonómica de cianobacterias a nivel supragenérico basada en el enfoque polifásico fue realizada por Hoffmann *et al.* (2005). Este sistema se diferencia de las clasificaciones tradicionales anteriores en que reconoce la naturaleza polifilética de las cianobacterias unicelulares. Se demostró que las características ultraestructurales, más específicamente la presencia y disposición de tilacoides, además de la capacidad de formar heterocistes, representan importantes marcadores filogenéticos. En base a estos criterios, se propusieron cuatro linajes principales de cianobacterias (subclases):

I. Gloeobacterophycidae, que comprende sólo un género unicelular que carece de tilacoides (Gloeobacter).

II. Synechococcophycidae, que comprende morfotipos unicelulares (orden Synechococcales) y filamentosos finos (orden Pseudanabaenales) con tilacoides paralelos a la superficie celular en sección transversal.

III. Oscillatoriophycidae, que comprende morfotipos unicelulares (orden Chroococcales) y generalmente filamentosos más grandes (orden Oscillatoriales) con tilacoides dispuestos radialmente en sección transversal.

IV. Nostocophycidae, que comprende morfotipos filamentosos heterocistosos con tilacoides dispuestos irregularmente (orden Nostocales y Stigonematales).

C. TAPETES MICROBIANOS

C.1 Biodiversidad microbiana

La riqueza de especies no es homogénea en la tierra. La variedad de elementos biológicos de un determinado ecosistema depende de una compleja interacción de factores abióticos, como la hostilidad del entorno o la frecuencia e intensidad de las perturbaciones, y bióticos, como la competencia y la depredación entre organismos (Antón-Pardo, 2019). El estudio de estas relaciones ha permitido establecer patrones de distribución de especies que permiten explicar las diferencias observadas entre los distintos ecosistemas. De acuerdo con el gradiente latitudinal de diversidad (Forster, 1778), existe una tendencia de la diversidad biológica a concentrase en las regiones

tropicales. Esto implica que la biodiversidad disminuye con el aumento progresivo de la latitud, con la temperatura como factor principal (Peters *et al.,* 2016), llegando a registros mínimos en las zonas polares más extremas (Rosenzweig, 1995; Gaston, 2000). De esta forma, las plantas vasculares y la macrofauna son escasos o están ausentes en la mayor parte de los ecosistemas polares. Sin embargo, un amplio rango de formas de vida microscópicas es relativamente frecuente, llegando a alcanzar diversidades incluso mayores que en zonas más meridionales del planeta (López-Bueno *et al.,* 2009), alterando la tendencia latitudinal de la disminución de la abundancia y la diversidad de especies (Yergeau *et al.,* 2007).

En la Antártida, como en todos los ecosistemas del planeta, los microorganismos forman la base de la red alimentaria (Cavicchioli, 2015). Estas comunidades contribuyen al ciclo de nutrientes y gestionan las redes tróficas. En conjunto, tienen un papel como reguladores de los procesos de los ecosistemas. Pero en la Antártida los organismos microscópicos son además los componentes más diversos y abundantes de las comunidades terrestres (Hughes *et al.,* 2015), habiendo desarrollado distintas estrategias que les permiten soportar las duras condiciones climáticas. Por ello se considera que la Antártida es un continente dominado por los microorganismos. Y es aquí donde las cianobacterias se convierten en una pieza clave debido a que son consideradas los principales productores primarios bénticos no marinos de la Antártida (Tang *et al.,* 1997).

C.2 Tapetes microbianos en la Antártida

Los tapetes microbianos son complejos microecosistemas donde las especies que los constituyen desarrollan una estrecha relación que lleva a la formación de comunidades dinámicas, y en ocasiones, muy estructuradas. Una gran diversidad de microorganismos, como son las diatomeas, algas verdes, bacterias, virus, ciliados, tardígrados, rotíferos y nemátodos, entre otros, conforman y contribuyen al desarrollo de estos microecosistemas.

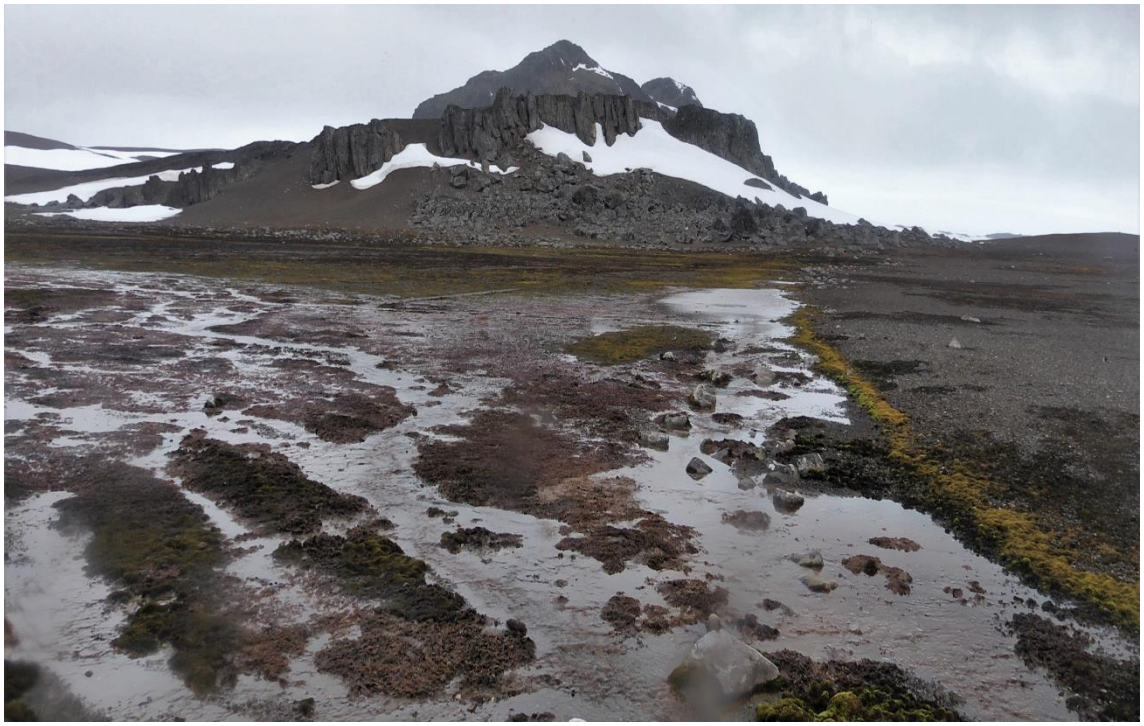


Figura 2. Los tapetes microbianos ocupan grandes extensiones del paisaje en la Península Byers (Islas Shetland del Sur, Antártida), cuando se dan las condiciones ambientales adecuadas para su establecimiento y desarrollo. Imagen tomada por P. Almela.

Los tapetes microbianos dominan los ecosistemas polares terrestres no marinos de la Antártida (Vincent y Quesada, 2012; Quesada y Vincent, 2012). Se les puede encontrar desde las zonas costeras más meridionales (**Figure 2**) hasta las zonas del continente con

las condiciones más extremas, como es el caso de Dufek Massif (84°S) donde constituyen una de las pocas formas de vida presentes (Hodgson *et al.,* 2010).

En general, los tapetes microbianos de cianobacterias de ambas regiones polares albergan una diversidad elevada de organismos procariotas y eucariotas. En la Antártida, estas comunidades están dominados principalmente por los géneros filamentosos *Leptolyngbya, Oscillatoria* y *Phormidium* (Izaguirre y Pizarro, 2000; De los Ríos *et al.,* 2004; Vincent y Quesada, 2012), siendo común la presencia de Nostocales (Quesada *et al.,* 2008). Chroococcales se encuentran con menor frecuencia. La mayoría de estos grupos excretan una serie de exopolisacáridos, mucílago orgánico, que contribuye a la estructuración y estabilización de los sedimentos, formando una estructura cohesiva que resulta en un tapete microbiano. La tupida malla formada por los filamentos de las cianobacterias, a veces de diámetro inferior a 1 µm, como el género *Leptolyngbya* (De los Ríos *et al.,* 2004), junto con sus vainas extracelulares de mucopolisacáridos, a menudo se mezcla con sedimentos del entorno o precipitados minerales como la calcita, que son atrapados o precipitados por la propia actividad bacteriana (Camacho y Fernández-Valiente, 2005), adquiriendo distinta consistencia dependiendo del lugar donde se desarrollen. Toda esta mezcla de materiales proporciona una cohesión global al tapete que lo protege de la desintegración y de las variaciones de la humedad. Por tanto, estos ecosistemas, con cierta capacidad autorreguladora, suponen verdaderos refugios para una gran variedad de organismos microscópicos, tanto fotótrofos como heterótrofos, donde las condiciones extremas de las regiones polares son atenuadas.

En los tapetes microbianos puede existir una estructura vertical diferenciada que denota una estratificación, y se hace patente a menudo por la diferente coloración de las

distintas capas, que es consecuencia del tipo de pigmentos de los organismos situados en ellas (Camacho y Fernández-Valiente, 2005; Rochera *et al.,* 2013). Esta estructura básica consiste en dos capas que difieren en composición, morfología y color. La capa superior se encuentra formada por un estrato compuesto en gran parte por vainas vacías o medio vacías de cianobacterias y frústulas de diatomeas, mientras que la capa inferior alberga la biomasa fotosintética más funcional (Rochera *et al.,* 2013). En cuanto a la composición microbiana, se ha podido comprobar como las cianobacterias dominan en las capas superficiales de los tapetes microbianos antárticos, mientras que las capas más profundas están constituidas por bacterias fototróficas de los phyla Chlorobi y Chloroflexi, así como por heterótrofos de la clase Epsilonproteobacteria. Al mismo tiempo, se demostró que Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria y Deltaproteobacteria habitan en todo el perfil vertical de los tapetes microbianos (Jungblut *et al.,* 2016).

A pesar de que estas comunidades son, en cierto modo, independientes del medio, se ha observado que los recuentos bacterianos, la actividad y la estructura de la comunidad, están relacionados con el tipo de suelo, contenido de nitrógeno, la abundancia de agua y el tipo de cubierta vegetal (Yergeau *et al.,* 2007). De hecho, la existencia de agua líquida constituye el principal factor que facilita la proliferación de los seres vivos en la Antártida (Kennedy, 1993), y por tanto en los tapetes microbianos, donde sólo durante unas pocas semanas al año, coincidiendo con el periodo estival, las temperaturas permiten la actividad de estos al derretirse el hielo.

La entrada de carbono mayoritaria tiene lugar de forma autóctona, es decir, a partir de la actividad fotosintética de los productores primarios fotótrofos como cianobacterias,

diatomeas y algas verdes, aunque también la entrada de C orgánico quimiosintético se ha descrito en estos sistemas. Por lo que respecta a la disponibilidad de nutrientes parece que son sistemas oligotróficos o ultraoligotróficos, aunque la influencia de la fauna marina, o incluso la influencia marina por medio del transporte de nutrientes por aerosoles (Camacho y Fernández-Valiente, 2005), puede aumentar su disponibilidad en zonas próximas a la costa. Por tanto, su estado trófico dependerá, entre otros factores, de su proximidad a la zona costera, al igual que ocurre en los sistemas lénticos antárticos (Villaescusa *et al.,* 2010).

Los tapetes microbianos tienen su óptimo metabólico a temperaturas muy superiores a las que habitualmente están expuestos en los ambientes polares (Velázquez y Quesada, 2011). A nivel de los productores primarios podemos apreciar las diferentes estrategias adaptativas a estas condiciones. Las cianobacterias presentan una actividad fotosintética muy reducida entre 0 ° y 10 °C, por lo que simplemente toleran las bajas temperaturas y pueden crecer a pesar de que dichas condiciones las mantienen por debajo de su óptimo de crecimiento (Tang *et al.,* 1997). Son, por tanto, organismos psicrotolerantes. La otra estrategia es convertirse en especialistas tolerantes al frío. En este sentido, las Chlamydomonas y otros integrantes de la comunidad algal eucarionte psicrófila presentan ciertas adaptaciones evolutivas, como un aparato fotosintético adaptado a temperaturas muy frías (Morgan *et al.,* 1998), que les permite máximos metabólicos a temperaturas próximas al punto de congelación (Velázquez, 2011) y crecimiento solo a temperaturas por debajo de los 15 °C. Por tanto, los rangos fisiológicos de temperatura son diferentes entre los organismos que forman la comunidad del tapete microbiano, pudiendo así ocupar una franja mayor de temperaturas que les permite una mayor tasa de producción.

OBJETIVOS Y ESTRUCTURA DE LA TESIS

1.4 Objetivos de investigación y estructura de la Tesis

Las cianobacterias contribuyen significativamente a la biomasa total del continente antártico. Son consideradas como los principales fotótrofos de la mayoría de los ecosistemas polares bentónicos de agua dulce, y como las arquitectas de los tapetes microbianos polares. Estos tapetes microbianos dominan muchos ecosistemas polares terrestres no marinos en la Antártida (Vincent, 1988; Jungblut *et al.,* 2009; Vincent y Quesada, 2012; Quesada y Vincent, 2012; Prieto-Barajas *et al.,* 2018), y se espera que su presencia siga aumentando conforme aumente la disponibilidad de agua en el ecosistema terrestre como consecuencia del derretimiento de hielo y el aumento de las precipitaciones (Nędzarek y Pociecha, 2010).

Sin embargo, hasta la fecha desconocemos qué papel juegan las cianobacterias en los procesos de sucesión primaria en la Antártida. No sabemos si existe un patrón entre la composición de sus comunidades que pueda relacionarse con factores abióticos o temporales, ni cómo se relacionan sus comunidades con el resto de la microbiota de los suelos. Tampoco sabemos cómo de similares son las comunidades de cianobacterias presentes en los suelos y los tapetes microbianos. Por otro lado, se desconocen cómo son las relaciones tróficas de su comunidad, o cuáles son los factores que las condicionan.

El objetivo general de la presente tesis doctoral es estudiar y entender con mayor detalle el microecosistema de los tapetes microbianos de cianobacterias, desde las primeras etapas de sucesión de las cianobacterias en los suelos recientemente deglaciados, hasta los cambios en las comunidades que conforman los tapetes microbianos frente a distintas condiciones ambientales.

Para lograr este objetivo general se propusieron los siguientes objetivos específicos, que se ordenan en función de la complejidad de la comunidad que los conforman:

- Conocer el rol que las cianobacterias juegan como colonizadores primarios de suelos recientemente deglaciados. Sabemos que son colonizadores importantes de estos suelos, pero no conocemos en profundidad cómo funciona la sucesión de estas bacterias fotosintéticas después de la retirada de los glaciares en la Antártida, ni cómo se relacionan con el resto de los organismos procariotas.
- Conocer las relaciones tróficas y la ecología funcional de los distintos grupos procariotas y eucariotas que conforman la comunidad 'microbiana' de los tapetes microbianos de cianobacterias.

La consecución de los objetivos planteados se realizó a través de cuatro estudios complementarios, presentados como cuatro capítulos diferentes, que corresponden a cuatro trabajos publicados o en proceso de revisión en revistas científicas internacionales. Una discusión general final intenta ofrecer una visión completa de la importancia que los tapetes microbianos de cianobacterias tienen en el ecosistema Antártico, así como de las consecuencias del cambio climático sobre dichas comunidades. La contribución de esta Tesis Doctoral al conocimiento del ecosistema no marino de la Antártida y de otros ecosistemas extremos supone un avance muy relevante en el conocimiento, habiendo descubierto y descrito aspectos muy novedosos como las interacciones tróficas en los tapetes de cianobacterias, así como la influencia de otros organismos sobre los tapetes. Además, las oportunidades de muestreo en

numerosos lugares de la Antártida han permitido descifrar comportamientos generales sobre la colonización de cianobacterias en suelos deglaciados y la formación de ecosistemas más complejos

Capitulo 1: "Heterogeneity of microbial communities in soils from the Antarctic Peninsula region".

Autores del artículo científico enviado: Pablo Almela, Ana Justel y Antonio Quesada.

Artículo publicado en la revista *Frontiers in Microbiology*.

Este segundo capítulo, que corresponde al primer artículo de la presente tesis doctoral, recoge el estudio de la diversidad y composición de las comunidades procariotas de suelos desnudos en tres escalas espaciales, que abarcan desde pocos centímetros a cientos de kilómetros. La hipótesis de partida es que en ecosistemas extremos, algunos microorganismos con mejores estrategias de supervivencia y mejores mecanismos de dispersión, ocurrirán en esas comunidades sin importar la distribución geográfica o aspectos menores locales, conformando así una comunidad común (core community). Para caracterizar la diversidad en las comunidades microbianas del suelo se realizó la secuenciación masiva, mediante Illumina, de la región V1-V3 del gen ARNr 16S de bacterias.

Capítulo 2: "Ubiquity of dominant cyanobacterial taxa along glacier retreat in the Antarctic Peninsula".

Autores del artículo científico enviado: Pablo Almela, Maria Cristina Casero, Ana Justel y Antonio Quesada.

Artículo enviado a la revista *FEMS Microbiology*.

Este tercer capítulo, que corresponde al segundo artículo de la presente tesis doctoral, aborda el análisis de las comunidades de cianobacterias en suelos recientemente

deglaciados a lo largo de la Peninsula Antártica. La hipótesis que se planteó es que las cianobacterias juegan un papel clave como colonizadores primarios de suelos recientemente deglaciados, siendo más abundantes aquellos grupos mejor adaptados a las condiciones climáticas dominantes. También planteamos la existencia de una comunidad de cianobacterias común entre los biotopos del suelo y los tapetes microbianos de cianobacterias, que destacaría el papel clave de estos procariotas fotosintéticos en el ecosistema antártico. Para caracterizar la diversidad en las comunidades de cianobacterias en el suelo se realizó la secuenciación masiva, mediante Illumina, de la región V1-V3 del gen ARNr 16S de bacterias, combinando el uso de cebadores universales (comunidad bacteriana completa) y específicos de cianobacterias.

Capítulo 3: "Carbon pathways through the food web of a microbial mat from Byers peninsula, Antarctica".

Autores del artículo científico enviado: Pablo Almela, David Velázquez, Eugenio Rico, Ana Justel y Antonio Quesada.

Artículo publicado en la revista *Frontiers in Microbiology*.

Este cuarto capítulo, que corresponde al tercer artículo de la presente tesis doctoral, presenta un estudio de las relaciones tróficas de un tapete microbiano de cianobacterias. La hipótesis inicial es que el flujo de carbono y energía entre los distintos compartimentos tróficos, desde los productores primarios hasta los consumidores 'top', tiene que ocurrir en cortos periodos de tiempo, adaptándose a la disponibilidad de agua líquida como principal limitante de estas comunidades microbianas. La comunidad

trófica se caracterizó mediante microscopía óptica y de fluorescencia, complementando estos resultados con la secuenciación masiva, mediante Illumina, de la región V1-V3 del gen ARNr 16S de bacterias. El estudio de las relaciones tróficas se realizó mediante el uso de isotopos estables como trazadores tróficos ($\delta^{13}C$ ‰ and $\delta^{15}N$ ‰), a partir de su abundancia natural, y del enriquecimiento con $NaH^{13}CO_3$ como fuente de carbono inorgánico para los productores primarios del tapete microbiano.

Capítulo 4: "Marine vertebrates impact the bacterial community composition and food webs of Antarctic microbial mats".

Autores del artículo científico enviado: Pablo Almela, David Velázquez, Eugenio Rico, Ana Justel y Antonio Quesada.

Artículo enviado a la revista *Environmental Microbiology*.

Este quinto capítulo, que corresponde al cuarto artículo de la presente tesis doctoral, recoge el estudio de los efectos de la macrofauna local en cinco tapetes microbianos muestreados a lo largo de la Península Antártica. La hipótesis inicial es que estos microecosistemas resultarán alterados por la presencia de fauna local, que condiciona la disponibilidad de nutrientes en el entorno, apareciendo cambios en las abundancias relativas de los grupos procariotas y eucariotas, que se verán reflejados en la estructura de sus redes tróficas. La comunidad trófica se caracterizó mediante microscopía óptica y de fluorescencia, complementando estos resultados con la secuenciación masiva, mediante Illumina, de la región V1-V3 del gen ARNr 16S de bacterias. El estudio de las relaciones tróficas se realizó mediante el uso de isotopos estables como trazadores tróficos ($\delta^{13}C$ ‰ and $\delta^{15}N$ ‰).

CAPITULOS

CAPITULO 1

HETEROGENEITY OF MICROBIAL COMMUNITIES IN SOILS FROM THE ANTARCTIC PENINSULA REGION

HETEROGENEIDAD DE COMUNIDADES MICROBIANAS EN SUELOS DE LA REGIÓN DE LA PENÍNSULA ANTÁRTICA

1.1 RESUMEN

Las áreas sin hielo representan menos del 1 % de la superficie antártica. Sin embargo, los modelos de cambio climático predicen un aumento significativo de las temperaturas en las próximas décadas, provocando una reducción relevante de la superficie cubierta de hielo. Los microorganismos, adaptados a las condiciones extremas y fluctuantes, son la biota dominante. En este artículo analizamos la diversidad y composición de las comunidades bacterianas del suelo en 52 muestras de suelo en tres escalas: i) escala fina, donde comparamos las diferencias en la comunidad microbiana entre suelos del estrato superior (0-2 cm) y suelos del estrato más profundo (5-10 cm) en el mismo punto de muestreo; ii) escala media, en la que comparamos la composición de la comunidad microbiana de suelos del estrato superior de diferentes puntos de muestreo dentro de la misma ubicación de muestreo; y iii) escala gruesa, donde comparamos comunidades de ecosistemas comparables ubicados a cientos de kilómetros de distancia a lo largo de la Península Antártica. Los resultados sugieren que en suelos sin hielo expuestos durante períodos de tiempo más largos (milenios), las comunidades microbianas son significativamente diferentes a lo largo de los perfiles del suelo. Sin embargo, en suelos recientemente deglaciados (décadas), las comunidades no son diferentes a lo largo del perfil del suelo. Además, las comunidades microbianas encontradas en los suelos en los diferentes lugares de muestreo muestran un alto grado de heterogeneidad, con una proporción relevante de variantes únicas de secuencia de amplicón (ASV) que aparecieron principalmente en baja abundancia, y solo en un solo lugar de muestreo. La comunidad Core90, definida como las ASV compartidos por el 90 % de los suelos de los 4 lugares de muestreo, estaba compuesta por 26 ASV, lo que representa un pequeño porcentaje de las secuencias totales. Sin embargo, la composición taxonómica de los

Core80 (ASV compartidos por el 80% de los puntos de muestreo por ubicación) de las diferentes ubicaciones de muestreo, fue muy similar, ya que fueron definidos en su mayoría por 20 taxones comunes, que representan hasta el 75.7 % de las secuencias de los Core80 comunidades, lo que sugiere una mayor homogeneidad de los taxones bacterianos del suelo entre ubicaciones distantes.

ABSTRACT

Ice-free areas represent less than 1 % of the Antarctic surface. However, climate change models predict a significant increase in temperatures in the coming decades, triggering a relevant reduction of the ice-covered surface. Microorganisms, adapted to the extreme and fluctuating conditions, are the dominant biota. In this article we analyse the diversity and composition of soil bacterial communities in 52 soil samples on three scales: i) fine scale, where we compare the differences in the microbial community between top-stratum soils (0-2 cm) and deeper-stratum soils (5-10 cm) at the same sampling point; ii) medium scale, in which we compare the composition of the microbial community of top-stratum soils from different sampling points within the same sampling location; and iii) coarse scale, where we compare communities between comparable ecosystems located hundreds of kilometres apart along the Antarctic Peninsula. The results suggest that in ice-free soils exposed for longer periods of time (millennia) microbial communities are significantly different along the soil profiles. However, in recently (decades) deglaciated soils the communities are not different along the soil profile. Furthermore, the microbial communities found in soils at the different sampling locations show a high degree of heterogeneity, with a relevant proportion of unique amplicon sequence variants (ASV) that appeared mainly in low abundance, and

only at a single sampling location. The Core90 community, defined as the ASVs shared by 90 % of the soils from the 4 sampling locations, was composed of 26 ASVs, representing a small percentage of the total sequences. Nevertheless, the taxonomic composition of the Core80 (ASVs shared by 80 % of sampling points per location) of the different sampling locations, was very similar, as they were mostly defined by 20 common taxa, representing up to 75.7 % of the sequences of the Core80 communities, suggesting a greater homogeneity of soil bacterial taxa among distant locations.

1.2 INTRODUCTION

Ice-free areas in Antarctica comprise less than 1 % of the continent (Cowan, 2014; Burton-Johnson *et al.,* 2016), constituting extremely cold and arid distant and isolated patches within a matrix of ice. These areas, which are not permanently covered by snow or ice, considered oases in the middle of a desert, are of an enormous ecological relevance, since are home to most of the continent's biodiversity.

Antarctica is warming. Although the rate of warming in maritime Antarctica seems to be slowing down (Turner *et al.,* 2016), Rignot *et al.* (2019) has determined an ice mass loss of billions of tons per year, for the period 1979 to 2017, in all regions of the continent due to climate change. The Antarctic Peninsula region has had the largest warming of any other terrestrial environment in the southern hemisphere in recent decades (Siegert *et al.,* 2019). The predictions for the end of the century suggest a 25 % increase of new ice-free areas in Antarctica, with more than 85 % emerging in the North Antarctic Peninsula bioregion (Lee *et al.,* 2017).

These ice-free areas have been exposed for a variable time span, being subjected to glacier retreats and advances. Thus, some areas have been only recently deglaciated and exposed for some decades, as Clark Nunatak (Oliva and Ruiz-Fernández, 2017), while others have been mostly deglaciated for millennia, as Byers Peninsula (Livingston Island, Antarctic Peninsula region) (Oliva *et al.,* 2016). They include islands, nunataks (exposed mountain tops), cliffs, plateaus, ice-free valleys and scree slopes, among others. In any of its forms, these ecosystems are governed by low temperatures, wide temperature fluctuations, low nutrient status, low water availability, high incident radiation, and high levels of physical disturbance (e.g. glaciofluvial activity, frost weathering and cryoturbation). These extreme conditions preclude the establishment of larger organisms (macrobes), resulting in environments dominated by microorganisms (Hughes *et al.,* 2015). Therefore, they constitute a perfect scenario for the study of distribution establishment and ecological functioning of soil microbial communities.

Although new 'omics' techniques have contributed greatly to a better understanding of communities inhabiting soils (Smith *et al.,* 2006; Krauze *et al.,* 2020), this knowledge is quite fragmented, and results obtained from different studies are hardly comparable among them to obtain a clear idea about the distribution of microbial communities in polar regions. It is well known in other latitudes that the microbial communities are different along the soil profiles, with relevant heterogeneity in the distribution of the soil microorganisms in the same biotope (Frey *et al.,* 2013). The highest abundance and diversity of microorganisms that inhabit soils are located in the most superficial centimetres (Brown and Jumpponen, 2014). Previous studies have examined the effects of depth on Antarctic soil bacterial communities (Herbold *et al.,* 2014). Nevertheless, this heterogeneous distribution of the microbial communities has not been as widely

studied on a wide geographical scale (Horrocks *et al.,* 2020), where cryoturbation and other physical process can alter the biotope.

In this paper we analyse the composition of the soil bacteria at three different scales, in order to determine at what level of sampling we were able to identify heterogeneity between ecologically comparable soils. At the fine scale, we compare the microbial community differences between the top-stratum soil (tss) and deeper-stratum soils (dss) at exactly the same sampling point at two sampling locations. In the medium scale, we study the heterogeneity of bacterial communities in top stratum soils at a local scale, comparing the diversity and structure of the communities obtained in sectors within the same geographical location. Finally, in the coarse scale we compare communities from the top stratum soils among sampling locations located in a wide range in the Antarctic Peninsula region.

Our main working hypothesis is that in extreme ecosystems, such as Antarctic soils, in which environmental constraints are the limiting factors, some microorganisms with better survival strategies and better dispersal mechanisms, will occur in those communities without reference to geographical distribution, or local minor aspects. Therefore, microbial communities will present common taxa in ecologically comparable ecosystems even at wide geographical scale.

1.3 MATERIAL AND METHODS

1.3.1 Study area

The study area included four locations along the Antarctic Peninsula (**Figure 1A**). These areas were selected based on their ecological similarity, remaining ice-free during the summer and vegetation free and free of megafauna disturbances, to the best of our knowledge (Plate S1). The physical and chemical characteristics of the soil were similar among locations (**Table S1**), with values of Total Organic Matter (TOM) and C/N ranging between 0.2-0.3 % (p/p) and 3.8-5, respectively. The apparent density of soils showed equal values for all locations (1.6 g/cm^3), and the soil texture classification was determined as 'sandy loam' and 'loamy sand'. The pH values showed variations that ranged between 4.6 and 7.9.

There are evidences indicating that the four sampling locations were ice covered for different time periods (from millennia to decades), following next gradient from the oldest to the most recent: Byers Plateau, Elephant Point, Clark Nunatak and Biscoe Point.

Three sampling locations were located in Livingston Island, the second largest island in the South Shetland archipelago: Byers Plateau, Clark Nunatak and Elephant point. Byers Peninsula, located at the western end of Livingston Island, is one of the largest ice-free areas in the Antarctic Peninsula and the largest in the South Shetland Islands. The coast itself and the ice margin of Rotch Dome glacier form a clearly defined and visually obvious boundary with the island, of approximately 60 km^2. The retreat of Rotch Dome glacier pre-dated 8.3 cal. ky in the westernmost third of the peninsula as a response to warmer climate conditions in the Antarctic Peninsula region during the Early Holocene, and continued eastwards, becoming ice-free the easternmost area probably before 1.8 cal. Ky (Oliva *et al.,* 2016). Today the Rotch Dome sits in contact with the moraine in the central plateau, therefore not being associated to a recent process of glacial retreat.

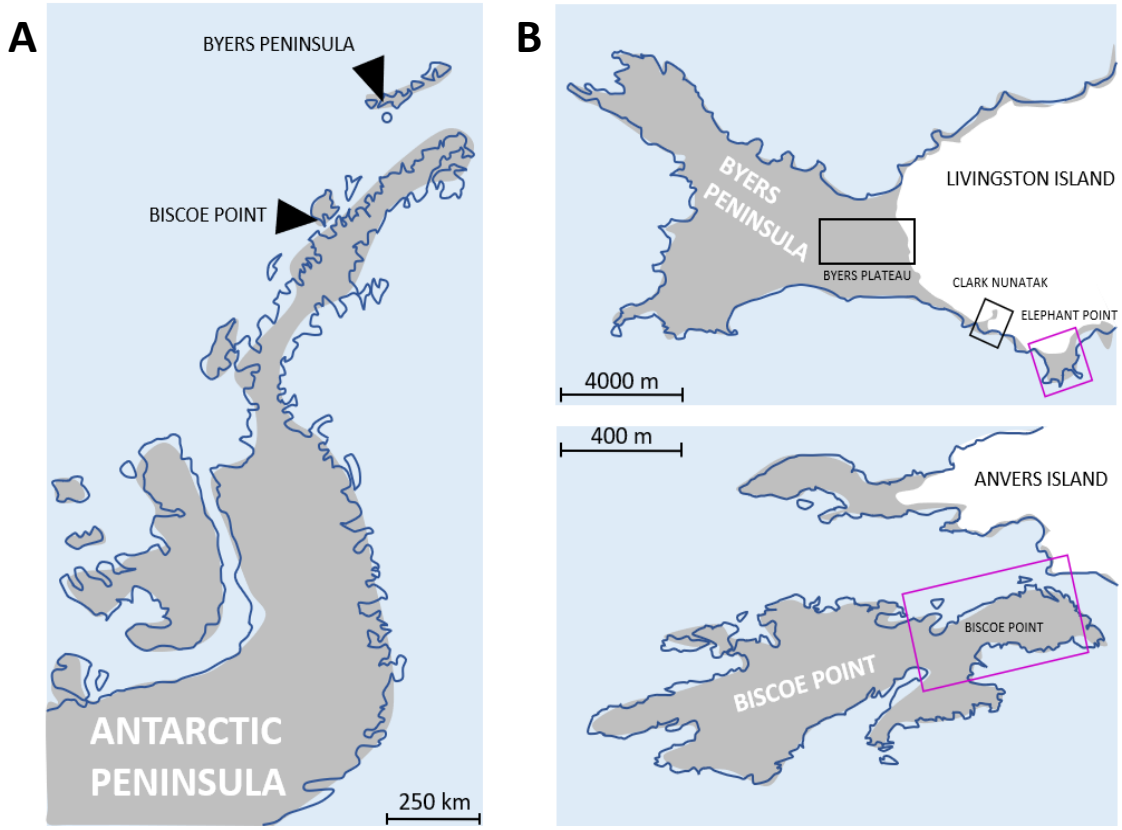


Figure 1. (A) Map of the Antarctic Peninsula. **(B)** Maps showing the 4 sampling locations at Byers Peninsula and Elephant Point in Livingston island (South Shetland Islands) and Biscoe Point (Anvers Island). The sampling locations have been highlighted by squares of different colors, depending on whether the samplings were of top-stratum soils (0–2 cm; purple square) or top- and depper-stratum soils (0–2 cm/5–10 cm; black square).

Soils from Byers Plateau (62°38'S, 60°58'W) were collected from an area of the plateau bordering the Rotch dome glacier front and moving away up to 500 meters (**Figure 1B**).

Clark Nunatak is a rocky peak located in the SE corner of Byers Peninsula, surrounded by the Rotch Dome glacier. It is estimated that the glacier has retreated from the moraine limits very recently, probably after 1950 (Oliva and Ruiz-Fernández, 2017; Palacios *et al.,* 2020). For this reason, it is not until 2002 when the Antarctic Treaty Consultative Meeting (ATCM) included it within ASPA 126, since in previous versions the small ice-free ground surface did not exist. Samples from Nunatak (62°40'S, 60°54'W) were

collected from an area bordering the Rotch Dome glacier front and moving away up to 200 meters (**Figure 1B**). Elephant Point (E) is an ice-free peninsula of 1.16 km^2 in the SW of Livingston Island. It is limited by the Rotch Dome glacier in the north and the sea encircling the rest of its margins. In this area there is evidence of glacial retreat, which has been accelerated over the last decades in response to the recent warming detected in the Antarctic Peninsula region (Steig *et al.,* 2009; Thomas *et al.,* 2009). It is estimated that 17 % of the total land surface exposed today in Elephant Point appeared between 1956 and 2010 (Oliva and Ruiz-Fernández, 2015). Soil samples from Elephant Point (62°40'S, 60°51'W) were collected from an area bordering the Rotch Dome glacier front and moving away up to 300 meters (**Figure 1B**).

The fourth sampling location was at Biscoe Point, an area of 0.59 km^2 located near the south-west coast of Anvers Island, in the Palmer Archipelago. Until recently, Biscoe Point formed a peninsula joined to Anvers Island by an ice ramp extending from the adjacent glacier. The ice ramp disappeared as the glacier retreated at least between 1985 and 2004 (ATCM 2004; ATCM 1985), and a narrow sea channel now separates Anvers Island from the island on which Biscoe Point lies (ATCM, 2014). Soil samples from Biscoe (64°48'S, 63°46'W) were collected from the area closest to the glacier front, now on Anvers Island, and moving away up to 400 meters (**Figure 1B**).

1.3.2 Sampling

Samplings were conducted during two different Antarctic campaigns: February 2018 in Plateau and Nunatak and January 2019 in Biscoe and Elephant. The sampling points were fixed previously to the field campaign to collect in each location two samples from each

of the 5 sectors (from I to V) in Plateau, Elephant and Biscoe, and 3 sectors (from I to III) in Nunatak, in order to test the potential heterogeneity due to patchy distribution of bacterial soil communities.

At each sampling point, we obtained samples comprised of 3 subsamples collected within at approx. 1 m distance of top-stratum soils (0-2 cm) to avoid the vertical heterogeneity in microbial communities attributable to soil horizon development, as recommended by Sigler *et al.* (2002) and Rime *et al.* (2015). It is in these first few centimeters that factors such as light, among others, could be critical for the C input in the ecosystem, and conditioning the composition of the communities. The soils considered into this study do not have another organic C input than microbial primary production (e.g. photosynthesis), since there are no plants and animal debris cannot reach the sites, besides the aerial transportation and eventual birds droppings. Additionally, in order to test the fine scale homogeneity due to the soil horizons development, deeper-stratum soil (5-10 cm) were collected in each sampling point of Plateau and Nunatak.

All soil samples were placed in sterile 50 ml Falcon® tubes and frozen at -20 °C for shipment and storage until processing in the laboratory. Every sample was obtained directly with the plastic tubes without any tool to avoid potential contamination.

1.3.3. DNA Extraction, Sequencing and Taxonomical Assignment

Total genomic DNA extraction was performed independently from the 4 different sampling locations and for soil strata, using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc.) according to standard procedures. DNA concentrations were

determined in a NanoDrop ND 1000 spectrophotometer (Thermo Fisher Scientific™). The 16S rRNA gene was amplified by PCR using barcoded primers set 341F (5'-CCTAYGGGRBGCASCAG -3') and 806R (5'- GGACTACNNGGGTATCTAAT -3') targeting the V3–V4 hypervariable regions (Otani *et al.,* 2014). This universal primer set is for bacterial community and the archaeal community was not included in the study. The pool of samples with the prepared libraries was sequenced by Illumina MiSeq platform. The sequencing was performed in two cycles, in the first cycle the Plateau and Nunatak samples were included, and in the second cycle the Elephant and Biscoe samples.

Microbiome 16S rRNA gene diversity was assessed with QIIME v2-2019.4 (Bolyen *et al.,* 2019). Briefly, cleaned and trimmed paired reads were filtered and denoised using DADA2 plug-in (Callahan *et al.,* 2016). For chimera identification, 250,000 training sequences were used. Identified amplicon sequence variants (ASVs) were aligned using MAFFT (Katoh *et al.,* 2002) and further processed to construct a phylogeny with fasttree2 (Price *et al.,* 2010). Taxonomy was assigned to ASVs using the q2-feature-classifier (Bokulich *et al.,* 2018) and blasted against the SILVA v132 99% 16S sequence database (Quast et al., 2012). Taxonomical assignation was carried out at the same time with all samples after the bioinformatics took place.

Sequences generated by this study were deposited to GenBank under the BioProject accession number PRJNA678471.

1.3.4 Statistical analysis

Some summary statistics of the ASVs obtained in each sampling location were calculated to obtain 'Total ASV' (total quantity), 'Different ASV', 'Predominant ASV' (more than

1000 copies per sampling location) and 'Unique ASV' (present in a sampling location and absent in the others). Also, ASVs were binned into 'core community' (highly persistent) if present in 90% (referred to as the Core90) or 80% (Core80) of soil samples. The Core90 community analysis was carried out for Plateau, Nunatak, Elephant and Biscoe sampling locations together. The Core80 community analysis was performed for the 4 sampling locations separately, thus obtaining the 'core community' of each location (if present in 80% of sampling points of a sampling location). The data obtained from each Core80, and their taxonomic assignments, were jointly compared between the 4 locations to determine their distributions. Results are shown in **Table S2**.

Alpha diversity indices (Richness and Shannon Index) and their rarefaction curves were estimated using the plugin q2-diversity (running 10 iterations and 1000 sequence steps up to the maximum number of sequences per sample). The lowest sample-specific sequencing depth (104,777) was used to compensate for the variation in read numbers. Beta diversity was assessed using Bray-Curtis dissimilarities between the community compositions of the sampling sites.

We have proposed different experimental design models, depending on the scale, for analysing the influence of the depth, sector or location factors on the diversity:

- At the fine scale, we adjusted two balanced block experimental design models in which the factor was the soil depth, and the block was the sampling point. One with data from 2 soil depths at 10 sampling points in Plateau, and the other with data from 2 soil depths at 6 sampling points in Nunatak.

- At the medium scale, we adjusted four balanced one-factor experimental design models, one for each location (Plateau, Nunatak, Elephant, and Biscoe), in which the

sector was the factor. Each model was fitted with data from two top-stratum soils of each of the sectors into which the sampling locations were divided.

- At the coarse scale, we adjusted a one-factor experimental design model, in which the factor was the location, with the data from all the top-stratum soils collected at the four locations.

We tested the homogeneity of alpha diversity indices between the two soil horizons, within site locations, and between locations, using one- and two-way ANOVA tests. To make the same comparisons using the information provided by the Bray-Curtis dissimilarity matrices, we used permutational multivariate analysis of variance tests (PERMANOVA). Differences are considered statistically significant if p-value < 0.05. When any of the hypothesis of equality of means is rejected, the corresponding multiple comparisons are made with Bonferroni correction with overall significance level $\alpha = 0.05$.

Plots of the two principal components of PCoA were used to visualized proximity in the community composition among samples. A heatmap of two-way cluster using Bray-Curtis dissimilarities was used to visualize the relative abundances of bacteria in Core80 of Plateau, Nunatak, Elephant and Biscoe. The heatmap was based on the predominant ASVs (≥ 1000 copies) present in any of the Core80.

Data visualization, alpha and beta diversity comparisons and multivariate statistics were performed using the R environment with the vegan (Oksanen *et al.,* 2015) and ggplot packages.

1.4 RESULTS

1.4.1 Diversity and microbial community at different soil strata (Fine scale)

Our sampling design focuses first on identifying the differences between the bacterial community in the two soil-strata defined at two sampling locations (Plateau and Nunatak). The alpha diversity indices have shown a markedly different pattern between the sampling locations (**Figure 2A**). At the sampling location Nunatak, there were no significant differences between the means of the Richness (1328 and 1238 for tss and dss, respectively) and Shannon indices (8.5 and 8.1 for tss and dss, respectively) in the communities found at the two soil-strata (**Table 1**). On the contrary, the means of both alpha diversity indices were significantly different at Plateau sampling location. The mean Richness in tss was 1729 (SD = 422), higher than in dss, where it was 1512 (SD = 373). The mean of Shannon index in tss was 9.4 (SD = 0.6), also higher than in dss, where it was 8.9 (SD = 0.6). Similar behaviour was observed in the bacterial community composition. The PERMANOVA tests (**Table 1**) showed that there were significant differences between tss and dss communities in Plateau ($p = 0.01$), while the differences were not significant in the soil profile at Nunatak ($p = 0.81$).

The differences between tss and dss community structures were smaller at Nunatak than at Plateau. The PCoA plot (**Figure 2B**) illustrates these two different results, showing closer similarity between samples in the same sampling point in the case of

Nunatak. In Plateau, samples from the same sampling point are as similar as those from different sampling points at the same or different depths points.

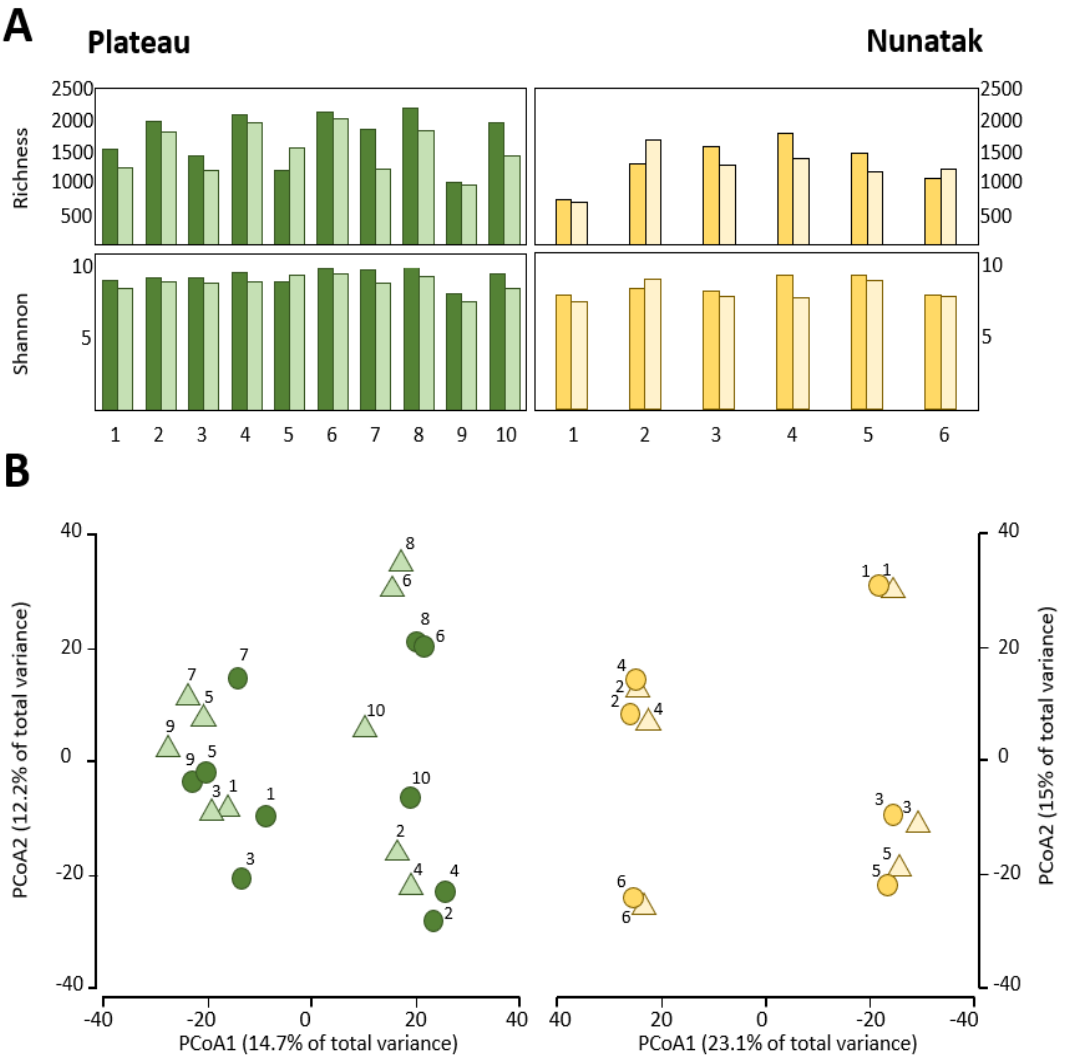


Figure 2. (A) Alpha diversity indices of the bacterial community of top-stratum soils (0–2 cm; dark colored plots) and deeper-stratum soils (5–10 cm; light colored plots) from Plateau and Nunatak. **(B)** Principal coordinate analysis (PCoA) of the bacterial community composition of top-stratum soils (0–2 cm; dark colored dots) and deeper-stratum soils (5–10 cm; light colored triangles) from Plateau and Nunatak. The principal coordinate analysis are based on Bray-Curtis dissimilarity matrices between the microbiome profiles. Distances between symbols on the ordination plot reflect relative dissimilarities in community structures. The variation in microbial community structures explained by each PCoA axis is given in parentheses.

1.4.2 Diversity and composition of prokaryotic communities within the different sampling locations (Medium scale)

The bacterial communities from top-stratum soils from Plateau, Nunatak, Elephant and Biscoe locations were analysed independently for each sampling location. There were not significant differences in terms of richness, diversity and community structure (**Table 1**). ANOVA and PERMANOVA tests showed no significant evidence of within location heterogeneity when comparing the different sampling sectors, with the glacial front of each site as a reference point, considered in Plateau, Nunatak and Biscoe. In location Elephant there were no significant differences between the sampled sectors in terms of richness and diversity; although a significant evidence of heterogeneity (PERMANOVA test $p = 0.01$) was observed in the structure of the prokaryotic communities among the sampled sectors.

1.4.3 Bacterial community diversity, richness and structure at different locations (Coarse scale)

From the results obtained in the medium scale, we can consider all the sampling sites within the same location as a representative sample of its soil microbial community. Comparing the four locations, we observe that median of ASVs richness and diversity was the highest in the Plateau samples and lowest in the Nunatak ones (**Figure 3A**). ANOVA and PERMANOVA tests (**Table 1**) indicated that both the diversity and composition of the bacterial community were different between locations, while there were no significantly clear differences between the means of Richness. For the Shannon index, the Bonferroni multiple comparison tests showed that there were only significant

differences between the Plateau mean and the Elephant and Biscoe means. There was no significant evidence of heterogeneity in the comparisons among Nunatak, Elephant and Biscoe, neither between Plateau and Elephant (**Figure 3A**).

The differences in the bacterial communities measured with the Bray-Curtis dissimilarity are represented in the plot of the two main principal coordinates obtained in a PCoA (**Figure 3B**). Ordinations based on this metric demonstrated a clear separation of bacterial communities among the sampling locations, except for the bacterial community from Nunatak which was slightly intermixed with Plateau and midway towards the other two communities, Biscoe and Elephant.

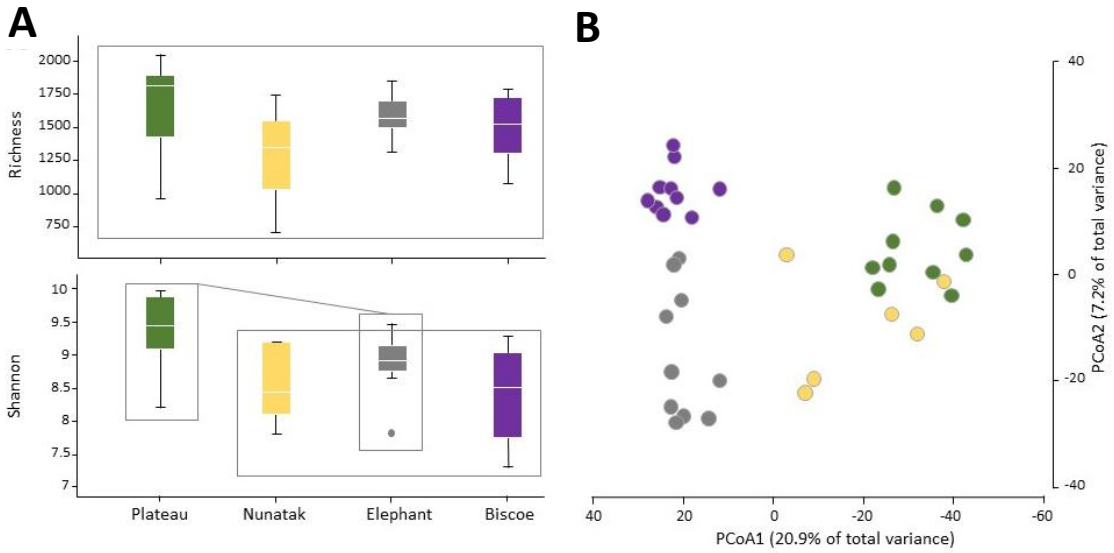


Figure 3. (A) Boxplots of the alpha diversity indices of the bacterial community composition of top-stratum soils (0–2 cm) for each of the sampling locations included in this study. **(B)** Principal coordinate analysis (PCoA) of the bacterial community composition of top-stratum soils (0–2 cm) from the samplings. The principal coordinate analysis is based on Bray-Curtis dissimilarity matrix. Distances between symbols on the ordination plot reflect relative dissimilarities in community structures. The variation in microbial community structures explained by each PCoA axis is given in parentheses.

Scale	Description of the samples	Richness	Diversity	Community	Anova/ Permanova	Sample size
Fine	Top-stratum soils (0-2 cm) and deeper-stratum soils (5-10 cm) from Plateau	**0.03**	**0.01**	**0.01**	Two-way	10 pairs
Fine	Top-stratum soils (0-2 cm) and deeper-stratum soils (5-10 cm) from Nunatak	0.52	0.26	0.82	Two-way	6 pairs
Medium	top-stratum soils (0-2 cm) from Plateau	0.75	0.45	0.66	One-way	2 samples, 5 areas
Medium	top-stratum soils (0-2 cm) from Nunatak	0.21	0.83	0.80	One-way	2 samples, 3 areas
Medium	top-stratum soils (0-2 cm) from Elephant	0.99	0.59	**0.01**	One-way	2 samples, 5 areas
Medium	top-stratum soils (0-2 cm) from Biscoe	0.21	0.15	0.38	One-way	2 samples, 5 areas
Coarse	All top-stratum soils (0-2 cm)	0.10	**0.00**	**0.00**	One-way	36 samples

Table 1. p-values of the ANOVA and PERMANOVA (Permutational Multivariate Analysis of Variance) tests for comparisons of richness and diversity, and composition of the bacterial community, respectively, among surface soil samples at different areas in each location (Plateau, Nunatak, Elephant and Biscoe).

1.4.4 Description of microbial communities and similarities in communities among sampling locations

The description of the communities found in the different sampling locations is illustrated in **Figure 4**. In Plateau the highest number of different ASV was found, which was almost 2-fold higher than the community from Nunatak location (11,566 and 6366 ASVs for Plateau and Nunatak, respectively), with the lowest amount of different ASVs. At Plateau location only 0.6 % of the different ASVs (70 ASVs) were found predominant with more than 1000 copies and represented over 41 % of the total ASVs, indicating that a low number of different sequences represented a large proportion of the community found at this location. Moreover, at Plateau location 11 % of the total sequences were found only at this sampling location (unique ASVs) and reached 68.9 % of the different sequences. Most of those unique sequences are found in low abundance, thus, only 2.7 % of the total sequences were unique and predominant (over 100 copies) representing 1.7 % of the different ASVs. A very similar pattern was found in the other locations, where the number of predominant ASVs (more than 1000 copies) reached 44, 45.7 and 52 % of the total ASVs for Nunatak, Elephant and Biscoe, respectively. These predominant sequences were composed of few different ASVs, representing 0.8, 1.8, and 2 % of the total different ASVs for Nunatak, Elephant, and Biscoe. In addition, 5.3, 10.9 and 9.9 % of the total ASVs found in Nunatak, Elephant and Biscoe were classified as unique ASVs, representing 48, 54.9 and 55.9 % of the diversity of the different ASVs determined in each location.

1.4.5 Core community

The microbial Core90 analysis revealed that 26 ASVs were found in at least 90% of the total sampling points from all locations (**Figure 4**). These results indicated that the soil bacterial communities at the highest level of taxonomic resolution were apparently not homogeneous between the 4 sampling locations. This ‘core community' represented a low proportion of ASVs from total ASVs sequenced.

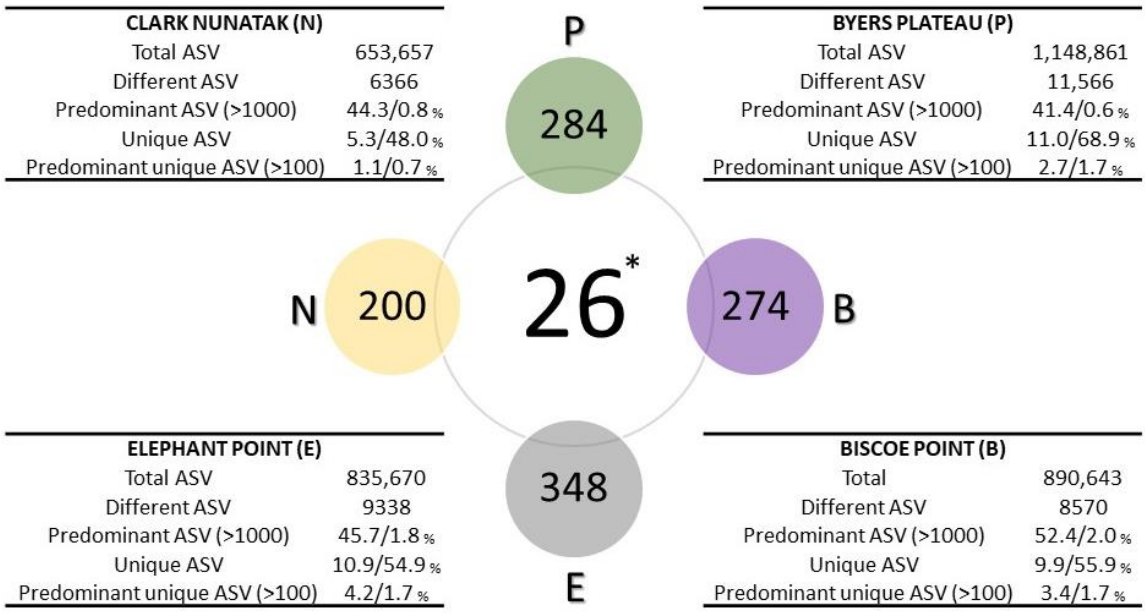


Figure 4. Diagram comparing the bacterial core communities. Colored spheres indicate the number of ASVs in at least 80% of top-stratum soils (core80) of each sampling location, while the central sphere (∗) indicates the Core90 (90% of all the top-stratum soils). For each location, in the tables are shown the number of total and different ASV of the bacterial community, and the percentages of the predominant ASV (at least 1000 copies), the unique ASV at each location (not present in other sampling areas), and the percentage of the predominant unique ASV (at least 100 copies), according to total and different ASV, respectively.

The 'Core80' was analysed for Plateau, Nunatak, Elephant and Biscoe, to describe its local composition and relative abundances (**Figure 4**). Thus, 284 ASVs in Plateau, 200

ASVs in Nunatak, and 348 and 274 ASVs in Elephant and Biscoe, respectively, were revealed. A PCoA plot, based on Bray-Curtis dissimilarity, was generated (**Figure 5B**) to visualise the proximities between the sampling points when considering only the ASVs found in any of the Core80. Results showed that ASVs from the four Core80 bacterial communities are well separated, except for one sample from Nunatak that is closer to some samples from Plateau than to the rest. A Heatmap (**Figure 5A**) was used to visualize the relative abundance of predominant ASVs (≥ 1000 copies) from Core80, regardless of taxonomic assignment. Results revealed that Plateau and Nunatak clustered together and separately from Elephant and Biscoe, that also clustered together, suggesting that core communities from those locations have more in common than the other locations.

The Core80 communities were also explored in detail in terms of taxonomic assignment. Family level was considered appropriate because of taxonomic resolution and ecological relevance for most of the bacterial groups. At this taxonomic level (or similar) the core communities were represented by a much lower number of identities, which ranged from 25 to 28. The ASVs included in those groups represented between 77.1 and 85.4 % of the total sequences within the Core80 community. The most part (20) of the identities were found in all the core communities and represented up to 75.7 % of the total ASVs in the Core80. These 20 groups belonged in the majority to Actinobacteria (8) and Proteobacteria (6), but Acidobacteria, Gemmatobacteria, Bacteriodetes, Cyanobacteria and Chloroflexi were also represented (**Table S2**).

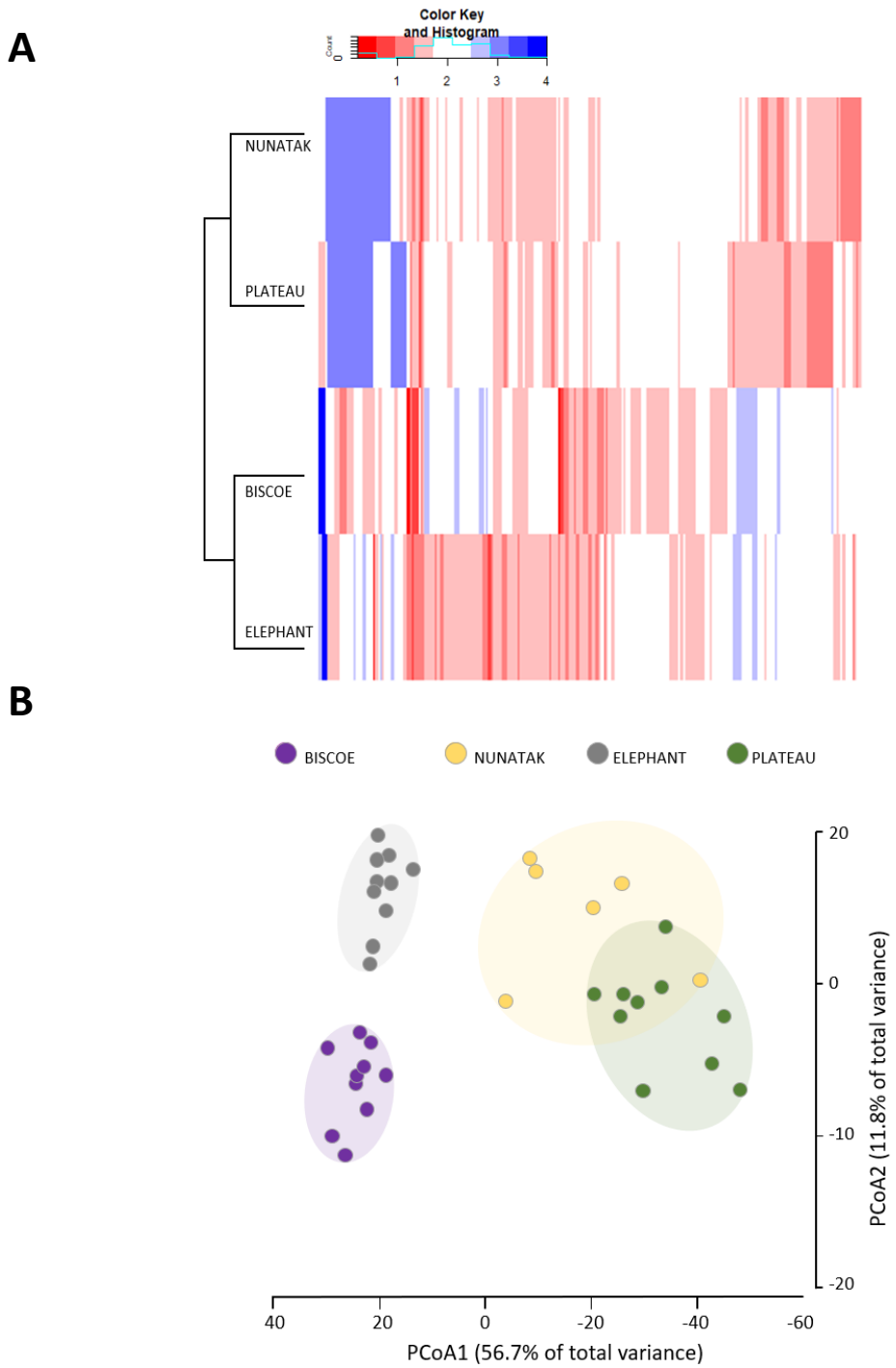


Figure 5. (A) Heatmap of two-way cluster analysis performed on most abundant Core80 bacterial community (only ASVs > 1000 copies) of all top-stratum sample soils from Plateau, Nunatak, Elephant and Biscoe, using Bray-Curtis dissimilarities. The color intensity in the cluster dendrogram correspond to the abundance of normalized reads. **(B)** Principal coordinate analysis (PCoA) for all the sampling points when considering only the ASVs in any of the Core80. The principal coordinate analysis is based on the Bray-Curtis dissimilarity matrix. Distances between symbols on the ordination plot reflect relative dissimilarities in community structures. The variation in microbial community structures explained by each PCoA axis is given in parentheses.

1.5 DISCUSSION

Microorganisms are the dominant biota and play a key role in the ecology of Antarctic terrestrial ecosystems (Cowan, 2014). Investigating the distribution of microbial diversity is essential to understanding ecological functioning of these ecosystems. The purpose of our analysis is to investigate the heterogeneity of the microbial community inhabiting ecologically comparable soils (no vegetation in the proximity of the sampling site, no megafauna presence, similar microtopographic characteristics, granulometry, etc) in the Antarctic Peninsula. This work considers different scales, starting at the fine scale, in which we compare, at the closest sampling locations (Plateau and Nunatak), the microbial community differences between the top-stratum (tss) and deeper-stratum soils (dss) at exactly the same sampling point. The medium scale brings into comparison the top-stratum communities found within the same sampling location (e.g. Plateau). Finally, the coarse scale compares the top stratum communities found among the four sampling locations (Plateau, Biscoe, Nunatak and Elephant) with diverse geographical distance.

The study revealed that comparisons of bacterial communities from different soil strata at Plateau resulted in low p-values ($p = 0.01$), indicating significant differences among the soil-strata, and therefore a non-homogeneous and differentiated distribution according to a vertical profile. Conversely, bacterial populations from Nunatak have shown a vertical homogeneity of their communities. These differences in the homogeneity of distribution among soil strata in the two locations are attributable to the length of the ice-free period, since ecologically the sampling locations are comparable. Oliva *et al.* (2016) have dated areas close to the soils studied in Byers

Peninsula in thousands of years, while the soil samples from Nunatak Clark have only been ice-free in summer for a few decades from the present. As described by Barrett *et al.* (2006a), vertical distributions of the soil microbiomes would be related to the variation in soil properties. Niederberger *et al.* (2008) showed how the highest microbial diversity in Antarctic soils was related with the highest levels of organic matter, while Chu *et al.* (2016) also related it to the N available in the different soil layers of the Tibetan Plateau. Such large temporal differences among Plateau and Nunatak should differentiate both locations in terms of soil properties established along the vertical profile. Therefore, the relative homogeneity in Nunatak among their prokaryotic communities would be related to the uniform characteristics of the soils, both in surface and depth. The opposite is shown in Plateau, where the microbial activity, over time, would have contributed by modifying the characteristics of the soil. Shannon diversity analysis indicated significant differences in microbial diversity between soil strata in Plateau (9.4 tss and 8.9 dss) and did not indicate relevant differences in Nunatak (8.5 tss and 8.1 dss). For both locations, bacterial communities in top-stratum soils have shown greater richness and diversity values than those from the deeper-stratum soils. Therefore, our results are similar to those reported from other Antarctic soils (Aislabie *et al.,* 2006; Bajerski and Wagner, 2013), showing that the highest abundance and diversity of soil microorganisms are located in the most superficial centimetres.

Previous studies have shown high levels of spatial heterogeneity in prokaryote biodiversity across terrestrial environments in Antarctica (Barrett *et al.,* 2006b; Chong *et al.,* 2010). This spatial heterogeneity is generated by physicochemical and trophic variations acting at all spatial scales. However, our results with the top-stratum soils of each sampling location showed no significant evidence of within locations

heterogeneity, both for alpha diversity and community composition. The diversity of prokaryotes is sensitive to local environmental conditions such as the availability of water and nutrients (Barrett *et al.,* 2006b; Chong *et al.,* 2010) and soil heterogeneity is expected in small spatial scales (Fraschetti *et al.,* 2005). However, our data, from samples taken from carefully chosen ecologically comparable sites and locations, offer another view about the microbial compositions of soils at different scales of analysis. When all sequences from the sampling sites are analysed jointly, data points from the same locations are closer supporting the heterogeneous distribution of the microbial communities. Differences in bacterial communities measured with Bray-Curtis provide insight into differences in community composition among samples, with the advantage of being based on ASV counts, regardless of taxonomic assignment as maximum sequencing resolution. The results showed that the bacterial communities from soils are again grouped by locations, suggesting that inter-site variations are greater than intra-site variations. Therefore, geological variables (e.g. spatial distance, climate conditions, geological features and historical context) are also of influence on microbial communities. These results are similar with previous studies such as that of Yergeau *et al.* (2007), which have reported significant differences in bacterial diversity in Antarctica on a continental scale.

Description of microbial communities from ASVs allows a different perspective of its composition, being able to analyse sequences with the maximum potential resolution, and with no risk of introducing deviations due to the taxonomic reference (Callahan *et al.,* 2016). However, one of the costs of this method leads to a possible loss of real sequences that would be present at very low levels. Our data indicate that a large proportion of the different sequences are unique. The amount of these unique ASVs

assigned to each location is high, ranging from 5.3 to 11 % of the total sequences, having a key role in the heterogeneity of the communities. However, clustering thresholds greater than 97 % identity can lead to an overestimation of the rare biosphere present in the samples (Kunin *et al.,* 2010). Most of these unique ASVs are in low abundance but represent between 48 and 69 % of the different sequences. At the moment, the ecological role of that rare biosphere is not well understood, but frequently neglected.

Analyses based on the core communities allow us to know the specific weight of those groups shared between different samples. The core at maximum resolution for the 4 sampling locations (Core90) makes up a common bacterial community in 90 % of sampled soils, of 26 ASVs which do not dominate the set sequences. These data, similar to those obtained in previous studies of core communities from different Antarctic environments (Murray *et al.,* 2020), show that at least a few dozen sequences are identical between ice-free areas separated by hundreds of kilometres along the Antarctic Peninsula. The possibilities of dispersal of microorganisms are numerous, ranging from air transport (Archer *et al.,* 2019; King-Miaow *et al.,* 2019; Cao *et al.,* 2020) and ocean currents (Fraser *et al.,* 2018), to the anthropogenic activities (Kirtsideli *et al.,* 2018). The dispersal capabilities of bacteria are evidenced in Antarctica showing that identical sequences are found in relatively distant regions. However, the ASVs heterogeneity between locations in the Core80 community is significant. Although the sampling locations Plateau, Nunatak and Elephant are located at the same island with a maximum distance of 10 km, bacterial community from location Elephant, located at more than 300 km away from location Biscoe, is more similar to that one than to the closer ones.

The previous analyses used ASVs as an expression of diversity, without considering the taxonomic assignment of those sequences. However, when the taxonomy is the purpose, it is necessary to find a balance between the genotypic diversity and the functional diversity, making in this case relevant to reduce the taxonomic resolution for the analyses. The detailed taxonomic assignment of the Core80 communities indicated that a small number of taxa, at Family level, conformed the bacterial core communities (less than 30 taxa) of each location. On average, these groups represented over 82 % of the sequences forming those core communities. In fact, 20 of those identities were present at all sampling locations and can be considered cosmopolitan taxa in this region and represented up to 75.7 % of the total sequences found at the Core80 community. Soils from extreme Antarctic environments present severely limited terrestrial productivity and, consequently, soil organic matter concentrations are very low (Burkins *et al.,* 2001). Nutrient inputs in these ecosystems have been attributed to aerial deposition (Reynolds *et al.,* 2001), abiotic processes driven by temperature changes (Parsons *et al.,* 2004), and microbial activity (Cary *et al.,* 2010). The presence of phototrophic bacterial families in the Core80 community suggests its key role in carbon and energy input to the ecosystem. Therefore, *Pseudanabaena* and *Tychonema* could be involved in the CO_2 photoassimilation and the fixation of N_2, as observed in high-elevated 'barren' soils from other latitudes (Freeman *et al.,* 2009). The presence of Chloroflexi could also be related to the CO_2 uptake. Cyanobacteria and Chloroflexi can utilize different portions of the radiation spectrum for photosynthesis (Ley *et al.,* 2006) and would be photosynthetically active at different microtopographic positions. The presence of *Thiobacillus* is also relevant, since all species described are obligate autotrophs (Boden, 2017), allowing an alternative energy input to the ecosystem.

However, the metabolic diversity of the Core80 community does not only include autotrophic bacteria. In fact, 16 of these 20 common taxa belonged to Proteobacteria and Actinobacteria phyla, typically described for glacier retreat areas (Brown and Jumpponen, 2014). Proteobacteria is a major player in soil microbial communities around the globe, due to its high metabolic versatility. This phylum, and especially *Burkholderia*, acquires a key role in organic matter decomposition processes in Arctic soils (Tao *et al.,* 2020), and its prevalence is related with increased soil carbon turnover upon warming in Antarctic soils (Thomson *et al.,* 2010; Yergeau *et al.,* 2012). Likewise, Actinobacteria groups are able to decompose organic matter, including recalcitrant polymers (Větrovský *et al.,* 2014). Therefore, most of the Core80 described for the 4 sampling locations would be associated to processes of degradation of recalcitrant organic matter, not accessible to other microorganisms (Hawes, 2008), with a probably key role in the subsequent colonization of these oligotrophic soils.

Our results indicate that the same functional taxa (with the taxonomical resolution used in this work) are inhabiting the ecologically comparable soils sampled in this study in the Antarctic Peninsula region, regardless of the potentially different environmental constraints and independently of their geographical proximity, or duration of the ice-free period. This is consistent with previous reports from Antarctic soils, where higher levels of similarity were observed between locations with similar physico-chemical characteristics (Chong *et al.,* 2010). Considering that the ice-free condition was acquired at different time scales at the different locations (millennia at Byers Peninsula Plateau, and few decades at Biscoe Point), it can be assumed that these taxa are first, highly transportable (most likely by the wind) and second, highly versatile. These characteristics confer those taxa a pioneer status in Antarctic soils, relating to potential

colonizers in the new deglaciated soils subjected to global change, due to a wider range of stress tolerance strategies than other microorganisms (Sigler *et al.,* 2002; Sigler and Zeyer, 2004). However, its presence in soils of such different ages, suggests that primary succession processes, in extreme ecosystems, could have an indeterminate duration.

Certainly, every sampling location, besides the central core community showed a unique fingerprint in terms of the microbial community inhabiting those soils. For instance, Biscoe showed a high diversity and abundance of Cyanobacteria which was obviously absent from other locations such as Plateau, while *Chthoniobacteraceae* were conspicuously abundant in Plateau (6.95 % of the total sequences in that core community) and absent or very scarce in the other locations. We suggest that those particularities are due to local environmental characteristics as higher humidity, or different mineral composition, or even to stochastic processes for the distribution that are out of the scope of this work.

1.6 CONCLUSIONS

In conclusion, our work is a contribution to understanding the distribution and dispersal characteristics of the microbial communities inhabiting Antarctic soils. While the heterogeneity of microbial communities can reach high levels in the soil profiles in older soils, this heterogeneity is clearer at geographical scales. However, only 20 common taxonomic groups formed the highest proportion of the ASVs sequenced from the Core80 communities, and most likely conform the Antarctic bare soil bacterial community identity. The potential metabolic diversity of the Core80 community could be linked to all the fundamental metabolic activities required for the acquisition and recycling of organic C, which would justify its presence in all the sampling locations studied.

1.7 SUPPLEMENTARY MATERIAL

Table S1. Soil physical and chemical characteristics from the 4 sampling locations. The values represent the mean of all sample points analysed.

Location	**pH**	**C/N**	**TOM** (%p/p)	**Soil density** (g/cm^3)	**Soil texture**
Plateau	4.6 ± 0.23	3.9 ± 0.96	0.3 ± 0.11	1.6 ± 0.02	Loamy sand
Nunatak	6.0 ± 0.43	3.8 ± 1.18	0.2 ± 0.05	1.6 ± 0.04	Sandy loam
Elephant	6.8 ± 0.17	5.13 ± 0.35	0.3 ± 0.03	1.6 ± 0.01	Sandy loam
Biscoe	7.9 ± 0.28	3.8 ± 0.80	0.2 ± 0.04	1.6 ± 0.04	Loamy sand

Table S2. Taxonomy summary of the dominant bacteria in Core80 analysis of the sampling locations. The total number of sequences belonging to the Core80 communities ranged from 120,209 in Nunatak to 500,390 in Biscoe.

Taxa	Plateau	Nunatak	Elephant	Biscoe
	% Core80			
Hydrogenophilaceae (Thiobacillus)	-	-	1.7	0.4
Iamiaceae (Iamia)	0.7	0.4	1.4	0.7
Tenderiaceae (Tenderia)	-	1.2	7.1	0.2
Pseudanabaenaceae (Pseudanabaena)	-	4.1	0.8	0.3
Phormidiaceae (Tychonema)	-	0.0	6.7	10.9
Intrasporangiacea (Oryzihumus)	11.8	9.9	3.6	1.2
Gaiellales	14.4	2.4	2.5	1.6
Chitinophagaceae (Chitinophaga)	7.1	5.3	5.0	7.4
Gemmatimonadacea (Gemmatimonas)	12.0	21.9	3.2	4.9
Chthoniobacteraceae (Udaeobacter)	7.0	0.5	-	-
Actinobacteria (MB-A2-108)	2.8	0.7	-	-
ilumatobacteriaceae (Ilumatobacter)	3.1	2.4	4.2	4.5
Frankiales	2.5	0.6	2.3	2.4
Chloroflexi- KD4	2.2	1.9	1.8	1.2
Pyrinomonadaceae (RB-41)	1.8	0.1	-	-
Sphingomonadaceae (Sphingomonas)	4.4	12.3	13.2	17.9
IMCC26256 (Ferrimicrobium)	2.1	0.8	0.1	-
Nocardiodiacaeaae (Nocardioides)	1.5	1.1	4.7	2.6
Xanthomonadaceae (Lysobacter)	1.4	7.2	5.0	7.4
Rubrobacteria	1.4	0.1	1.4	2.3

Solibacteraceae (Bryobacter)	1.3	0.2	0.6	0.4
Blastocatellaceae (JGI 0001001-H03)	1.8	0.6	0.7	3.0
Burkholderiaceae (Burkholderia-Caballeronia-Paraburkholderia)	1.6	3.2	4.3	5.2
Holophagae	1.0	0.3	0.2	0.4
Rhodobacteraceae	0.0	0.2	0.7	1.4
Rhizobiales+Xanthobacteraceae	1.4	1.0	1.3	1.3
Hymenobacteraceae(Hymenobacter)	0.0	0.0	1.5	1.0
Rhodanobacteraceae (Rhodanobacter)	0.4	0.9	0.7	1.0
Nitrosomonadaceae (Nitrosomonas)	1.0	1.5	1.1	0.0
Acetobacteraceae	0.7	0.4	0.2	0.0
Micrococcaceae	0.7	3.4	1.0	1.5
Total %	86.0	84.8	77.1	81.0

CAPITULO 2

UBIQUITY OF DOMINANT CYANOBACTERIAL TAXA ALONG GLACIER RETREAT AREAS IN THE ANTARCTIC PENINSULA

UBICUIDAD DE GRUPOS CIANOBACTERIANOS DOMINANTES A LO LARGO DE ZONAS DE RETROCESO GLACIAR EN LA PENÍNSULA ANTÁRTICA

2.1 RESUMEN

Las cianobacterias son organismos clave en el ecosistema antártico, pero la sucesión principal de sus comunidades en suelos recientemente deglaciados sigue siendo poco conocida. En este estudio, examinamos la sucesión primaria de comunidades de cianobacterias, empleando secuenciación de última generación, en tres áreas de retroceso glaciar en la Antártida que han quedado libres de hielo en los últimos 30 años. Oscillatoriales y Pseudanabaenales fueron los órdenes predominantes, seguidos de Nostocales. A pesar de las características fisicoquímicas similares de los suelos, no encontramos un patrón común en la distribución de las comunidades de cianobacterias en el nivel más fino de resolución taxonómica. Sin embargo, hubo una ligera tendencia a aumentar la homogeneidad de sus comunidades, a nivel de variante de secuencia de amplicón (ASV), en aquellas muestras relacionadas con etapas más avanzadas de desarrollo del suelo (por ejemplo, suelos más viejos). El análisis de metasecuenciación también reveló una comunidad común (Comunidad Central de Cianobacterias del Suelo) de 14 secuencias idénticas de cianobacterias en los suelos estudiados. Estos ASV se asignaron a los géneros *Pseudanabaena, Phormisdesmis, Phormidium, Microcoleus, Tychonema, Nostoc, Nodularia* y *Chroococcidiopsis*, y comprendieron una abundancia relativa dentro de la comunidad de cianobacterias del 51.5-81.7 % entre los tres lugares de retroceso glaciar. Estos 14 ASV encontrados en los suelos también se encontraron en dos tapetes microbianos de cianobacterias de la Península Antártica. Nuestros resultados sugieren que las interacciones (micro)bióticas actúan como un impulsor clave de la composición y dinámica de la comunidad de cianobacterias durante las primeras etapas de sucesión en suelos recientemente deglaciados de la Antártida. En los suelos, algunos géneros comunes pueden desempeñar un papel clave en el ecosistema debido

a su presencia ubicua, no solo en estos suelos, sino también en los tapetes microbianos, probablemente conformando los genotipos más ampliamente dispersos y dominantes en los suelos antárticos.

ABSTRACT

Cyanobacteria are key organisms in the Antarctic ecosystem, but the primary succession of its communities in recently deglaciated soils remains poorly understood. In this study, we surveyed the primary succession of cyanobacterial communities with an in-depth Next Generation Sequencing approach in three Antarctic glacier forefields which have become ice-free over much of their surface in the last 30 years. Oscillatoriales and Pseudanabaenales were the predominant orders, followed by Nostocales. Despite the similar physicochemical characteristics of the soils, we did not find a common pattern in the distribution of the cyanobacterial communities at the finest level of taxonomic resolution. However, there was a slight trend to increase the homogeneity of their communities at the amplicon sequence variant (ASV) level in those samples related to more advanced stages of soil development (e.g. older soils). The metabarcoding analysis also revealed a common community (Soil Cyanobacterial Core Community) of 14 cyanobacterial identical sequences in the studied soils. These ASVs were assigned to the genera *Pseudanabaena, Phormisdesmis, Phormidium, Microcoleus, Tychonema, Nostoc, Nodularia* and *Chroococcidiopsis*, and comprised a relative abundance within the cyanobacterial community of 51.5-81.7 % among the three glacial retreat locations. These 14 ASV found in the soils were also found in two cyanobacterial microbial mats from the Antarctic Peninsula. Our results suggest that (micro)biotic interactions act as a key driver of the community composition and dynamics of Cyanobacteria during the

early stages of succession in recently deglaciated soils of Antarctica. In the soils a few common genera might play a key role in the ecosystem, due to its ubiquitous presence not only in these soils but also in microbial mats, conforming probably the most widely disperse and dominant single genotypes in Antarctic soils

2.2 INTRODUCTION

Most of the glaciers from the Antarctic Peninsula have been retreating during the last decades (Silva *et al.,* 2020). The mass balance showed a net ice loss, as a response to the warming detected throughout the last twenty years (Turner *et al.,* 2005, Oliva *et al.,* 2017; Siegert *et al.,* 2019), causes differences among the accumulation of snowfall in the interior, surface ablation (wind transport and sublimation) and ice discharge, causing a backward movement of the glacier front. Terrestrial glacier retreat exposes new habitats which can be colonized by pioneering organisms which results in a spatio-temporal gradient of ecosystem development, also known as chronosequence. Thus, the distance from the glacier front can be used as a proxy for time since deglaciation (Nemergut *et al.,* 2007; Walker *et al.,* 2010), and therefore as a tool to understand primary succession and soil-forming processes.

Microbial populations with different abundances, community structures and diversities are the first organisms to colonize recently deglaciated areas all over the world (Nemergut *et al.,* 2006; Bradley *et al.,* 2014; Rime *et al.,* 2015; Fernández-Martínez *et al.,* 2017), playing an important role in primary succession, pedogenesis and biogeochemical cycling (Lazzaro *et al.,* 2009; Schutte *et al.,* 2009). Most Antarctic non-marine ecosystems are dominated by microbial communities (Wynn-Williams, 1996;

Aislabie *et al.,* 2006; Hughes *et al.,* 2015). Therefore, glacier forefields constitute an ideal setting for investigating changes in the composition and structure of the microbial community over time (Zumsteg *et al.,* 2012; Brown and Jumpponen, 2014; Jiang *et al.,* 2018), even in bare soils with thousands of years since deglaciation (Almela *et al.,* 2021).

Cyanobacteria are the main primary producers in the Antarctic ecosystems (Velichko *et al.,* 2021). They have developed some abilities to cope with the extreme polar conditions such as long periods of desiccation and wide temperature fluctuations (Varin *et al.,* 2012). An example would be the production of extracellular polymeric substances (EPS), which in addition to protecting cells against desiccation and freezing (Knowles *et al.,* 2008), constitute the structure of microbial mats, facilitating the establishment of complex communities (Almela *et al.,* 2019a). However, the success of this group of organisms lies in their survival strategies to withstand the harsh environmental conditions of polar environments (Quesada and Vincent, 2012), although they perform better in warmer conditions (Velázquez *et al.,* 2011).

In early succession stages from deglaciated soils, Cyanobacteria are thought to play an important role in incorporating C and N into topsoil layers through their phototrophic and eventually diazotrophic activities and, consequently, facilitating later colonization of other organisms (Turicchia *et al.,* 2005; Kastovská *et al.,* 2005; Schmidt et al. 2008; Frey *et al.,* 2013; Liu *et al.,* 2016). Also, investigations targeting whole bacterial communities showed that cyanobacteria dominate barren soils close to the glacier terminus in the Arctic (Kaštovská *et al.,* 2005; Fernández-Martinez *et al.,* 2017) and in Antarctica (Bajerski and Wagner, 2013). However, and considering that the polar microbiota has been extensively explored in recent years with next generation-high

performance-sequencing (NGS) (Chong *et al.,* 2015; Pessi *et al.,* 2015, 2016) specific insights into the successional dynamics of cyanobacterial communities in glacier forefields are limited (Pessi *et al.,* 2019; Pushkareva *et al.,* 2019; Knelman *et al.,* 2021).

Here, we conducted a specific insight into the primary succession of cyanobacterial communities in glacier forefields from Antarctica using Next-generation sequencing approaches and specific cyanobacterial oligonucleotide primers. By focusing on the composition of cyanobacterial communities in recently deglaciated soils, we described the early successional dynamics of its communities. We were interested in determining if cyanobacterial communities undergo any discernible compositional shift along the deglaciation gradients that allows assigning an ecological strategy to the different cyanobacterial taxa. Since the environmental and chemical properties of the soils are expected to be similar, we hypothesize that biotic interactions will play a key role in the primary succession processes of cyanobacterial communities among the three areas. We also intended to identify a central community shared between the different ecotypes studied (e.g. soils and mats), thus highlighting the role of some cyanobacterial groups in the Antarctic ecosystem. This approach allows us to perform a phylogenetically meaningful identification without pure cultures or molecular cloning, comparing sequences with a resolution of up to one nucleotide, enabling the study of the cyanobacterial taxa geographic distribution at a regional scale.

2.3 MATERIALS AND METHODS

2.3.1 Study Area

The study area included three glacier retreat locations along the Antarctic Peninsula: Clark Nunatak, Elephant Point and Biscoe Point. Clark Nunatak and Elephant Point are located in Livingston Island, the second largest island in the South Shetland archipelago. Clark Nunatak is a rocky peak located in the SE corner of Byers Peninsula, surrounded by the Rotch Dome glacier. It is estimated that the glacier has retreated from the moraine limits after 1950 (Oliva and Ruiz-Fernández, 2017; Palacios *et al.,* 2020). Whereas Elephant Point is an ice-free peninsula of 1.16 km^2 in the SW of Livingston Island. It is limited by the Rotch Dome glacier in the north and the sea encircling the rest of its margins. It is estimated that 17 % of the total land surface exposed today in Elephant Point appeared between 1956 and 2010 (Oliva and Ruiz-Fernández, 2015).

The third glacier retreat is located at Biscoe Point, an area of 0.59 km^2 located near the southwest coast of Anvers Island, in the Palmer Archipelago. Until recently, Biscoe Point formed a peninsula joined to Anvers Island by an ice ramp extending from the adjacent glacier. The ice ramp disappeared as the glacier retreated at least between 1985 and 2004 (ATCM XIII, 1985; ATCM, 2004), and a narrow sea channel now separates Anvers Island from the island on which Biscoe Point lies (ATCM, 2014).

Moreover, two cyanobacterial-based mats, collected from Byers Peninsula (BM) and Horseshoe Island (HM), were assessed in this study. BM sample was selected because of its proximity to NS and ES locations, while the HM sample was considered as an external location of the area defined by the three glacier retreat locations along the Antarctic Peninsula.

2.3.2 Sampling

Samplings were conducted during the austral summer in January 2018 and 2019. Soils from Elephant Point (ES) (62°40'S, 60°51'W) were collected in 2019 from an area bordering the southern Rotch Dome glacier terminus and moving away 30 and 70 m (ES1, ES2 and ES3, respectively). Soils from Clark Nunatak (NS) (62°40'S, 60°54'W) were collected in 2018 from an area located at 15 m from the Rotch Dome glacier and moving away 100 and 180 m (NS1, NS2 and NS3, respectively). Soil samples from Biscoe Point (BS) (64°48'S, 63°46'W) were collected in 2019 in an area located at 90 m from a glacier slope of the Marr Ice Piedmont, now on Anvers Island, and moving away 120 and 200 m (BS1, BS2 and BS3, respectively).

Byers Peninsula microbial mat (BM) (62°34'S, 61°13'W) was sampled in a flooding area (2–4 cm deep) at the Southern beaches, while Horseshoe Island microbial mat (HM) (67°48'S 67°18'W) was sampled from a stream located between Skua lake and the beach in the SW sector of the island.

Soil (0-2 cm depth) and microbial mat samples were obtained from collecting 3 subsamples within at approx. 1 m distance. For DNA analysis, samples were placed in sterile 50 ml Falcon® tubes and frozen at −20 °C for shipment and storage until processing in the laboratory. Every sample was obtained directly with the plastic tubes without any tool to avoid potential contamination. For chemistry analysis, 1 kg of soil near the diversity samples was collected and placed in zip bags by using a sterile spoon and frozen at −20 °C for shipment and storage until processing in the laboratory.

2.3.3 Glacier retreat estimation and Soil chemistry

The comparison of the satellite image of Google Earth (GE) from 1990 and 2020 was used to infer the area deglaciated over the last decades. The images were superimposed and the perimeters of the glacier front delineated, thus obtaining a final image with an approximation of the glacial retreat that occurred.

Soil physicochemical characteristics were determined through techniques accredited by the ENAC (Spanish Accreditation Agency). pH was measured in 1 M KCl solution (1:5) and conductivity in demineralized water (1:5). Soil organic carbon (SOC) content was measured by oxidation with dichromate according to UNE-EN 103204. Total nitrogen (TN) was obtained with the Dumas method according to UNE-EN 13654-2. Phosphorus (P), Potassium (K), Calcium (Ca) and Magnesium (Mg) concentration were determined by aqueous extraction and subsequent determination by ICP-AES, according to UNE-EN 16963:2019 (P) and UNE-EN ISO 14911:200 (K, Ca and Mg). C/N ratio was calculated from SOC and TN.

2.3.4 DNA Extraction, Sequencing and Taxonomical Assignment

Total genomic DNA was extracted from homogenized soils and biofilm using MoBio PowerSoil and PowerBiofilm DNA isolation kit (MO BIO, Carlsbad, CA, USA), according to standard procedures. To ensure an efficient DNA extraction from the soil samples, a high-speed benchtop homogenizer was used. DNA concentrations were quantified by fluorimetry with PicoGreen (Invitrogen, CA, USA). The DNA extractions from the subsamples were mixed in equal proportions in a single sample, thus obtaining 3 samples per glacier forefield and one per microbial mat. The 16S rRNA gene was

amplified by PCR using primers set 341F (5'- CCT AYGGGRBGCASCAG -3') and 806R (5'-GGACTACNNGGG TATCTAAT -3') targeting the V3–V4 hypervariable regions (Otani *et al.,* 2014), and primers specific for cyanobacteria with ample use in the literature (CYA359F/CYA781Ra and CYA781Rb) following reaction mix and PCR conditions from Nübel *et al.* (1997). The pool of samples with the prepared libraries was sequenced by the Illumina MiSeq platform.

All sequence processing and analysis were performed using QIIME (Quantitative Insights Into Microbial Ecology) software v2-2019.4 (Bolyen *et al.,* 2019). Cleaned and trimmed paired reads were filtered and denoised using DADA2 plug-in (Callahan *et al.,* 2016). For chimera identification, 500000 training sequences were used. Identified amplicon sequence variants (ASVs) were aligned using MAFFT (Katoh *et al.,* 2002) and further processed to construct a phylogeny with fasttree2 (Price *et al.,* 2010). Taxonomy was assigned to ASVs using the q2-feature-classifier (Bokulich *et al.,* 2018) and blasted against the SILVA v132 99 % 16S sequence database (Quast *et al.,* 2012). ASV's affinities reported as 'cyanobacteria' from the primers specific for cyanobacteria were re-aligned with sequences obtained from the NCBI GenBank using the Clustal W 1.4 software (Thompson *et al.,* 1994) for more precise taxonomic identification. 16S rRNA gene sequences from GenBank were selected using the NCBI MegaBlast tool (http://blast.ncbi.nlm.nih.gov/ Blast.cgi, accessed 16.06.21). The final alignment length was 385 bp. Phylogenetic tree of 16SrRNA gene sequences of cyanobacteria was constructed in MEGA X using the Maximum Likelihood (ML) method (Kumar *et al.,* 2016). The best-fitting evolutionary model, chosen as the one with the minimun BIC (Bayesian Information Criterion), was the Kimura 2-parameter model (Kimura, 1980). 1000 bootstrap replicates were performed for the phylogenetic tree.

After taxonomic assignment of cyanobacterial ASVs, the community was classified according to Hoffmann *et al.* (2005). Filamentous non-heterocystous cyanobacteria were clustered in the orders Pseudanabaenales (comprising generally thin filamentous taxa with parallel thylakoids such as *Leptolyngbya* and *Pseudanabaena*) and Oscillatoriales (larger filamentous taxa with radial thylakoids such as *Microcoleus, Phormidium* and *Phormidesmis*). Unicellular taxa were assigned to the orders Synechococcales (parallel thylakoids) and Chroococcales (radial thylakoids). Finally, all heterocystous taxa were grouped in a single order, the Nostocales.

Sequences generated by this study were deposited to GenBank under the BioProject accession number PRJNA678471.

2.3.5 Data Analysis

The correlation of the soil chemical composition and phylotype richness (order and genera level) along the deglaciation gradient was analysed with a one-way ANOVA test using the R-package Vegan 2.5–3. The analyses were carried out by clustering the soils into three groups according to the periods since deglaciation (e.g. soil stage 1: BS1, ES1 and NS1). 'Total N' and 'assimilable P' were not included in the analyses since their concentrations remained below the detection limits at least in one location.

Alpha diversity indices (Richness and Shannon Index) were estimated using the PAST 4.03 software (Hammer *et al.,* 2001). Beta diversity was assessed using Bray-Curtis dissimilarities between the community compositions of the sampling sites and visualized with non-metric MultiDimensional Scaling (NMDS) using the R package “phyloseq” (McMurdie and Holmes, 2013). A hierarchical cluster dendrogram was also used to

visualize similarities among the cyanobacterial communities (ASV level) from the studied soils. Differences in relative abundances among bacterial communities were corroborated statistically with PERMANOVA using the R-package Vegan 2.5–3. Differences are considered statistically significant if p-value < 0.05.

Relationships between abiotic (soil chemical parameters) and biotic (relative abundances of the bacterial community) variables, and soil microbial community structure (at ASV level) were examined and graphically represented using two distance-based redundancy analysis (db-RDA, Legendre and Anderson, 1999). A ORDISTEP analysis was performed to identify the subset of variables with the best correlation to community data also considering the possible collinearity between them. All analyses were conducted in R using the package "vegan" (Oksanen *et al.,* 2016).

To further examine the distribution of different ASVs among the different samples, a presence/absence data matrix was built to calculate the 'unique ASV' (present in a glacier forefield location and absent in the others) and 'shared ASV' (present in more than one glacier forefield location). Also, ASVs were binned into 'common community' if present in 100 % of soil samples from a glacier forefield location. Those 'common community' ASVs present in the three glacier forefield, constitute the 'Soil Cyanobacterial Core Community' (present in 100 % of the studied soils of the three glacier forefield). When the 'common community' analysis was performed for the soil and microbial mat samples, the resulting community was the 'Cyanobacterial Core 100' (present in 100 % of the studied samples).

2.4 RESULTS

2.4.1 Free-ice soil ages and their physichochemical characteristics change along the glacial forefields

The analysis of the GE satellite images taken between 1990 and 2020 evidenced the glacial retreat occurred in the sampled areas during the last 30 years (**Figure 1**). From these data, we determined that each one of the 3 soils sampled at each sampling location had been ice-free for a different period. On the one hand, the soils closest to each glacier front (BS1, ES1 and NS1) are estimated to be 5-20 years exposed. On the other hand, the most distant soils from the different glacier fronts (BS3, ES3 and NS3) would have more than 30 years, since they are located outside the estimated glacial retreat area in this time period. BS2, ES2 and NS2 samples would be related to an intermediate soil development stage. Therefore, a soil age gradient was estimated considering their distance to the studied glacier fronts and the time since they were ice-free.

Soil chemical composition fluctuated along the soil age gradient. SOC content shifted from 11.0–13.6 g kg^{-1} in younger (BS1, ES1 and NS1) to 12.8–16.7 g kg^{-1} in older (BS3, ES3 and NS3) samples ($F = 0.2$, $p = 0.8$). Weak increasing trends were also observed for K ($F = 0.4$, $p = 0.7$) and Mg ($F= 0.1$, $p = 0.9$), while C/N ratio decreased from 4.1-5.0 (in BS1, ES1 and NS1) to 2.7-5.5 (in BS3, ES3 and NS3) ($F = 0.6$, $p = 0.6$). pH shifted from 7.7-8.0 (BS1, ES1 and NS1) in younger to 5.5-6.2 in older (BS3, ES3 and NS3) samples ($F = 0.1$, $p = 0.9$) (**Figure S1**).

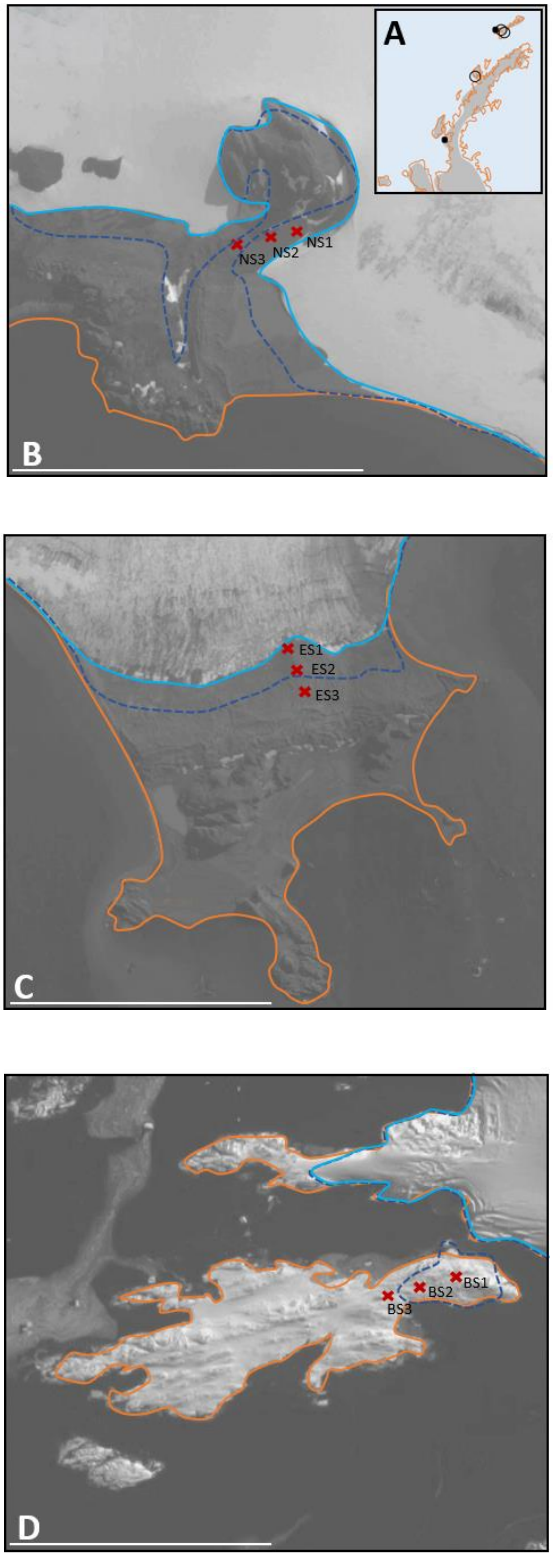


Figure 1. (A) Location of the sampling sites along the Antarctic Peninsula. Circles indicate the studied glacier retreat areas, while dots indicate microbial mats. A more detailed representation of the sampling sites: Clark Nunatak **(B)**, Elephant point **(C)** and Biscoe point **(D)**. The dashed blue line indicates the glacier front in 1990, while the solid light blue line indicates the glacier front in 2020. The red crosses represent the points where the soils included in this study were collected. The white line indicates a 1 km scale.

2.4.2 Relative abundances of cyanobacterial sequences in the overall bacterial community differed among the three sites

The bacterial communities were dominated by Proteobacteria, Actinobacteria and Bacteroidetes, accounting for 66-72 % of the total sequences of each glacier forefield (**Figure S2**). The relative abundance of Cyanobacteria in the overall bacterial community differed among the three sites. At Biscoe Island (BS) glacier forefield Cyanobacteria made up 19 % of the overall bacterial communities on average (mean), 14.2 % in ES, and 7.1 % in NS glacier forefield. Variations were also found throughout the three chronosequences, moving from 22.4 % in BS1 to 2.7 % in BS3. In ES and NS glacier forefields, the maximum relative abundances were reached in the intermediate soils with 8.3 and 8 % (for ES2 and NS2, respectively) of the total sequences of the bacterial community.

2.4.3 Cyanobacterial communities are dominated by filamentous taxa

A total of 1,771,375 reads with an average length of 403 bp were obtained (average of 196,819 sequences per sample) for the soil samples. These initial reads were sorted out in 4010 different ASV. After removal of low-quality, chimeric, and non-cyanobacterial sequences (including chloroplasts sequences), 475,834 sequences remained (26.9 % of the initial reads) and were grouped into 315 different ASVs. The analysis of the composition of the cyanobacterial communities considering only the dominant ASVs (defined as those sequences with relative abundance > 0.8 % over the total community across all samples) revealed that non-heterocystous filamentous cyanobacteria (Pseudanabaenales and Oscillatoriales; sensu Hoffmann *et al.,* 2005) dominated all

sampling locations (**Figure 2A**). The cyanobacterial community composition along the age gradient showed a similar shift for Clark Nunatak (NS) and Elephant point soils (ES), where Oscillatoriales dominated in NS1 and ES1 (59 and 60.8 %) and NS3 and ES3 (69.7 and 79.8 %), while Pseudanabaenales prevailed in NS2 and ES2 (72 and 48.5 %). However, in Biscoe Island (BS), Oscillatoriales dominated the three samples along the soil age gradient (70, 72.5 and 87.2 % for BS1, BS2 and BS3, respectively) while Pseudanabaenales ranked as the third most abundant order (8.2, 7.7 and 6.1 % for BS1, BS2 and BS3). Sequences assigned to Nostocales in BS and ES were more abundant in the younger soils (21.8 and 14.2 % for BS1 and ES1) and decreased in those related to older development stages (6.7 and 2.4 % for BS3 and ES3). In NS2, Nostocales reached 2.4 % of the total cyanobacterial sequences, remaining below 1 % in NS1 and NS3. Sequences assigned to Synechococcales appeared in ES and NS but remained with a relative abundance < 0.5 %.

For Oscillatoriales and at the genera level (**Figure 2A**), *Microcoleus* dominated but decreased throughout BS glacier forefield (from 53 to 31 %, for BP1 and BP3), while *Phormidium* (6.4-28.6 %) and *Phormidesmis* (9.7-24.2 %) increased. In ES and NS, sequences assigned to *Microcoleus* increased in older soils (29.0-44.3 % in ES and 3.3-27.0 % in NS), while sequences assigned to *Phormidium* were more abundant in younger soils (5.9-2.3 % and 4.9-4.1, respectively). *Phormidesmis* showed opposite trends in ES and NS (22-32 % and 43.7-36.3 %, respectively). For Pseudanabaenales, the genera *Pseudanabaena* showed the same pattern in the three sampling locations, with maximum relative abundances in intermediate soils, and being the dominant genus in ES2 (48.1 %) and NS2 (71.9 %).

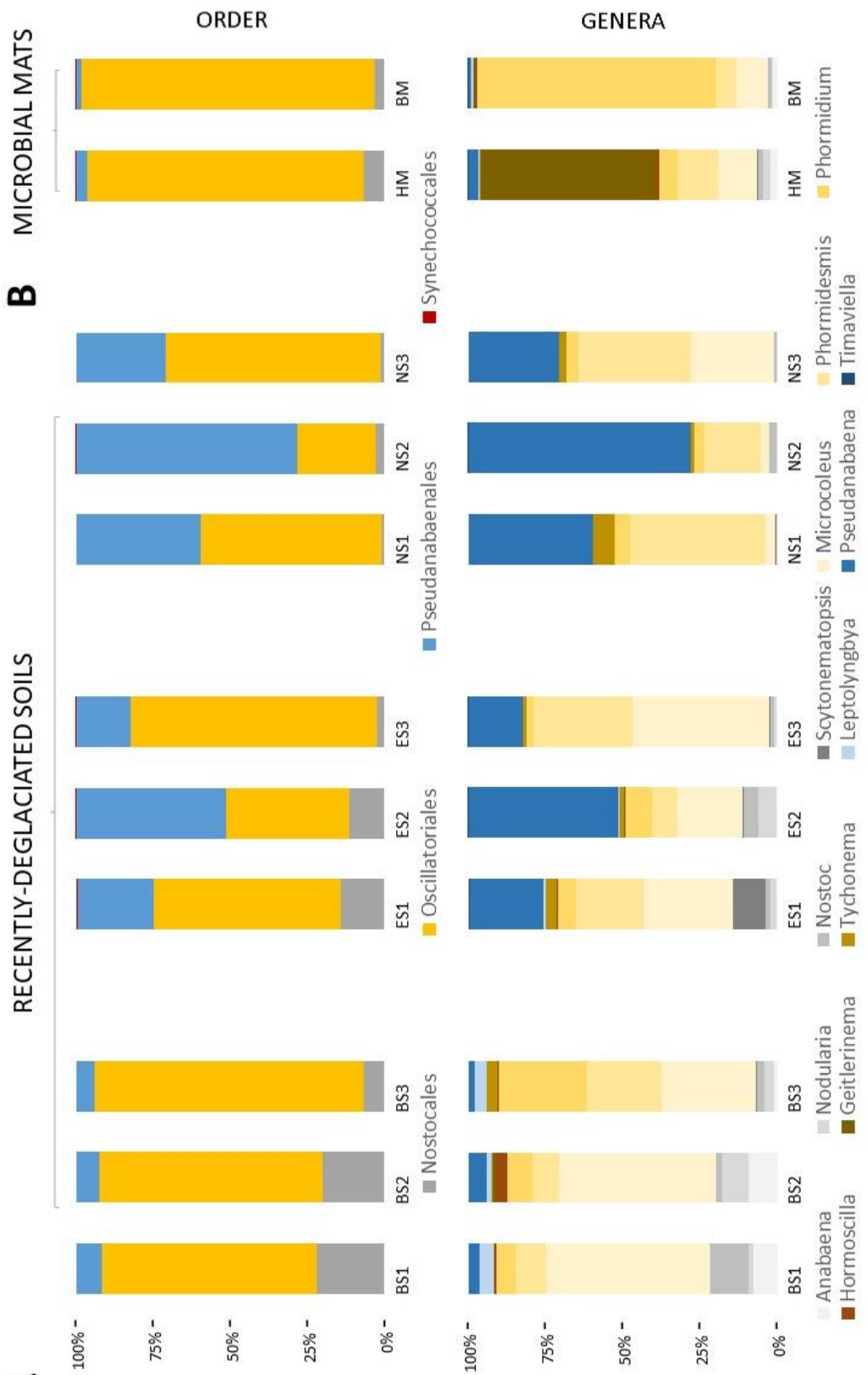


Figure 2. Cyanobacterial community composition **(A)** along the soil age gradient and **(B)** in the studied microbial mats.

When the variation of the relative abundance of the different cyanobacterial groups was studied, Synechococcales (F = 0.1, p = 0.9) and *Timaviella sp.* (F = 0.1, p = 0.9) showed significant differences, decreasing from young to older soils. For the other groups, a considerable fluctuation within and among locations appeared (**Figure S3**).

In microbial mats, the sequences assigned to Oscillatoriales were also predominant in both samples, showing a relative abundance of 90.0 and 95.5 % of the cyanobacterial community for HM and BM, respectively (**Figure 2B**). They were followed by Nostocales (6.3 and 3.0 % for HM and BM) and Pseudoanabaenales (3.6 and 1.6 % for HM and BM). At the genus level, *Phormidium* widely dominated BM (77.7 %), while *Geitlerinema* (57.0 %) dominated in HM, followed by *Phormidesmis* (13 %).

2.4.4 Cyanobacterial richness shifts with soil age

For the alpha diversity indices (**Figure 3**), cyanobacterial phylotype richness decreased 12-20 % with increasing soil age in ES and BS locations, respectively. NS showed a different trend, with a maximum ASV richness in NS2 (112 ASVs) and with similar values in the other two sampling sites (90 and 85 ASVs) respectively). The Shannon index showed similar values along the glacial forefield in BS. For ES, the diversity index decreased with the soil age (from 3.6 in ES1 to 2.8 in ES3), while in NS the highest value was reached in the older soil (3.2).

For microbial mats, the richness of cyanobacterial ASVs (84 and 106 for HM and BM) remained within the range shown by BS, ES and NS. However, the Shannon index values were much lower than those shown for soils (0.9 and 1.6, respectively).

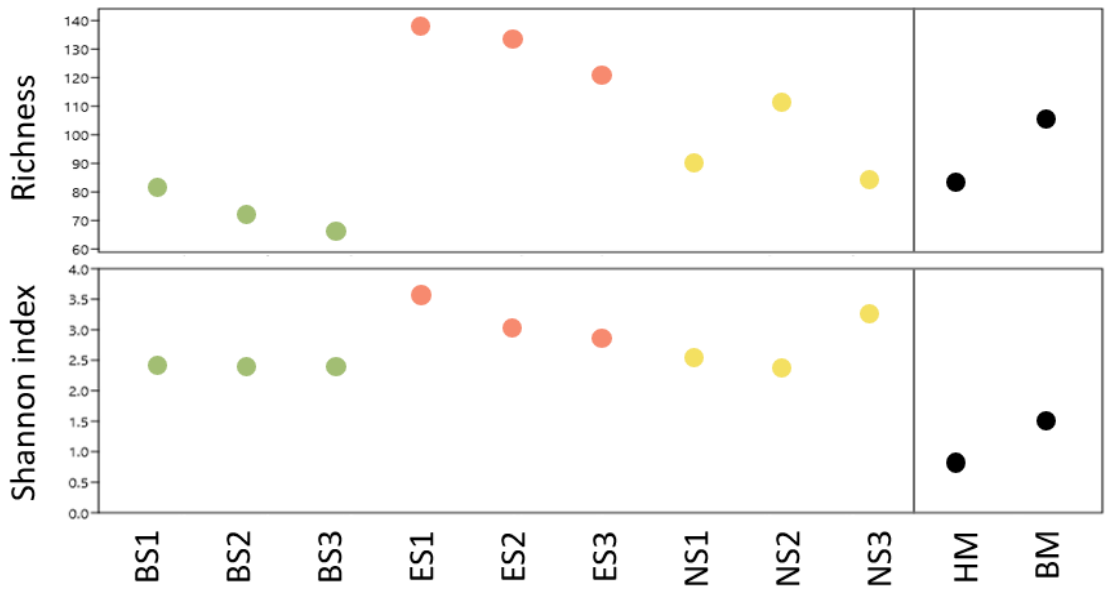


Figure 3. Alfa diversity indices of soils and microbial mats included in this study.

Multidimensional scaling based on Bray-Curtis similarities among cyanobacterial communities (using ASVs) showed a clear separation in their structures among the three glacier forefields (**Figure 4A**). Community differentiation among locations was corroborated statistically using PERMANOVA (F = 2.0, p = 0.00). However, the cyanobacterial community inhabiting soils that remained ice-free for the longest time period in each glacial forefield (BS3, ES3 and NS3) were more similar to each other than to the other sites at the same locations, appearing in the same cluster when a dendrogram of their communities was performed (**Figure 4B**). Among soil chemical parameters (abiotic variables), pH was the only significant predictor of the community structure, accounting for 46% of the variation (F = 6.1, p = 0.00) (**Figure S4A**). When biotic variables were analysed, the RDA graph revealed that Acidobacteria,

Armatimonadetes and Verrucomicrobia were significant predictors of cyanobacterial community structure, accounting for 60 % of the variation (F = 2.4, p = 0.01) (**Figure S4B**).

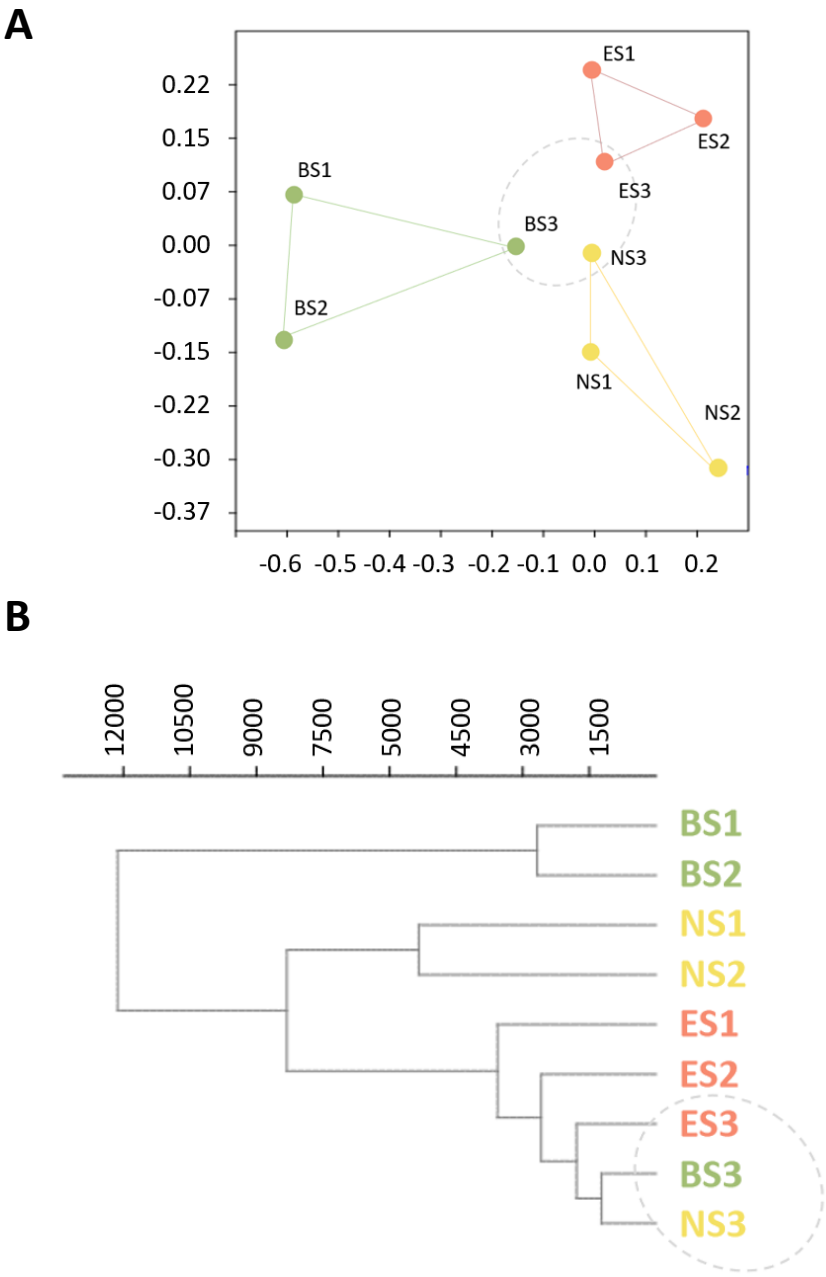


Figure 4. (A) Non-metric multidimensional scaling (NMDS) of the cyanobacterial community composition in the soils from the studied glacier forefields. The multivariate analysis is based on Bray-Curtis dissimilarity matrices between the community profiles at ASV level. Distances between symbols on the ordination plot reflect relative dissimilarities in community structures. **(B)** Hierarchical cluster dendrogram of the cyanobacterial community (ASV level) of the soils from the studied glacier forefields. The relative abundances were used to evaluate the relationships between cyanobacterial communities, using weighted pair clustering based on Barry-Curtis measurements.

2.4.5 The Core Community is constituted by 8 genera of Cyanobacteria

The analysis of the ASVs present at each glacial retreat location (**Figure 5A**) revealed the presence of 67, 23 and 38 'unique ASVs', not shared among the different locations in ES, BS and NS, respectively. In addition, 44 ASVs were shared by at least one soil sample from each location. When the ASVs present in 100 % of the soil samples from each glacial retreat location (e.g. BS1, BS2 and BS3) were compared (**Figure 5B**), only 1 'unique ASV' emerged in BS and NS, while in ES contained 4. These ASVs were defined by sequences assigned to Oscillatoriales and Nostocales for ES, while in BS it was assigned to Synechococcales. In NS, the 'unique ASV' did not match with any known sequence from the database. 14 ASVs were present in all 3 glacial retreat locations and all its sampling points, henceforth referred to as the Soil Cyanobacterial Core Community, and represents a total relative abundance of 68.2-81.7 %, 60-78.7 % and 51.5-81.7 % in BS, ES and NS glacial retreat locations.

The Soil Cyanobacterial Core Community was constituted by sequences assigned to Oscillatoriales (8 ASVs), Pseudanabaenales (3 ASVs), Nostocales (2 ASVs) and Chroococcales (1 ASV), belonging to the genera *Pseudanabaena, Phormisdesmis, Phormidium, Microcoleus, Tychonema, Nostoc, Nodularia* and *Chroococcidiopsis* (**Figure 5C**). The 14 ASVs from the core community were also found in HM and BM microbial mat samples (Cyanobacterial Core 100), where represents a relative abundance of 29.8 % and 17.4 %, respectively, thus constituting a core community present in both the studied soils and microbial mats from the Antarctic ecosystem.

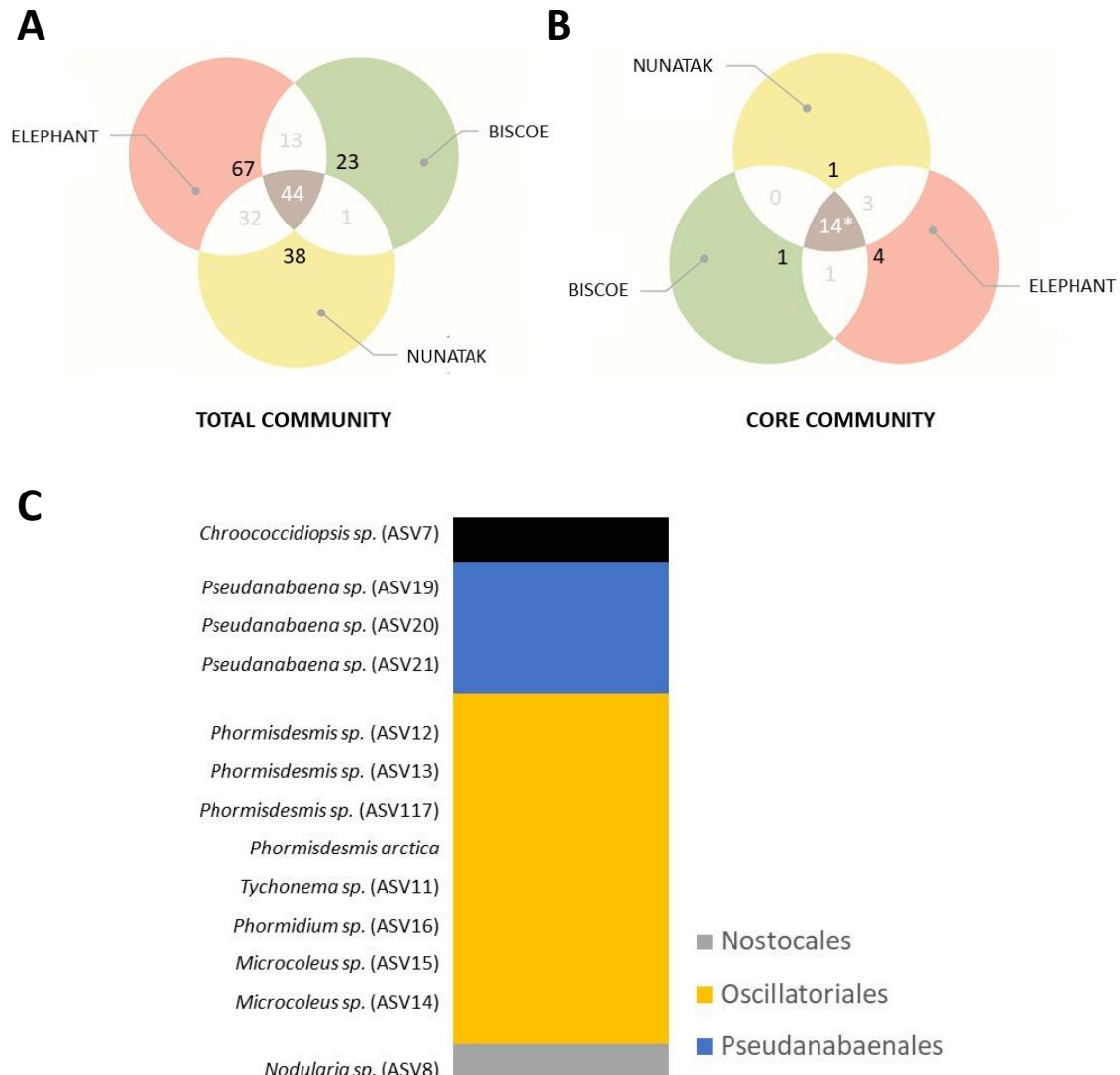


Figure 5. Venn diagram of cyanobacterial amplicon sequence variants (ASVs) from the glacier forefields studied. **(A)** Unique and shared ASVs among sampling locations determined from the analysis of the entire cyanobacterial community. Pie charts represent the taxonomic ascription of those unique cyanobacterial ASVs (number again in the middle) of each sampling location. The central brown area of the Venn diagram indicates the number of ASV found in at least one soil sample of each sampling site. **(B)** Unique and shared ASVs among sampling locations determined from the analysis of the 'common community' (sequences present in 100 % of the soil samples from each sampling location). The central area of the diagram indicates the ASV found in 100 % of the studied soil samples from all the studied sampling location (Soil Cyanobacterial Core Community). **(C)** Taxonomical adscription of the 14 ASV which constitute the Soil Cyanobacterial Core Community.

2.5 DISCUSSION

Our main results focus on cyanobacterial communities in the earliest stages of primary succession along three glacier forefields in Antarctica. Since Cyanobacteria play the role of principal primary producers in some Antarctic terrestrial ecosystems, a more thorough knowledge on how these photosynthetic bacteria respond to changing environmental conditions is key to better understand the functioning of polar ecosystems.

The analysis of sequencing data obtained from 16S rRNA gene with universal primers revealed that Proteobacteria and Actinobacteria dominated the studied soils, as occurs in other (non-)polar and alpine retreating glacier forefields (Pessi *et al.,* 2015; Fernández-Martínez *et al.,* 2017; Jiang *et al.,* 2018). These phyla include several phototrophic, photoheterotrophic and chemolithotropic taxa able to thrive in the oligotrophic environments represented by recently exposed soils (Rime *et al.,* 2016; Wei *et al.,* 2016). For Cyanobacteria, their relative abundance in the overall bacterial community differed among the three glacier forefields and within them. Higher relative abundances in areas close to the glacier front, as shown in BS, had been previously reported (Bajerski *et al.,* 2013; Fernández-Martinez *et al.,* 2017). This trend to decrease their presence in later succession stages has been related to their role as pioneer microorganisms (de Caire *et al.,* 1997; Vincent 2000; Elster 2002; Frey *et al.,* 2013; Rime *et al.,* 2015). However, in ES and NS locations, Cyanobacteria was almost absent in the most recently exposed soils and reached a considerable relative increase in the next sample following the chronosequence. Similar results have been reported in recently deglaciated soils from temperate regions (Sattin *et al.,* 2009), suggesting the presence

of 'ancient' or allochthonous organic matter (Hodkinson *et al.,* 2002) that would limit the competitive advantages of (photo)autotrophic microbes in the youngest soils. However, SOC values suggest lower organic carbon concentrations in the younger soils compared to the older soils along the three glacier forefields surveyed. Therefore, the study of Cyanobacteria at a level of higher taxonomic resolution is necessary to delve into the structure of their communities and understand the dynamics of the different taxa.

Considering that primer choice can alter reported values of relative abundance and dominance of bacterial communities from environmental samples (Fredriksson *et al.,* 2013), insights into the cyanobacterial community by using specific primers is highly recommended to avoid over or underestimations of certain groups' abundance. From this approach, our results did not show a consistent linkage between cyanobacterial community structure and soil age, as previously reported from the Arctic (Pessi *et al.,* 2019). The abiotic factors have been considered of a greater influence on the colonization and settlement of organisms during primary succession processes (Raab *et al.,* 2012). But based on the chemical properties analysed in this study, no significant edaphic development appears along the three chronosequences for the studied variables, besides the variation of pH values observed. Similar results have been reported from recently exposed areas in Antarctica (Vega-Garcia *et al.,* 2021), with soil development processes mainly related to characteristic non-vascular plant communities (e.g. mosses and lichens) and natural fertilization processes (Boy *et al.,* 2016). Our results suggest that, while abiotic factors probably shape cyanobacterial communities, as shown for pH values, their composition was influenced by relative abundances of Acidobacteria, Armatimonadetes and Verrucomicrobia. Previous publications have

pointed to diverse bacterial communities associated with Cyanobacteria, as heterotrophic satellite bacteria which supply indispensable growth factors (Cornet *et al.,* 2018). Also, processes of competition, facilitation, tolerance and/or inhibition with the different bacterial communities should occur (Raab *et al.,* 2012), as suggested for Cyanobacteria and chlorophytes in glacier retreat areas from the Artic (Pessi, 2017). Although most of these interactions have been studied in temperate freshwater ecosystems and under *in vitro* conditions (Eiler and Bertilsson, 2004; Dziallas and Grossart, 2011; Shao *et al.,* 2014; Zhu *et al.,* 2016), and very little is known for the natural soil ecosystems. Therefore, and considering the little alteration in chemical properties through the studied bare soils, given the short timeframe, biotic factors should be considered as a key driving force for the changes in the distribution of cyanobacterial phylotypes we observed in the three glacier forefields. Also, other unmeasured abiotic and biotic variables could be responsible of the differences among communities. Further studies are required to understand the relationship between Cyanobacteria and soil microbial community composition, including also the eukaryotic portion. In addition to exploring structural differences among communities, enhanced knowledge on their functions (e.g. metatranscriptomics) would provide valuable insights.

Common dominant cyanobacterial taxa at the order level were observed throughout the three glacial forefields. Oscillatoriales dominated cyanobacterial communities in relative abundances, with Pseudanabaenales comprising the second most abundant group at each site, except in ES2 and NS2 where this order dominates. These results are consistent with previous studies of bacterial succession in disparate glacier forefields (Frey *et al.,* 2013; Pessi *et al.,* 2019; Knelman *et al.,* 2021), adding more evidence to the potential role of non-heterocystous filamentous cyanobacteria as prominent colonizers

of the earliest primary succession soils. The majority of the Oscillatoriales sequences we retrieved were assigned to *Microcoleus sp.* and *Phormidium sp.*, but also *Phormidesmis sp.* was widely present in the soils. Since this later genus traditionally included species originally included in the genera *Leptolyngbya* and *Phormidium* (Turicchia *et al.,* 2009; Komarek *et al.,* 2009; Raabova *et al.,* 2019), there could be a misunderstanding in their role in primary succession processes, despite its distribution in the Antarctic, Arctic, and Alpine environments (Chrismas *et al.,* 2015; Raabova *et al.,* 2019). Our results suggest an important role of *Phormidesmis sp.* as a pioneering autotrophic organism in recently deglaciated soils in Antarctica, even dominating the cyanobacterial community of younger bare soils, as shown in NS. Regarding the dominant non-heterocystous filamentous cyanobacteria showed along the sampling locations, which also include *Pseudanabaena sp.*, are known to develop environmental adaptations and stress responses to cope with cold temperatures and osmotic stress (Lange *et al.,* 1994; Belnap and Lange, 2001; Chrismas *et al.,* 2016) thanks to the extracellular polymeric substances (EPS). These EPSs may help to retain water during dry periods and are essential for the stabilization of the substrate in recently deglaciated areas (Mataloni *et al.,* 2000, Belnap and Lange, 2001).

Nitrogen-fixing cyanobacterial groups were also an integral part of the investigated communities. These groups, mainly constituted by Nostocales (*Nostoc sp., Nodularia sp., Anabaena sp. and Scytonematopsis sp.*), were more abundant in the youngest soils and decrease in those areas furthest from the glacier terminus. Previous studies showed that the relative abundance of N_2-fixing Nostocales is limited in early stages of succession increasing with soil age (Frey *et al.,* 2013; Nemergut *et al.,* 2007; Schmidt *et al.,* 2008, 2009), due to their association with some level of early vegetation (Arróniz-Crespo *et*

al., 2014), and in some cases limited by phosphorus availability. Phosphorus appeared as the most relevant nutrient limiting primary succession in the earliest stages along glacial chronosequences in the Central Andes and central Alaska (Darcy *et al.,* 2018), especially controlling Nostocales (Knelman *et al.,* 2021). But P concentrations were below our detection limit (10 g kg^{-1}) in BS and NS glacier forefields, while remained fairly stable along ES soils. The decrease of N_2-fixing cyanobacteria relative abundance with soil age observed could be related to the presence of other diazotrophic microorganisms, which would explain the slight increase of the amount of N accumulated in older soils. Within the diverse community of diazotrophic bacteria in glacier retreat soils, Cyanobacteria do not appear as the predominant group (Duc *et al.,* 2009). Therefore, their role as pioneer organisms would be highlighted by generating organic carbon inputs and fixing nitrogen in the early stages, facilitating the settlement of microbial communities in more mature soils.

The disparity of cyanobacterial communities was evident when the analyses were carried out at the ASV level, with beta diversity analyses discriminating between three general community types according to the glacier forefield. This result is in concert with previous studies, which showed high levels of spatial heterogeneity in prokaryote biodiversity across terrestrial environments in Antarctica (Barrett *et al.,* 2006a; Chong *et al.,* 2010; Almela *et al.,* 2021; Fernández-Martínez *et al.,* 2021). However, in those soils that have remained ice-free for a longer period, the structure of their communities differs slightly from the other samples within each glacier forefield, resembling more with the older soils from the other locations. Older soil samples from all three locations have shown the lowest values of richness of ASVs, the lowest values of diversity in BS and ES, and the lowest relative abundances of cyanobacteria within the whole bacterial

community for BS and NS. Considering these data and the traditional knowledge on primary succession, we could suggest that a less diverse cyanobacterial community of generalist taxa tends to prevail against pioneer taxa and establish on soils related to more advanced stages of primary succession.

A consistent Soil Cyanobacterial Core Community was found in all sampled soil and sampling location. These ubiquitous cyanobacterial sequences dominated the cyanobacterial community, representing from 51.5 to 81.7 % of the total cyanobacterial sequences. Analysis of communities considering the ASVs, which differ from each other by a single nucleotide (Callahan *et al.,* 2016, 2017), offers an approach to gain insight into the global distribution of microorganisms (del Moral *et al.,* 2021). Our detection of identical cyanobacterial ASVs in the three glacier forefields, 280 km apart, served to confirm their distribution on Antarctic non-marine ecosystems, and probably the high mobility of these genotypes among the different locations. Although an important proportion of them may correspond to globally dispersed species since more than half of these sequences were not associated with polar or cold ecosystems (data not shown). In previous studies, it has been suggested the prevailing exclusive nature of pioneering organisms in these regions (Frey *et al.,* 2013; Pessi *et al.,* 2019). Our results suggest that the more we expand our understanding of cyanobacterial communities around the world, the better we understand their ability to cope with multi-extreme conditions (Mehda *et al.,* 2021), and most likely provided of a high dispersal capacity through the atmosphere as airborne microorganisms even for large filaments (Galbán *et al.,* 2021).

Members of this core community may also be important keystone species involved in microbial mats development since this cyanobacterial core community was also present

in the studied mats. It is considered that the survival and development of Cyanobacteria in extreme environments are mainly due to their ability to produce biofilms, especially microbial mats (Convey, 2013; Chong *et al.,* 2015). Most of the sequences belonged to non-heterocystous filamentous cyanobacteria (e.g. *Microcoleus sp., Phormidium sp., Phormidesmis sp., Pseudanabaena sp., Tychonema sp.*), related to the production of EPS. Therefore, they would not only be involved in the stabilization of the soil but in improving structural conditions that lead to the consolidation of complex microbial mat communities. Furthermore, most of these AVS were associated with genera that have been described as true psychrophiles (Fritsen and Priscu, 1998; Nadeau and Castenholz, 2000; Vincent and Quesada, 2012; Chrismas *et al.,* 2015), compared to a large majority of psychrotolerant cyanobacterial species in the Antarctic ecosystem (Roos and Vincent, 1998; Singh and Elster, 2007; Vincent, 2007; Zakhia *et al.,* 2008), with optimal metabolic growth rates from 15 °C to 35 °C (Tang *et al.,* 1997). Nostocales appeared within this group, underscoring their contribution to the diazotrophic community and their role in introducing nitrogen in environments that are mostly nutrient-poor. Despite unicellular cyanobacteria are in general not commonly found in soils due to their low mobility (Pushkareva *et al.,* 2016), *Chroococcidiopsis sp.* was also involved in this central community. This cyanobacterium has been described as extremely resistant to ionizing radiation and desiccation (Meslier *et al.,* 2018; Crits-Christoph *et al.,* 2016; Billi *et al.,* 2000), dominating endolithic microbial communities of the hyper-arid zone of the Atacama Desert (Casero *et al.,* 2021). Therefore, this core community would be associated with those organisms that better cope with the Antarctic environmental conditions. In addition, given that these taxa remained stable and dominant throughout the three locations, and considering their relative abundances as an indicator of fitness

(Evans and Wallenstein, 2014), the plasticity of these taxa needs to be considered as key to withstand the different abiotic and biotic variables that control the composition of the studied soils.

2.6 CONCLUSIONS

Our results confirm the role of Cyanobacteria as important pioneers and phototrophs in recently deglaciated forefield soils. Sequences assigned to filamentous non-heterocystous cyanobacteria dominated the three locations, with Nostocales as an integral part but with higher abundances in younger soils. While chemical properties of soils remained almost stable with the range of age investigated, the interaction with other microbial communities appeared as a fundamental factor in determining the structure of cyanobacterial communities. The presence of a core community with high relative abundances suggests the existence of a central community, essential in processes of primary succession of recently deglaciated soils, as well as in the conformation of microbial mats. These results are of special interest given that deglaciated areas are likely to expand in the future according to general circulation models which predict enhanced warming.

2.7 SUPPLEMENTARY MATERIAL

Figure S1. Soil chemical composition along the sampled soils from the deglaciation gradients. Black dots correspond to the median values of each location.

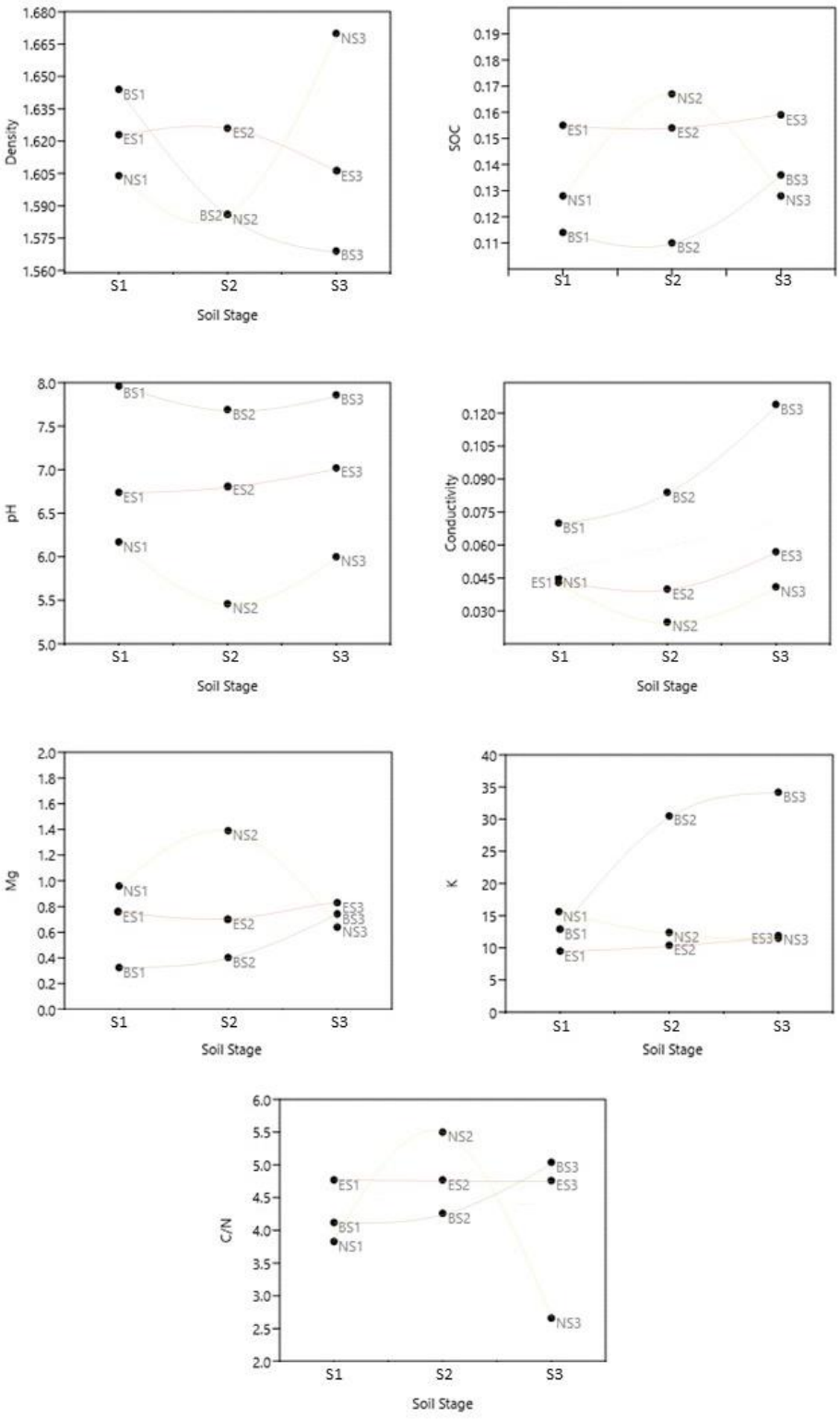


Figure S2. Bacterial community composition in the soils from the studied glacier forefields.

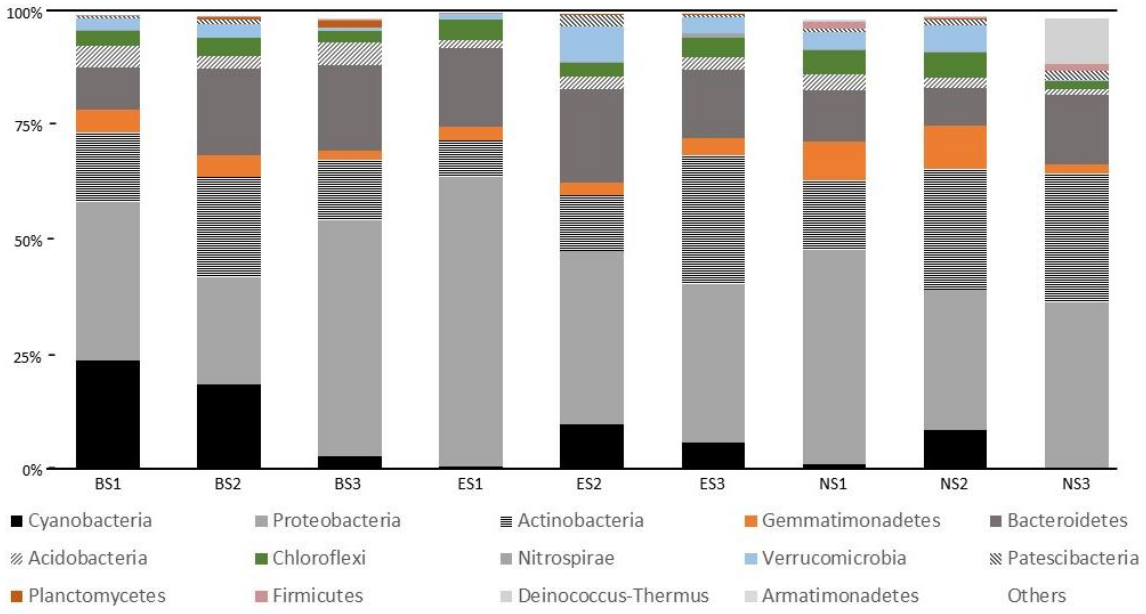


Figure S3. Cyanobacterial relative abundances determined along the sampled soils from the deglaciation gradients. Black dots correspond to the median values of each location.

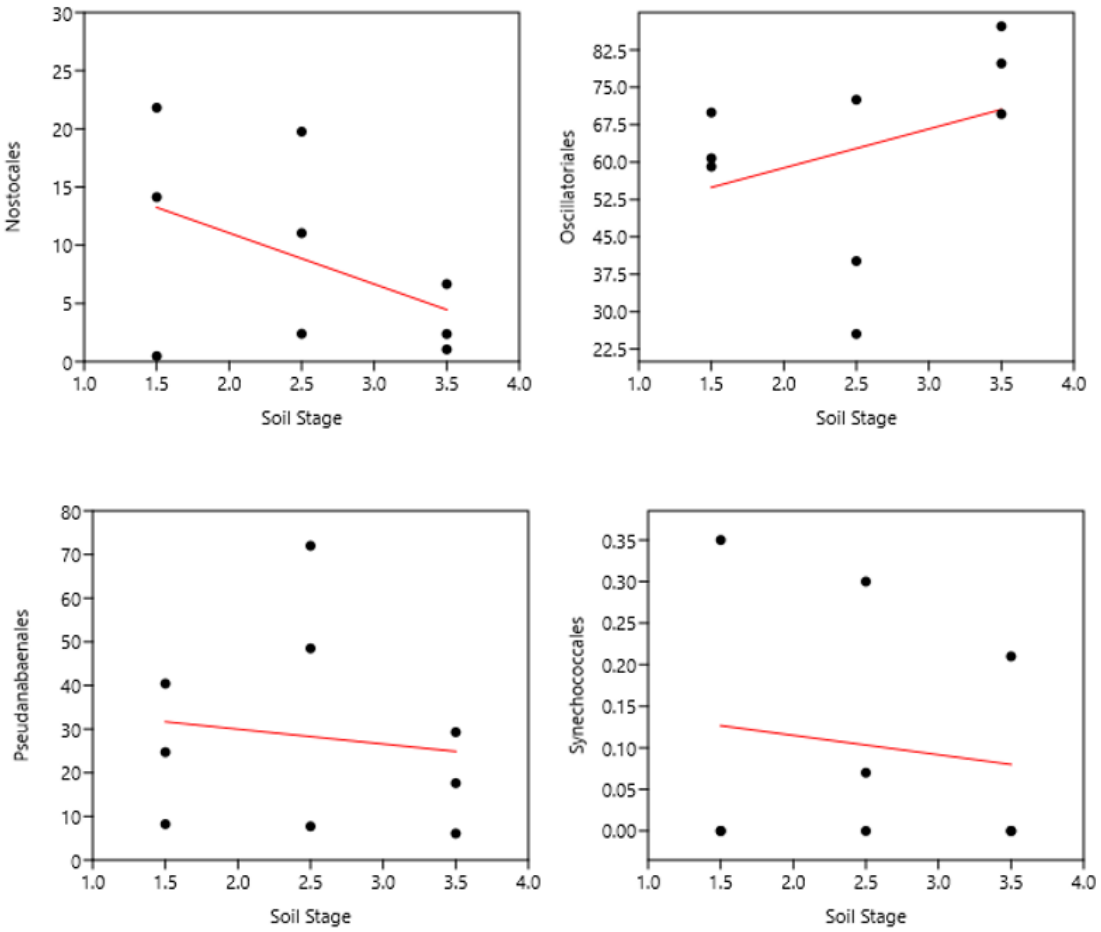
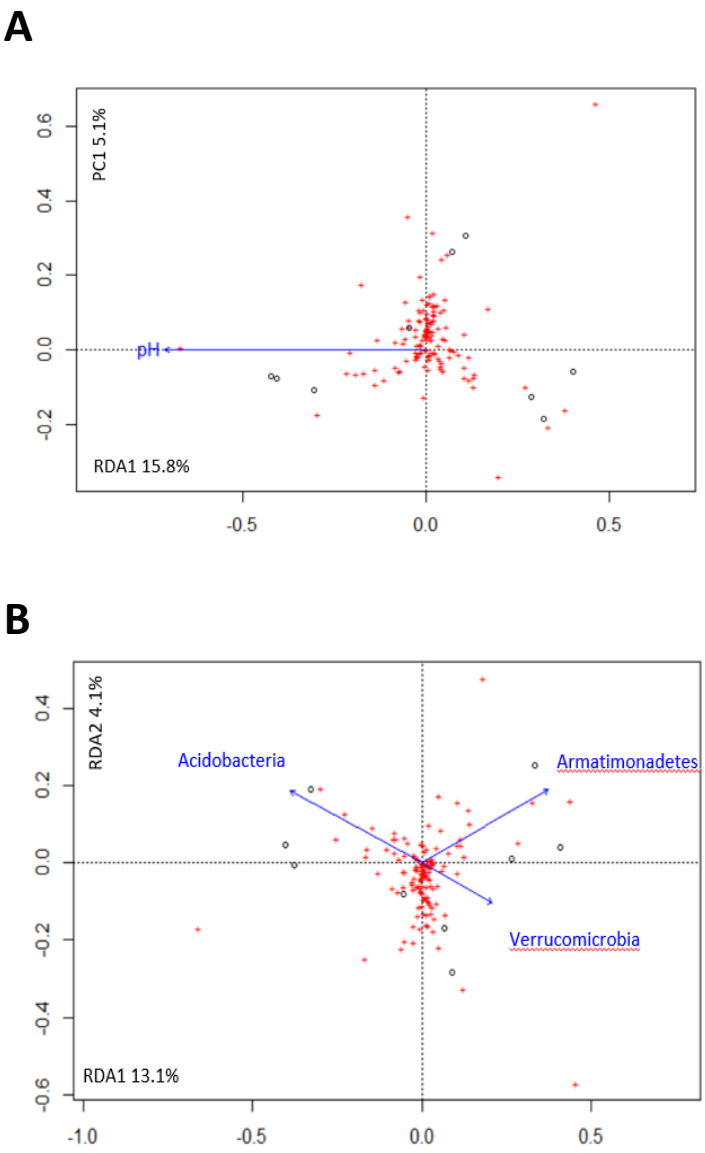


Figure S4. Redundancy analysis (RDA) of the associations of soil microbial community composition with **(A)** abiotic and **(B)** biotic factors.



CAPITULO 3

CARBON PATHWAYS THROUGH THE FOOD WEB OF A MICROBIAL MAT FROM BYERS PENINSULA, ANTARCTICA

FLUJOS DE CARBONO A TRAVÉS DE LA RED TRÓFICA DE UN TAPETE MICROBIANO DE BYERS PENINSULA, ANTÁRTIDA

3.1 RESUMEN

Las esteras microbianas son comunidades complejas que representan una gran fracción de biomasa en los ecosistemas antárticos no marinos. Confieren estructura a los suelos y constituyen, por sí mismos, intrincados microecosistemas, donde una gran variedad de microorganismos y microfauna contribuyen a las funciones del ecosistema. Aunque en los últimos años se han investigado a fondo los tapetes microbianos antárticos, las relaciones tróficas dentro de las comunidades siguen sin resolverse. Por lo tanto, realizamos un estudio de las relaciones tróficas de un tapete microbiano de la Península Byers, Antártida, utilizando análisis de ADN e isótopos estables como trazadores tróficos. Nuestros resultados sugirieron, basados en un modelo de mezcla bayesiano, que al menos cuatro niveles tróficos están presentes dentro de este microecosistema: productores primarios (cianobacterias y diatomeas), consumidores primarios (rotíferos y tardígrados), consumidores secundarios (nematodos) y descomponedores (hongos). Los nematodos jugarían un papel clave como principales consumidores de la comunidad, conectando las dos entradas de carbono descritas en el sistema, como omnívoros en el nivel trófico secundario. Además, las vías del carbono desde el nivel trófico primario hasta los consumidores se producen rápidamente durante las primeras 24 h posteriores a su incorporación a través de los productores primarios, dispersándose por todos los niveles tróficos y llegando a los consumidores secundarios en menos de 11 días. Esto sugiere que, dadas las condiciones físicas cambiantes y los cortos períodos de actividad, existe un fino acoplamiento temporal entre los organismos de la comunidad, minimizando la redundancia en el desempeño funcional entre los niveles tróficos.

ABSTRACT

Microbial mats are complex communities that represent a large biomass fraction in non-marine Antarctic ecosystems. They confer structure to soils and constitute, by themselves, intricate microecosystems, where a great variety of microorganisms and microfauna contributes to the ecosystem functions. Although in recent years Antarctic microbial mats have been thoroughly investigated, trophic relationships within the communities remain unresolved. We therefore conducted a study of the trophic relationships of a microbial mat from Byers Peninsula, Antarctica, using DNA analysis and stable isotopes as trophic tracers. Our results suggested, based on a Bayesian mixing model, that at least four trophic levels are present within this microecosystem: primary producers (cyanobacteria and diatoms), primary consumers (rotifers and tardigrades), secondary consumers (nematodes) and decomposers (fungi). Nematodes would play a key role as top consumers of the community, connecting the two carbon inputs described into the system, as omnivores at the secondary trophic level. In addition, carbon pathways from primary trophic level to consumers take place quickly during the first 24 h after its incorporation in the primary producers, dispersing across all the trophic levels and reaching secondary consumers in less than 11 days. This suggests that, given the changing physical conditions and presumably short periods of activity, there is a fine temporal coupling among the organisms in the community, minimizing the redundancy in function performance among trophic levels.

3.2 INTRODUCTION

Microbial mats, with a ubiquitous distribution throughout Antarctica, are the most widespread microbial consortia in terrestrial landscapes. They constitute the largest non-marine biomass concentrations in these regions (Quesada *et al.,* 2008) and accumulate the greatest biodiversity in inland waters, being recognized as hotspots for biological productivity and diversity. The organisms that inhabit these microecosystems range from viruses to green algae, rotifers, diatoms, nematodes and tardigrades, with cyanobacterial species as the most common organisms (Vincent, 2000; Jungblut *et al.,* 2005). A regular feature in these microecosystems is the presence of differently colored layers due to the different pigmentation of phototrophic microorganisms (Vincent *et al.,* 1993), resulting in a layered vertical structure. These cyanobacteria-based ecosystems have shown a considerable level of community stability throughout time, with some structures almost unchanged over the last 100 years (Jungblut and Hawes, 2017), which suggests a resilience capacity that assures its function as refugia for biological diversity (Chown *et al.,* 2015).

Previous works on microbial mats from polar regions have mostly focused on structural aspects, biodiversity of microbial community and their relationship with the environment (de Los Ríos *et al.,* 2004; Jungblut *et al.,* 2012), but little is known about trophic relationships within these microbial mat ecosystems (Velázquez *et al.,* 2017). Freshwater food webs in Antarctic regions are simpler than those in temperate regions (Hansson and Tranvik, 2003). Soil fauna biodiversity is reduced to 1.1–2.6 % of temperate soils (Freckman and Virginia, 1997), and this is also reflected in the complexity of trophic relationships. However, some microbial ecosystems from

Antarctica showed larger biodiversity than the same ecosystems from lower latitudes (López-Bueno *et al.,* 2009).

Byers Peninsula is one of the largest ice-free areas in the Antarctic Peninsula region, with a well-developed network of water bodies, especially during the snow-melting season. It has been described as one of the main Antarctic hotspots of biodiversity (Convey *et al.,* 1996; Toro *et al.,* 2007) and proposed as a key observing spot to monitor the effects of climate change on freshwater and terrestrial ecosystems (Quesada *et al.,* 2009). Microbial mats are extremely abundant in Byers Peninsula, particularly in the central plateau and associated to its large freshwater network (Toro *et al.,* 2007). These micro-ecosystems are, therefore, essential to understand the diversity, the community structure and dynamics of these ecosystems to forecast future biological responses to perturbations as, e.g. climate change, human activity or invasive species (Velázquez *et al.,* 2017).

Previous analyses indicate different structural organization of microbial mats depending on their community composition (De Los Ríos *et al.,* 2004; Velázquez *et al.,* 2017). Moreover, community carbon assimilation diverges according to relative abundances of chlorophytes and cyanobacteria (Velázquez *et al.,* 2011), with green algae adapted well to cold temperatures and cyanobacteria performing better in warmer conditions. The present study tests the trophic position and the trophic relationships among living organisms that shape the structure of a cyanobacterial microbial mat from Byers Peninsula during the austral summer, by using the stable isotopes of N and C. Accumulation rates of nitrogen isotope can be used to estimate the trophic position of the organisms because of the differential isotopic enrichment of the organism

depending upon its diet (Peterson and Fry, 1987), while ^{13}C isotope can be used to describe the origin of the incorporated C. Moreover, there is a natural isotopic discrimination against ^{13}C and in favor of ^{12}C (Post, 2002), so the trophic relationships (e.g. C transfer) among primary trophic level and consumers in a microbial mat can be determined by incubating the community with $\delta^{13}C$ enriched substrates. Nematoda, Tardigrada and Rotifera, the main microfaunal groups in Antarctic soils (Sohlenius *et al.,* 2004), were the studied consumers, besides the main primary producers presented during the study.

All the community components were sampled at different time points to track the carbon pathways along the trophic web. To the best of our knowledge, this "enriching and hunting" methodology has never been used for microbial mats at these high-latitudes. These results were analysed and tested by a Bayesian mixing model and completed by a small sub-unit of the RNA (SSU RNA) meta-barcoding approach of the community to characterize the bacterial and eukaryotic populations that might interplay along the trophic web.

3.3 MATERIALS AND METHODS

3.3.1 Study site and sampling

Sampling was conducted on a microbial mat from Byers Peninsula (**Figure 1**) during the austral summer in January 2013. The study site is located at the western end of Livingston Island, Antarctica (South Shetland Islands; 62°34'35''S, 61°13'07''W). Byers Peninsula, with a central plateau length of 18.2 km from northwest-southeast, is the largest ice-free area of South Shetland Islands. It is an Antarctic Specially Protected Area

(ASPA N° 126), because of the importance of their biological communities and its geological and archeological values.

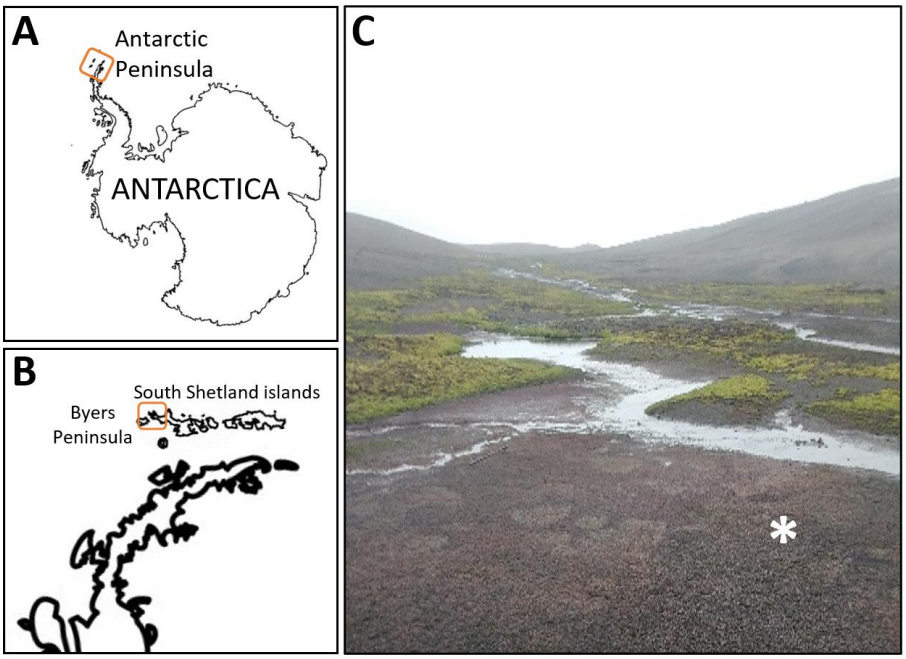


Figure 1. (**A,B**) Location details of Byers Peninsula at Livingston Island in the South Shetland Islands, Antarctica. (**C**) Picture showing common microbial mat types from Byers Peninsula. The symbol "∗" indicates where exactly enrichment experiments were conducted.

The microbial mat was sampled in a flooding area (2–4 cm deep) at the Southern beaches of Byers Peninsula, which was almost 100 % covered by microbial mats (**Figure 1C**). It displayed a vertical structure of bi-layered microbial mats that has been previously described in this area by Rochera *et al.* (2013), due to the pigmentation of the different layers. Six samples were haphazardly collected with metal spatulas from different areas of the wetland to cover a sampling area of ca. 10 m^2. These six samples were placed in the experimental trays (about 600 cm^2) and the sampling within each experimental tray was haphazardly made again. The "enriching and hunting" experiment, explained below, was carried out on-site using the collected samples.

Replicates of all samples were stored in Whirl-pak bags at -20 °C until further analysis at the laboratory.

3.3.2 Microscopic identification of the community composition

The composition of the community involved in the food web of the microbial mat, grouped in primary producers and consumers (microfauna), was studied using a stereozoom microscope (Leica MZ75). Photosynthetic organisms' observations were conducted under white illumination and using epifluorescence microscopy with an Olympus® blue filter set (EF 400–490 nm, DM 570, FB 590) where chlorophyll-a was excited, while differential cyanobacterial phycobilins were excited with an Olympus® green filter set (EF 530–545 nm, DM 570, FB 590).

3.3.3 Biomass determination

Estimations of relative abundances of the microbial mat trophic community were calculated from total biomass from the ash free dry weight (AFDW). After obtaining the carbon content per surface unit of each of the studied groups (consumers, primary producers and fungi), as described below, the biomass value was subtracted from the total carbon calculated for the microbial mat and expressed as percentage.

Phototrophic community biomass (cyanobacteria, green algae, and diatoms) was estimated by chlorophyll-a (Chla) concentration and assuming a proportion of Chla per wet weight unit (ww) of 0.9 % (Kasprzak *et al.,* 2008). Chlorophyll-a was extracted in triplicates from 7 mm in diameter and 2–3 mm in thickness core samples using ethanol,

according to European Union standard ISO 10260, which prescribes 90 % (v/v) ethanol for chlorophyll extraction and measurement at 665 nm. Extracts were measured using a Hitachi U2000 spectrophotometer. A specific density of 1 g cm^{-3} was assumed for transforming the biovolume of the phototrophic community into dry weight (Wieser, 1960). Carbon content was estimated as 40 % of the dry weight, as determined by Feller (1988).

Ergosterol, a biochemical marker of fungal active biomass, was quantified using HPLC equipped with a UV detector (282 nm). For ergosterol extraction cores (7 mm in diameter and 2–3 mm thick according to microbial mat thickness) were placed in triplicates in 12 ml glass culture tubes then 10 ml of KOH 0.14 M were added. Tubes were sealed with rubber caps and placed in a temperature-controlled bath (80 °C) for 30 min while shaking. After cooling at room temperature, two additional extractions were carried out with 10 ml methanol and sonication cycles to maximize the ergosterol retrieval. Extracts were mixed and eluted with a 1 ml min^{-1} flow through the solid-phase extraction cartridges (ExtraBond Cartridge C18 1000 mg. Scharlab). The ergosterol in the eluted product was quantified by HPLC, as described by Gessner (2005). The concentration of ergosterol determined by HPLC is transformed to fungal biomass assuming that 5.5 mg of ergosterol are found in 1 g of fungal biomass (Gessner and Chauvet, 1993). Finally, C in the fungal biomass was estimated as 43 % of the dry weight (Baldy and Gessner, 1997).

Relative abundance of consumers (rotifers, tardigrades and nematodes) was estimated by direct counting in core samples and interpreted per surface unit. Total biomass of each consumer was estimated measuring length and width from microscope

photographs using the image analysis system SigmaScan Pro 5, following the approaches proposed by Ruttner-Kolisko (1977) for rotifers, Benke *et al.* (1999) for tardigrades, and Burgherr and Meyer (1997) for nematodes. A specific density of 1 g cm^{-3} was assumed for transforming biovolume into dry weight (Bottrell *et al.,* 1976). The dry weight was converted into carbon content, which was assumed to be 40 % of the dry weight (Feller, 1988).

3.3.4 ^{13}C and ^{15}N natural abundance analysis and microbial mat enrichment incubations

At the laboratory, microbial mat samples were manually disaggregated, and microorganisms were sorted using a stereozoom microscope (Leica MZ75) for $\delta^{13}C$ ‰ and $\delta^{15}N$ ‰ natural abundance signal analysis. Individuals of different trophic levels were manually separated under the microscope by microdissection and encapsulated in triplicates in 175 pl zinc cases and dried at 65 °C for 48 h. A minimum of 0.02 mg of dry weight biomass was needed (Shaw *et al.,* 2018), achieved by collecting approximately 80–100 live individuals per zinc case. Particulate organic matter (POM) between 30 and 0.22 µm was separated by sequential filtering and dissolved organic carbon (DOC), considered as the supernatant < 0.22 µm, were analyzed for $\delta^{13}C$ ‰ and $\delta^{15}N$ ‰ as above. The sequential filtration was determined by triplicate as follows: a piece of mat was pressed gently against a Nytal sieve of 30 µm pore size diameter, the filtrate was filtered again through a 5 µm filter and the filtrate was then filtered through 0.5 µm pore membrane. The matter retained in the filters was manually recovered for isotopic analyses. The 0.5 µm filtrate was then filtered through a 0.22 µm hydrophilic membrane

and concentrated by evaporation at 40 °C under vacuum. Samples were analyzed by a mass spectrometer of isotopic ratios (MEIR; 20-20 PDZ Europa mass spectrometer, Sandbach, United Kingdom).

Stable isotopes of carbon were also used as tracers of the food web, providing information about matter transfers within the microbial mat by an on-site "enriching and hunting" experiment. The community was labeled with ^{13}C (98 % ^{13}C. Isotec) by exposing the community under the sunlight to $NaH^{13}CO_3$ which was photoassimilated by the primary producers. The incubations were launched by adding the ^{13}C tracer to the microbial mat inside the experimental trays at an approximate concentration of 10 % of the natural concentration of dissolved inorganic carbon (DIC). DIC was estimated from water alkalinity considering pH and temperature and measured after titration with HCl using phenolphthalein as pH shift double indicator. After an incubation period of 24 h *in situ*, autotrophic organisms, mostly photoautotrophs, should have assimilated the isotope, and excess of unassimilated isotope was removed as described in Velázquez *et al.* (2017). Then, microbial mat in incubation trays were placed again at the wetland, fixing them to the ground so to avoid water exchange with the surrounding wetland. Samples were obtained at time periods of 0, 8, 24, 48, and 168 (7 days) and 264 h (11 days), with sterile 7 mm diameter brass cylinders from the incubation trays and kept frozen until the organisms were manually separated under stereozoom microscope. The different compartments of the community were encapsulated as described previously for natural abundance analysis, at the different time-lapses previously mentioned. The capsules were analysed by a MEIR (20–20 PDZ Europa mass spectrometer, Sandbach, United Kingdom). So, carbon isotopic ratios were measured in the different ecological compartments of the community throughout the study period, thus determining the

trophic relationships between them by Stable Isotope Analysis in R (SIAR; Parnell *et al.,* 2013) (see below). Stable isotopes enrichment of the fungal community, as an ecological compartment of the food web, could not be finally analysed due to the small amount of biomass recovered.

3.3.5 Data analysis and modeling

Trophic pathways within the community were studied using a Bayesian isotopic mixing model, available as an open source R package, SIAR (Parnell *et al.,* 2013). The main carbon sources of the studied consumers were determined by comparison between their ^{13}C signatures to the groups previously identified as sources. Nematodes were compared to the rest of the community, including rotifers and tardigrades as sources, due to their variety of feeding strategies described by Shaw *et al.* (2018). The fungal community was considered as decomposers, and was analysed with those groups considered trophically related and closest after natural abundance analyses.

Also, we assumed similar stoichiometry for C and N fractionation through the food web to implement SIAR modeling. A quadratic function was fitted with data obtained from ^{13}C ‰ enrichment experiment at different time intervals. Trophic enrichment factors (TEF) were based on mean trophic fractionations with standard deviations that are considered global averages (TEF $\delta^{13}C$ – 0.4 ± 1.3; TEF $\delta^{15}N$ – 3.4 ± 1) (Post, 2002). No parameters were modified between consumers according to their trophic habits, assuming that each group was composed of different species with different characteristics.

3.3.6 16S and 18S rRNA analysis

Bar-coding profiles using high-throughput sequencing were carried out to better assess the microscopic identification of the community composition. Total genomic DNA was extracted separately from three microbial mat cores using the MoBio PowerBiofilm DNA extraction kit (Carlsbad, CA, United States) following the manufacturer's instructions. The 16S rRNA gene was amplified by PCR using barcoded primers set 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 534R (5'-ATTACCGCGGCTGCTGG-3') targeting the V1–V3 hypervariable regions, according to Crits-Christoph *et al.* (2013). This universal primer set is for bacterial community and the archaeal community was not included in the study. For 18S rRNA gene, genomic DNA was amplified according to the protocol of Bates *et al.* (2013), using the eukaryotic-specific primer set 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 1119R (5'-GGTGCCCTTCCGTCA-3'), targeting the V4 region. The pool of samples, three for each targeted gene, with the prepared libraries was sequenced by Illumina MiSeq platform.

High-quality sequences were trimmed by "cutadapt" (Martin, 2011) to 300 bp on average and then checked for chimeras using UCHIME (Edgar *et al.,* 2011). Chimeras were discarded for downstream analyses. Using the same rationale, in the case of 18S rRNA results, sequences lower than 150 nts were removed from the analysis. Operational Taxonomic Units (OTUs) were delineated based on 97 % sequence by using MOTHUR (Schloss *et al.,* 2009). BLASTN search (Altschul *et al.,* 1990) to SILVA reference database for 16S and 18S rRNA gene sequences was performed to determine the closest cultured and uncultured match. Sampling effort was assessed by calculation on

rarefaction curves. Sequences generated by this study were deposited to GenBank under the BioSample accession number SAMN10505289.

3.4 RESULTS

3.4.1 Community relative abundances

Relative abundances (**Table 1**) were calculated from total biomass of the microbial mat, estimated in 6667 µg C cm^{-2}. From there, and for each studied group of the community, relative abundances calculated were: primary producers (8.8 %, equivalent to average 587 µg C cm^{-2}), fungi community (0.9 %, equivalent to average 55 µg C cm-2), and consumers (2.4 %, equivalent to average 159 µg C cm^{-2}). For tardigrades, rotifers and nematodes, relative abundance was estimated in 1.8 % (equivalent to average 122 µg C cm^{-2}), 0.001 % (equivalent to average 0.04 µg C cm^{-2}) and 0.6 % (equivalent to average 37.2 µg C cm^{-2}), respectively. The remaining portion not assigned to any of the studied trophic groups, was named as "remaining biomass," reaching 87.9 % of total estimated carbon.

	µg C/cm²	**% C/total C**
Primary producers	587 ± 91.2	8.8
Fungi	55 ± 4.3	0.9
Nematodes	37 ± 0.1	0.6
Rotifers	0.04 ± 0.1	0.001
Tardigrades	122 ± 0.1	1.8
Remaining biomass	-	87.9
Total biomass	6667 ± 1886	100

Table 1. Carbon relative abundances of biological compartments from the studied microbial mat (Byers Peninsula, Antarctica). From total biomass, calculated by the ash free dry weight (AFDW), the carbon amounts represented by each of the organisms were subtracted, finally leaving the percentage of remaining biomass. Primary producers are represented by the photosynthetic organisms (algae and cyanobacteria). Consumers community are represented by tardigrades, nematodes and rotifers. Decomposers community is only represented by the fungi.

3.4.2 16S and 18S rRNA amplicon sequencing

Approximately 400,000 valid sequences were obtained, clustered in 1121 OTUs. OTU richness for Bacteria was 979 OTUs obtained from the V1–V3 hypervariable region of the 16S rRNA gene, and for Eukarya 142 OTUs from V4–V5 region of the 18S rRNA gene. Rarefaction curves indicate that almost the plateau of detection of the bacterial and eukaryotic OTU diversity has been reached, so an increase in the number of sequences will not impact the number of OTU detected.

The bacterial community (**Figure 2**) was dominated by Proteobacteria, with 39.4 % relative abundance, followed by Bacteroidetes (27.8 %), and Cyanobacteria (11.1 %). The sequences assigned to Betaproteobacteria were dominated by members of the Burkholderiales (55.2 %) order, while Micrococcales (60.3 %) and Flavobacteriales (53.2 %) were the most abundant for Actinobacteria and Bacteroidetes OTUs, respectively. The cyanobacterial fraction was dominated by Nostocales, with a relative abundance of 8.3 %, followed by Oscillatoriales (1.9 %) and Chroococcales (0.4 %). For eukaryotic community, the group with a relatively greater abundance of OTUs was Chlorophyta (19.4%), followed by Ciliophora (13.8 %), Bacillariophyta (11.9 %), Cercozoa (10.5 %), and Heterokontophyta (6.4 %). Fungi-related OTUs (mainly Ascomycota and Basidiomycota-affiliated ribotypes) represented on average 13 % of the eukaryotic datasets. Nematoda, Tardigrada and Rotifera showed relative abundances of 2.7, 2.3, and 1.4 %, respectively.

As the diversity of the studied trophic groups was the main interest of the study, their relative abundances per families were scaled to total number reads per phylum (**Figure 2**). Cyanobacteria was dominated by *Nostocaceae* (44.0 %), followed by *Oscillatoriaceae* (26.5

%) and *Chroococcaceae* (17.6 %). Bacillariophyta's OTUs matched *Naviculaceae* (16.2 %) and *Pinnulariaceae* (15.9 %), followed by *Bacillariaceae* (12.7 %) and *Fragilariaceae* (12.0 %). The nematode community was dominated by *Plectidae* (27.0 %) and *Monhysteridae* (25.2 %). The predominant families for tardigrades were *Hypsibiidae* (34.1 %) and *Macrobiotidae* (33.4 %), especially the genera *Macrobiotus, Isohypsibius, Acutuncus* and *Calohypsibius*. Rotifer community was dominated by OTUs matching *Philodinidae, Lecanidae* and *Lindiidae* families, with very similar relative abundances. Fungal community, which represents an important portion of the decomposers fraction, was dominated by Ascomycota (38.5 %) and Basidiomycota (25.6 %) OTUs.

3.4.3 $\delta^{13}C$ ‰ and $\delta^{15}N$ ‰ natural abundances of the community

According to $\delta^{13}C$ ‰ and $\delta^{15}N$ ‰ natural abundance analysis of the studied groups and their ecology, a classification into three different trophic categories was established. Therefore, primary producers, the consumers category, and fungi community as decomposers, were defined. Primary producers included cyanobacteria and diatoms, while consumers category consisted of tardigrades, rotifers and nematodes. The isotopic $\delta^{13}C$ ‰ signatures of primary producers (**Figure 3**) were -17.30 ± 0.3 for cyanobacteria and -21.84 ± 0.9 for diatoms, whereas $\delta^{15}N$ ‰ signals were 11.75 ± 0.6 and 11.76 ± 0.1, respectively. DOC isotopic values (-18.5 ± 0.1 $\delta^{13}C_{vpdb}$ and 12.5 ± 0.1 $\delta^{15}N_{air}$) were assumed to reflect cyanobacterial exudates and/or dissolved exopolymeric substances (EPS). POM smaller than 30 µm includes chlorophytes, partially decomposed matter and some ciliates (-20.5 ± 0.1 $\delta^{13}C_{vpdb}$ and 10.4 ± 0.1 $\delta^{15}N_{air}$). The POM fraction between 0.5 and 5 µm (-20.5 ± 0.3 $\delta^{13}C_{vpdb}$ and 12.1 ± 0.6 $\delta^{15}N_{air}$), which includes a fraction of microbes of the microbial mat, due to the size range filtered, has been plotted above the larger fraction of POM, with a $^{13}C/^{12}C$ ratio 2 % higher.

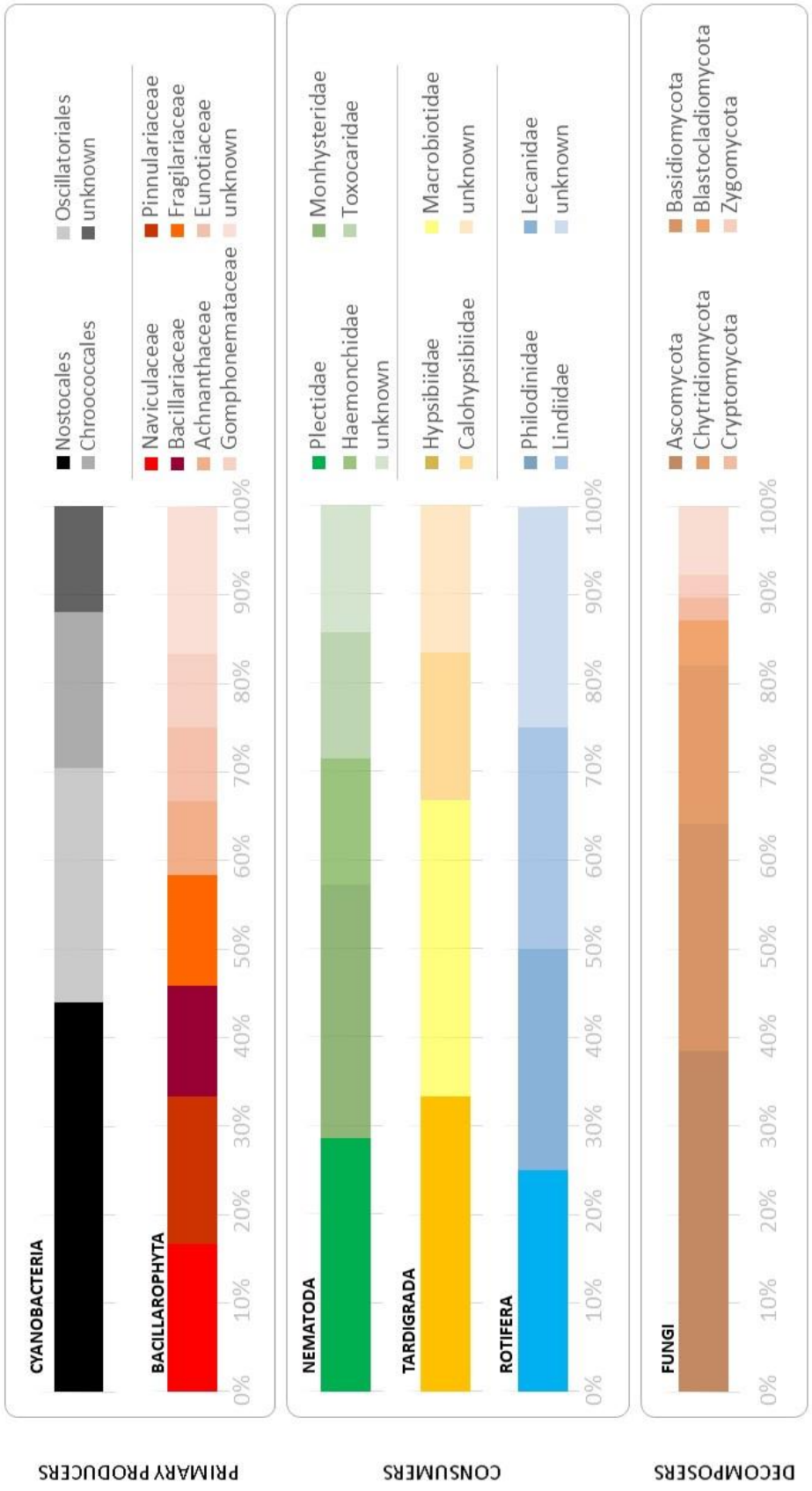


Figure 2. Relative abundance of the studied taxa, including microbial and eukaryotic community, in a microbial mat from Byers Peninsula (South Shetland Islands. Antarctica).

Fungi $\delta^{13}C$ ‰ displayed the most negative values within the food web. Tardigrades appear midway between the fungi and rotifers regarding to carbon ratios, and with similar values to those shown by diatoms. Rotifers appear around POM fraction smaller than 30 µm and below tardigrades regarding to nitrogen ratios. In nematodes, there seems to be a tendency toward less positive values of $\delta^{13}C$ ‰ that suggest differences in carbon sources, and toward more positive nitrogen ratios in comparison with the other studied consumers (**Figure 3**).

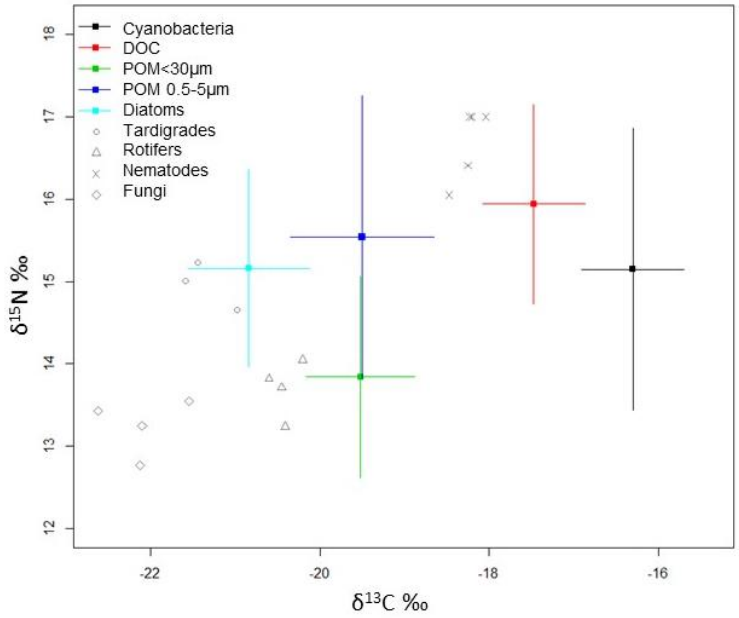


Figure 3. Bivariate model plot of isotopic signatures ($\delta^{13}C$ and $\delta^{15}N$), of organisms in the community and potential food sources of a microbial mat from Byers Peninsula, Antarctica. For sources, points are mean trophic enrichment factor, and error bars are ±1 standard deviation of the trophic enrichment factor. For consumers, shapes around the plots represent the average of each group.

3.4.4 Carbon tracking among primary producers, consumers and decomposers

The circulation of C through different trophic levels was determined from changes in $^{13}C/^{12}C$ signatures through the food web at different time points (**Figure 4**).

Cyanobacteria and diatoms showed similar behavior, incorporating the labeled inorganic carbon during the first 24 h after the incubation. The enrichment peak appeared earlier in cyanobacteria, with of 64.8 ± 56.8 $\delta^{13}C$ ‰ for cyanobacteria and 59.1 ± 33.1 $\delta^{13}C$ ‰ for diatoms. Then, $\delta^{13}C$ ‰ values decreased gradually for both, due to the natural predominance of ^{12}C in the ecosystem and its incorporation, diluting and re-establishing the initial $\delta^{13}C$ ‰ in the next 2 days. Rotifers and tardigrades were the first consumers that incorporate the labelled inorganic carbon. Rotifers showed the enrichment peak earlier than tardigrades, with values of 33.8 ± 10.6 $\delta^{13}C$ ‰ and 22.9 ± 14.9 $\delta^{13}C$ ‰, respectively. As for primary producers, $\delta^{13}C$ ‰ decreased gradually for both along the experiment. Nematodes showed a gradual enrichment of ^{13}C since the beginning of incubation, reaching the highest $\delta^{13}C$ ‰ at 168 h, becoming the last organism of the food web to assimilate the added inorganic carbon. At the end of the study, nematodes $\delta^{13}C$ ‰ signal values continued almost at the same level than from the enrichment peak.

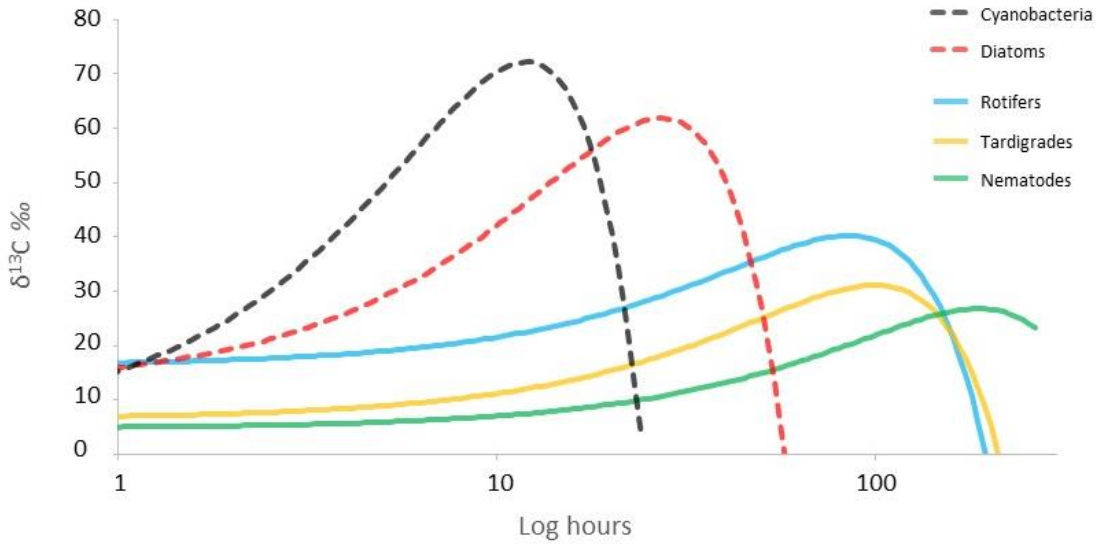


Figure 4. ^{13}C signal through the different organisms along time in a microbial mat from Byers Peninsula, Antarctica. Lines represent ^{13}C data adjusted to a quadratic function. Dashed lines represent primary producers and solid lines consumers.

3.4.5 Food web modeling

Stable Isotope Analysis in R model interpretation displayed important trophic interactions between tardigrades and rotifers to diatoms (52 and 36 %, respectively), POM <30 µm (35 and 5 %) and POM 0.5–5 µm (4 and 35 %), respectively, and practically not related to cyanobacteria and DOC (**Figure 5**). So, a deviation of the tardigrades toward largest POM fraction related to green algae of the microbial mat can be observed while the rotifers would be related to POM fraction of smaller size. Carbon sources of fungal fraction were influenced mainly by POM < 30 µm (37 %) (**Figure 5**). Nematodes were included in the model as omnivores, at the top of the food web, including consumers as available C sources. In this case food sources were more dispersed (**Figure 5**), appearing trophically closer to cyanobacteria (42 %) and DOC (21 %), but also to tardigrades (7 %) and rotifers (10 %).

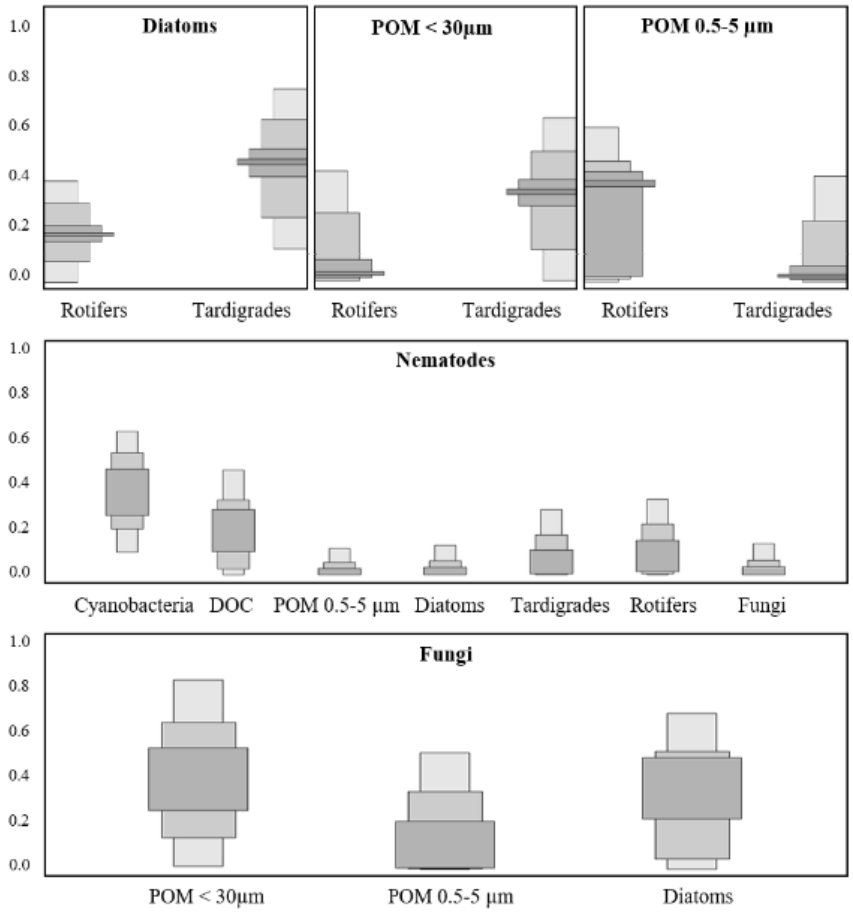


Figure 5. Results of SIAR Bayesian mixing model showing the contribution of each C source to the different fractions of the microbial mat community from Byers Peninsula, Antarctica. Different gray colored boxes indicate confidence intervals of 25, 75, and 95 %. (**A**) Results for tardigrades and rotifers by their main sources diatoms, Particulate Organic Matter fraction < 30 µm and POM fraction between 0.5 and 5 µm, of the microbial mat community. (**B**) Results for fungi by their main C sources diatoms, POM fraction < 30 µm and POM fraction between 0.5 and 5 µm. (**C**) Results for nematodes by their main C sources, where the entire community was included as potential C source.

3.5 DISCUSSION

The high diversity of microbial communities inhabiting Antarctic soils (Barrett *et al.,* 2006a) is well known. Benthic microbial mats appear as aggregated microecosystems favoring the establishment of high diversity and complex communities because of the highest nutrient availability and protection against the harsh environmental conditions

(De Los Ríos *et al.,* 2014; Chown *et al.,* 2015). Understanding trophic structure of microbial mats from Antarctica is critical to studies of biotic interactions and therefore ecosystems functioning, due to the important role they play in biomass accumulation and productivity. For the first time, we have carried out an on site enrichment experiment of an Antarctic microbial mat with ^{13}C, that has provided insights into their trophic interactions, together with natural abundance ratios of C ($^{13}C/^{12}C$) and N ($^{15}N/^{14}N$). The stable isotope composition of microbial mat's compartments revealed a food web with at least four trophic levels: a basal level of primary producers divided into diatoms and cyanobacteria as different organic carbon inputs, a secondary level of consumers composed of rotifers and tardigrades, then nematodes occupying a higher trophic position, and a 4th level where fungi would act as part of the decomposer's community. POM less than 30 µm measured in this study contained a mixture of primary producers and detritus.

Diatoms and cyanobacteria displayed different isotopic signatures (**Figure 3**) with different C assimilation ratios along the incubation period (**Figure 4**). It has been considered that cyanobacteria success at high latitudes is due to their wide range of tolerance to conditions and to maintaining slow but constant growth rates, despite the frigid ambient temperatures (Taton *et al.,* 2003; Quesada and Vincent, 2012). However, we have seen how they are metabolically comparable to photosynthetic eukaryotes as diatoms, considered as crucial in colonization and in primary and secondary succession processes (Rahmonov *et al.,* 2015). Therefore, cyanobacteria could be competing at the same level as the other primary producers within the microbial mat community, with comparable photosynthetic efficiency, which is relevant in a cyanobacteria-based microecosystem where they also provide structural integrity to the microbial mat.

The secondary trophic level is composed by rotifers and tardigrades, with similar natural abundance of isotopic composition between each other, but differences in trophic preferences. Results extracted from trophic modeling of the community showed that tardigrades are more trophically related to diatoms and to POM < 30 μm than to cyanobacteria (**Figure 5**). Microbial mat community progressively adjusts its photosynthetic metabolism to environmental conditions (Velázquez *et al.,* 2011), and it is during the initial stages of the spring period when green algae have an important role as a carbon source to the system. During this period, melting occurs from soil to the upper layer of the microbial mat, and the community awakens at a very low temperature and light conditions. Only a few groups of psychrophiles can be metabolically active, and as long as the ice remains, this community enjoys optimal conditions. When temperatures rise, cells differentiate into resistant spores (Hoham *et al.,* 2008). This study was conducted when the ice and snow completely melted out, and not many chlorophytes were found in the microbial mat, despite being the group with the highest relative abundance of 18S rRNA sequences; therefore, they could not be physically separated and thus included directly in the study. The low abundance of green algae in the community, as well as the dominance of *Nostocaceae* versus other filamentous cyanobacteria (**Figure 2**), compared to previous studies in the same area (Fernández-Valiente *et al.,* 2007; Rochera *et al.,* 2013; Velázquez *et al.,* 2017), suggests the seasonality during the ice-free period of the community of primary producers, as has been published previously (Velázquez *et al.,* 2011). Even so, we consider that a portion of POM $\delta^{13}C$ ‰ signal comes from Chlorophytes. The abundance of *Nostocaceae* could also represent relevant differences in terms of N source, because of the potentiality of N_2-fixation by this family (Fernández-Valiente *et al.,* 2007). However, N_2-fixation

relevance was not evident from the data of natural abundance of the N isotopes. According to our results, tardigrades act as the main grazers of the microbial mat, feeding mainly on diatoms and green algae upon their presence in the community. Rotifers seems to feed preferentially on POM fraction between 0.5 and 5 µm, due to its size and mastax anatomy observed in the analysed specimens. Considering that this is the estimated size for bacteria and some microeukaryotes, it seems reasonable to consider that rotifers would feed on the small POM fraction with high content in detritus and bacteria, also related to dead organic matter as has been previously described for Antarctic bdelloids specimens (Iakovenko *et al.,* 2015). The ^{13}C enrichment differences between tardigrades and rotifers (**Figure 5**) associate consumers of the second trophic level to multiple C sources and diet preferences, as it has been previously described in mats of maritime Antarctica (Velázquez *et al.,* 2017), and the model plots them in different positions within the trophic network.

According to the Bayesian mixing model, nematodes would be related trophically to cyanobacteria and DOC in 42 and 21 %, respectively (**Figure 5**). In a cyanobacteria-based microbial mat, most part of DOC is derived from cyanobacterial exudates and/or EPS (Velázquez *et al.,* 2017). So, we conclude that nematodes might feed mostly on cyanobacteria, also considering cyanobacteria as a direct driver of the food web in the microbial mats on Byers Peninsula. Bactivorous nematodes are common in soils (Nielsen *et al.,* 2011; Shaw *et al.,* 2018; Caruso *et al.,* 2019) and microbial mats from Antarctica (Velázquez *et al.,* 2017), suggesting that by feeding on cyanobacteria, nematodes obtain proteins that would compensate for the lack of polyunsaturated fatty acids in their diet (Gaudes *et al.,* 2006). But also, nematodes probably feed on rotifers and tardigrades, as shown by the enrichment peak along the time frame of the study. The theoretical

trophic enrichment of 3.4 ‰ for ^{15}N (Peterson and Fry, 1987) between nematodes and rotifers are almost fulfilled, and the Bayesian mixing model relates them trophically. For tardigrades this enrichment is not so evident, and nematodes only increase its $\delta^{15}N$ around 2 ‰. Even so they appear related, and this could be due to the variability of isotopic signals in the ecosystem, with differences even between tissues of the same organism. Protozoa may also be a possible food source (Bamforth *et al.,* 2005), but the difficulty in collecting enough biomass for isotope measurement did not allow them to be included in this study. We can expect some bias in dietary proportions, considering that some carbon sources have not been included in the model, and TEF values were made without taking into account the dietary variability within consumers. Assuming this uncertainty in some aspects of the analysis, as well as the caveats of isotopic mixing models, we are simplifying trophic relationships as much as possible to define a baseline for future studies. So, according to our isotope and modeling results, nematodes in the microbial mat from Byers Peninsula should be considered as omnivores, feeding on bacteria and other consumers. With these feeding habits and attending to their trophic position in the microbial mat food web, nematodes play a key role as top consumers of the community, connecting the two described carbon inputs into the ecosystem. In addition, by feeding on cyanobacteria and DOC, they would also contribute to the decomposition of the organic matter accumulated in these microbial mats.

Cyanobacterial mats from Byers Peninsula accumulate high standing-stocks of carbon (Velázquez *et al.,* 2017), but its proportion had never been estimated. According to our results, during the study almost 90 % of the accumulated organic carbon in the microbial mat would not be part of any of the studied trophic levels. Considering that the accumulated biomass per unit area is two to three-fold less in drylands, like Antarctica,

compared to temperate ecosystems (Bay *et al.,* 2018), this represents a huge carbon stock. This accumulated biomass, mostly EPS, represents several seasons of growth (Vincent and Howard-Williams, 1986), and is considered as an ecological adaptation of the microbial mat community to overcome fluctuating conditions across seasonal scales (Moyer *et al.,* 1994), providing protection against temperatures and desiccation (De Los Ríos *et al.,* 2014).

Microbial mats from Byers Peninsula have been described as a self-contained ecosystem with extremely low matter inputs (Velázquez *et al.,* 2017). Therefore, and considering the large amount of accumulated biomass, the microbial- and myco- loops should be key to system functioning (Velázquez *et al.,* 2016). Fungi as members of the decomposer community have an important role in the maintenance of these ecosystems. The relationship of the fungi with the POM < 30 µm (**Figure 5**), where a large proportion of the susceptible matter to be decomposed would be concentrated, confirms their role as decomposers within the community. Sequences related to fungi (mainly Ascomycota and Basidiomycota affiliated ribotypes) represented on average 13 % of the OTUs assigned to eukaryotes in this study with high-throughput sequencing. However, only 0.8 % has been reported in previous studies by using different approaches (Velázquez *et al.,* 2016). In addition, if we estimate total dry weight from calculated fungal biomass, we determined the fungal community is 10 times higher than reported in our previous study of microbial mats from Byers Peninsula, and they approximate to those areas defined as “blighted patches” (Velázquez *et al.,* 2016), where fungal community seem to be triggering the decay of the community. Thus, an intermediate scenario is proposed, where the relative abundance of fungi would be higher than that considered in other studies, despite not being truly cold adapted (Ruisi *et al.,* 2007). Their role as

decomposers of organic matter, in a system where most of the biomass accumulates outside the main trophic groups, makes its study a key element to understand the functioning of these microbial ecosystems. Even so, a more detailed study on the relative abundance and ecological role of fungi in Antarctic microbial mats is required, taking into account the potential limitations of the analyses carried out in this study.

3.6 CONCLUSIONS

Antarctica is not biologically isolated, and global change has the potential to allow the establishment of diverse new species (Fraser *et al.,* 2018). Understanding trophic positions and biotic interactions is critical for predicting future changes in species distributions and interactions. We have verified how the food web of the microbial mat from Byers Peninsula has at least four trophic levels, and each of the studied organism seems to have a specific role, with a low redundancy in ecosystem function that would be a consequence of the low diversity in these high latitudes. Nematodes have shown a key ecological role within the community, connecting the two organic carbon inputs described during the study. The presence of liquid water becomes the limiting factor for Antarctic microbial mats, and only during a few weeks in the austral summer, temperatures allow ice-free conditions. Carbon flows through the different trophic levels studied have been completed during the study, moving from primary producers to top consumers. During mid-January the environmental conditions are the most propitious for the microbial mat community, and they could be in an optimal metabolic stage (Velázquez *et al.,* 2017). So, time intervals showed in the study could represent the fastest one, likely increasing toward the end of the summer season. Future studies

are necessary to deepen the functioning and evolution of these ecosystems and their resilience to physicochemical changes.

CAPITULO 4

MARINE VERTEBRATES IMPACT THE BACTERIAL COMMUNITY COMPOSITION AND FOOD WEBS OF ANTARCTIC MICROBIAL MATS

LOS VERTEBRADOS MARINOS IMPACTAN LA COMPOSICIÓN DE LA COMUNIDAD BACTERIANA Y LAS REDES TRÓFICAS DE LOS TAPETES MICROBIANOS DE LA ANTÁRTIDA

4.1 RESUMEN

Los vertebrados marinos representan un aporte relevante de nutrientes en el ecosistema terrestre antártico, con importantes consecuencias para su biodiversidad. Aunque los tapetes microbianos reúnen la mayor parte de la diversidad biológica de la Antártida no marina, los efectos de la macrofauna local en estos microecosistemas siguen sin resolverse. Utilizando la secuenciación del gen ARNr 16S, isótopos estables ^{13}C y ^{15}N, y caracterizando la disponibilidad de nutrientes en el microecosistema, evaluamos los efectos de la macrofauna local en cinco tapetes microbianos ubicadas a lo largo de la Península Antártica. Nuestros resultados muestran que las proporciones C/N y N/P en los tapetes microbianos están altamente correlacionadas con la presencia de fauna local, mientras que NO_3^-, NH_4^+ y N/P contribuyen significativamente a la variación entre las comunidades procariotas. Los tapetes microbianos de lugares con densas poblaciones de vertebrados marinos fueron menos diversos y mostraron una menor abundancia relativa de cianobacterias que las muestras de lugares oligotróficos. Las relaciones tróficas y el papel de los diferentes grupos como principales consumidores (nematodos y tardígrados) en las redes tróficas microbianas también se vieron influidos por la proximidad a las colonias de macrofauna. Estos cambios en las comunidades también son relevantes en el escenario de cambio global, ya que las alteraciones en la distribución de los vertebrados marinos debido al aumento de las temperaturas antárticas podrían cambiar la disponibilidad de nutrientes en el ecosistema.

ABSTRACT

Marine vertebrates represent a relevant input of nutrients in the Antarctic terrestrial ecosystem, with relevant consequences for their biodiversity. Even though microbial mats gather most of the biological diversity of the non-marine Antarctica, the effects of the local macrofauna on these microecosystems remain unresolved. Using 16S rRNA gene sequencing, ^{13}C and ^{15}N stable isotopes, and by characterizing the availability of nutrients in the microecosystem, we evaluated the effects of local macrofauna on five microbial mats located along the Antarctic Peninsula. Our results show that C/N and N/P ratios in microbial mats are highly correlated with the presence of local fauna, while NO_3^-, NH_4^+ and N/P contribute significantly to the variance among the prokaryotic communities. Microbial mats from locations with dense marine vertebrates populations were less diverse and showed lower relative abundance of Cyanobacteria than those from oligotrophic locations. The trophic relationships and the role of different groups as top consumers (nematodes and tardigrades) in the microbial food webs were also influenced by the proximity to macrofaunal aggregations. These community shifts are also relevant in the global-change scenario, since alterations in the distribution of marine vertebrates due to increases in Antarctic temperatures could change the availability of nutrients in the ecosystem.

4.2 INTRODUCTION

The Antarctic climatic conditions impose severe restrictions on living organisms. Low temperatures, low availability of liquid water, long periods with continuous irradiance and months of nearly complete darkness during winter (Thomas *et al.,* 2008), result in

an ecosystem dominated by microorganisms. The harsh environmental conditions together with the prevalence of rocky sites with low nutrient levels and continental geographic isolation, make microscopic organisms the most diverse and abundant components of terrestrial Antarctic communities (Hughes *et al.,* 2015). This provides a unique setup to validate ecological hypotheses on microorganism-based food webs in a more 'controlled' scenario (Hansson and Tranvik, 2003). Cyanobacteria become key players at these high latitude ecosystems (Vincent and Quesada, 2012; Quesada and Vincent 2012), because of their extreme resilience resisting desiccation, freeze-thaw cycles and high levels of UV radiation, which contribute their dominance in polar aquatic ecosystems (Bonilla *et al.,* 2005). Cyanobacterial microbial mats accommodate high biodiversity and represent one of the highest concentrations of non-marine biomass with a ubiquitous distribution throughout Antarctica (Quesada and Vincent, 2012), and therefore constitute an important carbon reservoir.

Recently, Velázquez *et al.* (2017) and Almela *et al.* (2019a) described in detail the trophic relationships among the different elements of the community within Antarctic microbial mats. The authors described at least four trophic levels in the microbial mats and a community adapted to a short growing season by virtue of a fine temporal coupling, where fungal and bacterial activity represent the main connectors between consumers and producers. The influence of local fauna on polar ecosystems is well known (Jakubas *et al.,* 2008; Zawierucha *et al.,* 2016; Zawierucha *et al.,* 2019; Bokhorst *et al.,* 2019; Wang *et al.,* 2020), but the influence of vertebrate fauna on the ecology of microbial mats (diversity and trophic interactions) is understudied. Seal haul-outs and seabirds colonies on Antarctic coastal ecosystems are not considered part of the terrestrial biota (Laybourn-Parry, 2009), but they influence the non-marine ecosystem, where they

remain a considerable portion of their time. Therefore, local vertebrate fauna can provide a relevant input of nutrients into this ecosystem by transferring nitrogen and phosphorus from sea to land (Bokhorst *et al.,* 2019; Wang *et al.,* 2020).

The Antarctic Peninsula region has shown rapid warming, similar in magnitude to the Arctic and substantially faster than the global mean (Robinson *et al.,* 2020). Because average temperatures are close to the freezing point (Rochera *et al.,* 2013) and small temperature changes can trigger pronounced effects, this region is considered as one the most sensitive on Earth to climate warming. Climate change in the region is already causing an alteration in marine vertebrates' communities with shift in species and abundance in their populations (Lynch *et al.,* 2012; Clucas *et al.,* 2014; Dunn *et al.,* 2016; Ropert-Coudert *et al.,* 2019; Morley *et al.,* 2019), suggesting major implications for local terrestrial biodiversity patterns in Antarctica (Bokhorst *et al.,* 2019).

The aim of this study was to identify the influence of nutrient availability derived from marine vertebrates on benthic microbial mat communities from the Antarctic freshwater ecosystem. We compared microbial mats along the Antarctic Peninsula within an environmental gradient caused by macrofauna pressures, focusing on the community composition of prokaryotes and on the trophic relationships among the main primary producers and consumers. Since to date it is unknown how the presence of local macrofauna influences these polar microecosystems, the results presented here will shed light on microbial community function understanding and assessing the potential risks on freshwater microbial communities derived from changes on populations of marine vertebrates.

4.3 MATERIALS AND METHODS

4.3.1 Study site and sampling

Samplings were conducted during two different Antarctic campaigns in different locations of the Antarctic Peninsula (**Figure 1**): Byers Peninsula Coast (BC), Byers Peninsula Plateau (BP), Avian Island (AI) and Cierva Point (CP) microbial mats were sampled in February 2016, and Lagotellerie Island (LI) microbial mat in February 2019. These sampling areas were selected considering the apparent nutrient availability derived from the presence of local macrofauna.

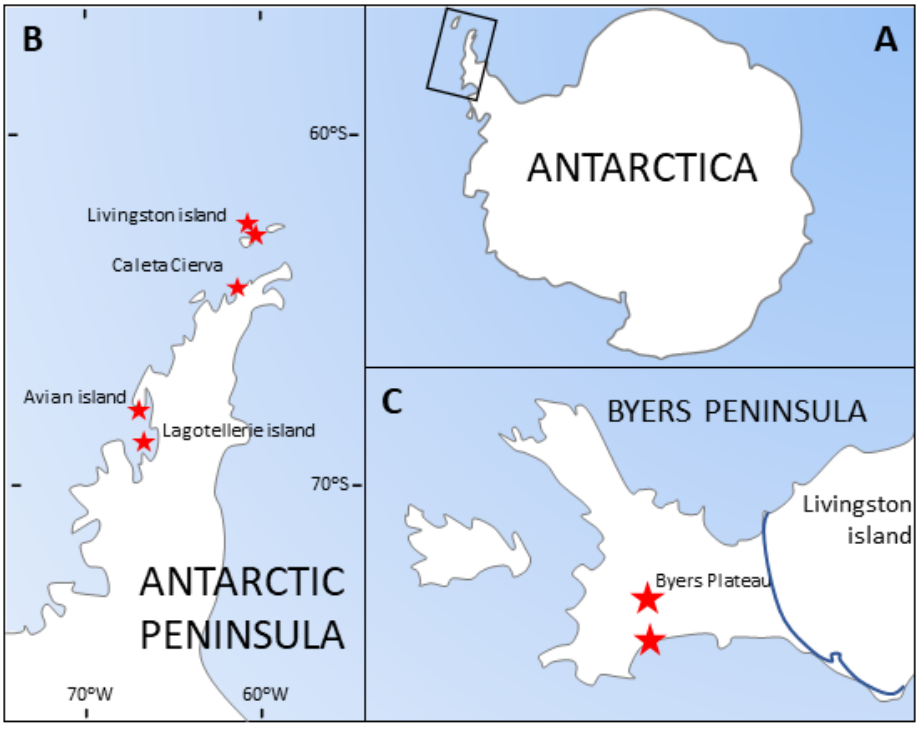


Figure 1. Map indicating the location of the sampling areas. (**A**) Detail of the area at a continental scale. (**B**) Distribution of the sampled microbial mats along the Antarctic Peninsula. (**C**) Location details of Byers Peninsula (Livingston Island, South Shetland Islands) where two microbial mats were sampled.

Byers Peninsula is one of the largest ice-free areas in the Antarctic Peninsula. It is located at the western end of Livingston Island (South Shetland archipelago). It is designated as the Antarctic Specially Protected Area (ASPA) No. 126 to protect its terrestrial and lacustrine habitats and it is considered one of the main Antarctic hotspots of biodiversity (ATCM, 2011). In this area, we sampled two microbial mats: BC was located near a stream 100 m away from the coast at 2 m above sea level (masl), and BP microbial mat was located over the plateau, 1.5 Km away from the coast and 60 masl. Mosses were observed around the growth areas of the studied microbial mats but with a clear physical delimitation, suggesting independent microecosystems.

Cierva Point, designated as ASPA No. 134, is located in the northwestern portion of the Antarctic Peninsula. The site comprises the ice-free area between the southwest coast of Cierva Cove and the northeast coast of Santucci Cove. It has great scientific value due to its unusual biodiversity, which includes numerous species of birds, flora, and invertebrates (ATCM, 2013). The sampled microbial mat (CP) was located within the ASPA, 200 m away from the coast and 34 masl.

Avian Island (ASPA No. 117) is sited 400 m south of Adelaide Island at Marguerite Bay on the western side of the central Antarctic Peninsula. The island comprises 0.49 km^2, which usually remains ice-free during the summer seasons. The values described under its ASPA designation aims to protect the abundance and diversity of breeding seabirds on the island (ATCM, 2002). The sampled microbial mat (AI) was collected in a pond at less than 30 m from the coast and 10 masl.

Finally, we also collected samples from Lagotellerie Island, which sited at Marguerite Bay as well. It has been designated as ASPA No. 115 because of its relatively diverse flora

and fauna, which is characteristic of the southern Antarctic Peninsula (ATCM, 2017). The studied microbial mat from this island was collected in an outlet stream to the coast at 2 mamsl.

The presence of local fauna in the surroundings of the studied microbial mats was different among the sampling areas. In Byers Peninsula area, BC mat was located nearby a haul-out site of elephant seal (*Mirounga leonina*), while BP sample was collected from an area with no influence of local vertebrate fauna, far from the coast, and therefore without any known extra input of nutrients due to its presence. AI mat was located in the middle of an Adelie penguin (*Pygoscelis adeliae*) breeding area, and CP mat was collected upslope of Papua penguin (*Pygoscelis papua*) rookery, but without such direct influence of their biological activity. LI mat had a remarkable influence of different marine mammals and seabirds, such as different seals, skuas, gulls and penguins. Although the colonies are not very extensive, the suitable place for the animals is narrow and faunal accumulation is rather high. The five sampling areas are not subjected to almost any direct human influence, and visitors can only access the sites for scientific purposes and with permits. Both access and sampling were specifically permitted by Spanish Polar Committee.

All samples were collected as follows: six replicates of ca. 6 cm^2 were randomly collected using sterilized metal spatulas from each sampling spot, covering a sampling area of ca. 2 m^2 of the wetland. These samples were stored in sterile Whirl-Pak bags (Nasco Ltd.) at -20 °C until further analysis at the laboratory.

4.3.2 Environmental data collection

The averages of 21 years of number of days per year with temperatures above freezing point (TAF) were considered a good proxy to summarize the climatic characteristics of the sampling sites. This parameter was estimated with the hourly data obtained from the Modern-Era Retrospective Analysis for Research and Applications (MERRA) (Rienecker *et al.,* 2011).

The nutrient content of the microbial mats was commercially determined by techniques accredited by the ENAC (Spanish Accreditation Agency). Briefly, 10-15 grams of fresh material were used from each microbial mat. After drying out for 48 h at 65 °C, samples were homogenised by screening through a 0.5 mm sieve. The extraction process was carried out by distilled water. For ammonia nitrogen ($mg \cdot g^{-1}$) and nitrate ($mg \cdot g^{-1}$), the concentration in aqueous solution was determined by ion chromatography, according to UNE-EN-ISO 14911 and UNE-EN-ISO 12014, respectively. Phosphorus concentration ($mg \cdot g^{-1}$) was determined after a digestion procedure (Matula *et al.*, 2011) by inductively coupled plasma emission spectroscopy (ICP-AES), according to UNE-EN 15510.

C/N ratio was analysed from dry-homogenised samples, as described above, by a LECO CHNS-932 Analyzer (Model NO: 601-800-500) according to UNE-EN 103204. Analysis was performed in triplicates. N/P ratio was calculated from % N (from C/N ratio) and phosphorus concentrations previously determined.

Estimations of total biomass were calculated from the ash free dry weight (AFDW). After obtaining the carbon content, in triplicates, from 8 mm diameter cores per sample, the biomass value was subtracted from the total carbon calculated for the microbial mat and expressed as total organic matter (TOC) ($mg \cdot cm^{-2}$).

4.3.3 Phototrophic and fungal community estimations

Phototrophic community abundance (Cyanobacteria, Chlorophyta and Bacillariophyta) was estimated by Chlorophyll-a (Chla) concentration. Chla was extracted in triplicates from 8 mm in diameter core samples (and thickness of 0.5, 1.0, 0.5, 0.4 and 1.3 cm for LI, BC, AI, CP and BP, respectively) using 90 % (v/v) acetone (Ritchie, 2008). Extracts were measured at 665 nm using a Hitachi U2000 spectrophotometer.

Ergosterol, a biochemical marker of fungal active biomass, was quantified using HPLC equipped with a UV detector (282 nm) as described by Gessner (2020). For its extraction, the procedures described by Romaní *et al.* (2009) were followed, from 8 mm diameter core samples (same thicknesses as described above) and by triplicate. The concentration of ergosterol in the eluted product was then transformed to fungal biomass assuming that 5.5 mg of ergosterol are found in 1 g of fungal biomass (Gessner and Chauvet, 1993).

4.3.4 Microscopic identification of the community composition and ^{13}C and ^{15}N natural abundance analysis

The composition of the trophic community was studied from 8 mm diameter cores, which were manually disaggregated. Relative abundance of the meiofauna (rotifers, tardigrades and nematodes) was analysed by using a stereo-zoom microscope (Leica MZ75) and interpreted per surface units. Photosynthetic organisms were determined using epifluorescence microscopy equipped with an Olympus® blue filter set (EF 400–490 nm, DM 570, FB 590) and an Olympus® green filter set (EF 530–545 nm, DM 570, FB 590) to excite Chla and cyanobacterial phycobilins, respectively.

For $\delta^{13}C$ ‰ and $\delta^{15}N$ ‰ natural abundance signal analysis, individuals of different trophic levels were manually separated under the microscope by microdissection and encapsulated in triplicates in 175 µl zinc cases and dried at 65 °C for 48 h. A minimum of 0.02 mg of dry weight biomass was required (Shaw *et al.,* 2018), which was achieved by collecting approximately 50–100 living individuals per zinc case. Particulate organic matter (POM) smaller than 30 µm was determined in triplicate and per sample, as follows: a piece of mat was pressed gently against a Nytal sieve of 30 µm mesh size diameter. From this filtrate, and after standing for 1 hour, the POM fraction was obtained with that heavier material that precipitated in 50 ml corning tubes. Therefore, we obtained the fraction of organic matter that mostly contains exopolysaccharides (EPS) from cyanobacterial community, but also organisms smaller than 30 µm. From the supernatant, and after filtering through a 0.22 µm hydrophilic membrane and concentrating by evaporation at 40 °C under vacuum, dissolved organic matter (DOM) fraction was obtained and encapsulated. The isotopic ratios from all samples were analysed by mass spectrometry (MEIR; 20-20 PDZ Europa mass spectrometer, Sandbach, United Kingdom).

4.3.5 DNA extraction, sequencing, and ribotype diversity analysis

Total genomic DNA was extracted by triplicate for the studied microbial mats. The DNAeasy Power Biofilm kit (Qiagen) was used for DNA extraction, using the protocol supplied with the kit. Bacterial 16S rRNA marker gene was amplified from the cellular fractions using the set of primers 8F15B (5'-AGAGTTTGATCCTGG-3') and 515R14AM (5-

TTACCGCGGCTGCT-3') (Aguirre de Cárcer *et al.,* 2011). The pool of samples with the prepared libraries was sequenced by Illumina MiSeq platform.

Bacterial 16S rRNA gene diversity was assessed with v2-2019.4 (Bolyen *et al.,* 2019). Briefly, cleaned and trimmed paired reads were filtered and denoised using DADA2 plug-in (Callahan *et al.,* 2016). For chimera identification, 250,000 training sequences were used. Identified amplicon sequence variants (ASVs) were aligned using MAFFT (Katoh *et al.,* 2002) and further processed to construct a phylogeny with fasttree2 (Price *et al.,* 2010). Taxonomy was assigned to ASVs using the q2-feature-classifier (Bokulich *et al.,* 2018) and blasted against the SILVA v132 99 % 16S sequence database (Quast *et al.,* 2012).

Sequences generated by this study were deposited to GenBank under the BioProject accession number PRJNA678484.

4.3.6 Data analyses

To assess the trophic pathways within the microbial mat community, a Bayesian isotopic mixing model, available as an open-source R package, simmr (Parnell and Inger, 2016), was used. The model is fitted via Markov Chain Monte Carlo (MCMC) method, producing simulations of plausible values of dietary proportions of sources consistent with the data using a single data point prior distribution (Parnell *et al.* 2010). The main carbon sources of the studied consumers were determined by comparison of their ^{13}C signatures to the groups that were previously designated as sources. We assumed similar stoichiometry for C and N fractionation through the food web in the model. Trophic enrichment factors (TEF) were based on mean trophic fractionations with standard deviations (TEF δ ^{13}C: –

0.4 ± 1.3; TEF δ ^{15}N: –3.4 ± 1) (Post, 2002). No parameters were modified between consumers according to their trophic habits, assuming that each group was composed of different species with different characteristics.

Alpha diversity indices and their rarefaction curves were estimated using the PAST software (Hammer *et al.,* 2001). Shannon index ($H = -\sum_{i=1}^{s} pi \ln pi$, where 'p' is the proportion (n/N) of individuals of one particular species found (n) divided by the total number of individuals found (N), 'ln' is the natural log, 'Σ' is the sum of the calculations, and 's' is the number of species) and Margalef's richness index (formula: $\frac{(S-1)}{\ln(n)}$, where 'S' is the number of taxa, and 'n' is the number of individuals) were estimated. Beta diversity was assessed using Bray-Curtis dissimilarities between the community compositions of the sampling sites and visualized with non-metric MultiDimensional Scaling (NMDS) using the R packages phyloseq. A hierachical cluster dendrogram was used to visualize similarities among the bacterial communities (order level) from the studied microbial mats. Differences among bacterial communities were tested with permutational multivariate analysis of variance tests (PERMANOVA) using the R-package Vegan 2.5–3.

Pearson correlation analysis was performed to evaluate the relation between the nutrient characteristics of microbial mats and different parameters related to the richness and abundance of the biological communities within the samples. A correlogram was used to visualize these results, by using the PAST software (Hammer *et al.,* 2001). To explore the relationships between microbial communities and environmental variables, a redundancy analysis (RDA) was performed. Variables were only retained in the analyses if they explained a significant ($p < 0.01$) variation in the

microbial data. All significance testing was assessed by Monte Carlo permutation of full models with 999 unrestricted permutations using the R-package Vegan 2.5–3.

4.4 RESULTS

4.4.1 Local fauna represents a relevant source of nutrients in the microbial mats

Redfield ratios (C/N and N/P) and $\delta^{15}N$ signatures were used to determine the influence of the local macrofauna, from the nutrient enrichment of the microbial mats. On the one hand, BP microbial mat displayed the highest C/N and N/P ratio (10.11 and 12.82), and the lowest $\delta^{15}N$ signature (-3.9 ‰) and values of inorganic nitrogen (28 and 24 $mg \cdot kg^{-1}$ for ammonium and nitrate respectively) (**Table 1**). Therefore, BP microbial mat was associated with the most oligotrophic environment, without the influence of local fauna and with less availability of nutrients. On the other hand, LI, BC, AI and CP samples showed different influence of marine vertebrates that was reflected in their physicochemical characteristics. LI microbial mat displayed the lowest C/N and N/P ratio (5.42 and 0.6, respectively), and the highest $\delta^{15}N$ signature (22.3) and nitrate value (130 mg/kg). Therefore, it was associated with an environment with greater nutrients availability due to the presence of local macrofauna. BP, AI and CP showed intermediate values of nutrients ratios, standing in an intermediate position between BP and LI microbial mats.

The faunal accessibility to the sampled areas was defined as the distance between microbial mats and sea. When this parameter was analysed, a significant positive correlation was obtained for C/N and N/P ratio (**Figure 2**). Also, a significant negative

correlation was obtained for $\delta^{15}N$ signatures of microbial mats and the faunal accessibility.

	Lagotellerie Island (LI) --- 67°53'16"S 67°24'02"W	Byers Coast (BC) --- 62°66'76"S 61°10'46"W	Avian Island (AI) --- 67°46'15"S 68°53'10"W	Cierva Point (CP) --- 64°09'00"S 60°57'50"W	Byers Plateau (BP) --- 62°65'49"S 61°11'41"W
PHYSICOCHEMICAL CHARACTERISTICS					
Marine vertebrates					-
Faunal accesibility (m)	10	100	30	200	1500
TAF (days per year)	83	190	83	13	190
C ($mg \cdot g^{-1}$)	115.6 (12.2)	285.9 (0.6)	190.3 (12.8)	212.9 (20.8)	115.6 (12.2)
N ($mg \cdot g^{-1}$)	21.3 (2.4)	44.9 (0.4)	26.3 (1.8)	21.1 (2.4)	21.3 (2.4)
P ($mg \cdot g^{-1}$)	33.7 (0.9)	57.2 (2.5)	11.0 (0.6)	1.8 (0.1)	33.7 (0.9)
$\delta^{15}N$ (‰)	22.3 (0.2)	12.4 (0.1)	8.8 (0.1)	-3.9 (0.3)	22.3 (0.2)
C/N	5.4 (0.0)	6.4 (0.1)	7.2 (0.2)	10.1 (0.2)	5.4 (0.0)
N/P	0.6 (0.1)	0.8 (0.0)	2.4 (0.3)	12.0 (2.2)	0.6 (0.1)
NH_3-NH^+_4 ($mg \cdot kg^{-1}$)	255	257	513	459	28
NO_3 ($mg \cdot kg^{-1}$)	130	27	41	88	24
TOC ($mg \cdot cm^{-2}$)	3.9 (0.2)	5.7 (1.2)	4.9 (0.5)	3.4 (0.7)	5.8 (1.9)
Chla ($\mu g \cdot cm^{-2}$)	26.8 (0.3)	15 (0.8)	8.1 (1.0)	5.2 (1.1)	11.6 (1.7)
Fungal Biomass ($\mu g \cdot cm^{-2}$)	109 (31.1)	127 (10.1)	61 (27.2)	37 (18.2)	9 (3.6)
MEIOFAUNA					
Nematodes ($ind \cdot cm^{-2}$) /%	0.0 (0) /0	203.7 (37.2) /6	22.9 (13) /6	48.9 (34) /52	96.7 (55) /50
Tardigrades ($ind \cdot cm^{-2}$) /%	644.9 (588) /86	2932.8 (532.4) /87	169.3 (69) /45	7.6 (11) /8	70.3 (32) /37
Rotifers ($ind \cdot cm^{-2}$) /%	106.9 (122) /14	249.5 (54.3) /7	184.6 (29) /49	37.7 (25) /40	28.5 (15) /13

Table 1. Physicochemical characteristics and meiofaunal relative abundances estimations (per surface) of the five microbial mats included in this study.

4.4.2 Fungal community showed positive correlation with the trophic status

Relative abundance of fungi, represented by ergosterol concentrations (**Table 1**), showed a negative correlation with C/N ratio (**Figure 2**). Therefore, fungi would be more abundant in those microbial mats from environments with higher proportions of N and P due to marine vertebrates and, therefore, related to more eutrophic status. No

consistent pattern was observed between none of the physicochemical environment parameters and the relative abundance of photosynthetic primary producers (**Figure 2**), represented by Chla concentration per area surface. TOC, related to total biomass of cyanobacterial microbial mats, showed significant positive correlation with climatic parameter of days per year with temperatures above freezing (0.95, $p < 0.05$).

The microscopic analyses indicated that the microbial community matrix was dominated by different Oscillatoriales and Synechococcales (Cyanobacteria) morphotypes. *Wilmottia sp.* (5.5-7 µm in diameter), previously classified as *Phormidium* (Anagnostidis and Komárek, 1988), was common in all the studied microbial mat. Morphotype I *sensu* Broady and Kibblewhite (1991) (2 µm in diameter, *Leptolyngbya cf. antarctica*), dominated the cyanobacterial community of BP and BC mat, but also occurred in all the mats. Abundant microcolonies (20-100 µm in diameter) of Nostocacean morphotypes (3 µm in diameter cells) appeared mostly in BP mat, but also in BC mat, below photosynthetically active layers. Unicellular cyanobacteria (e.g. *Synechococcus spp.,* 1.5 µm in diameter) were relatively abundant and occurred intermixed within the EPS matrix. The photosynthetic community also included Chlorophyta, although with an apparent relative lower abundance than the cyanobacterial community. *Prasiola sp.* constituted a top layer in AI and CP microbial mats, where *Chlamydomonadaceae* were also found. *Chlamydomonas sp.* (16-20 µm in diameter) was very common within the matrix of LI. Large amounts and high diversity of Bacillariophyta were found in BC mat and were observed in AI and LI samples. In all microbial mats *Mougeotia sp.* (22-27 µm in diameter) occurred.

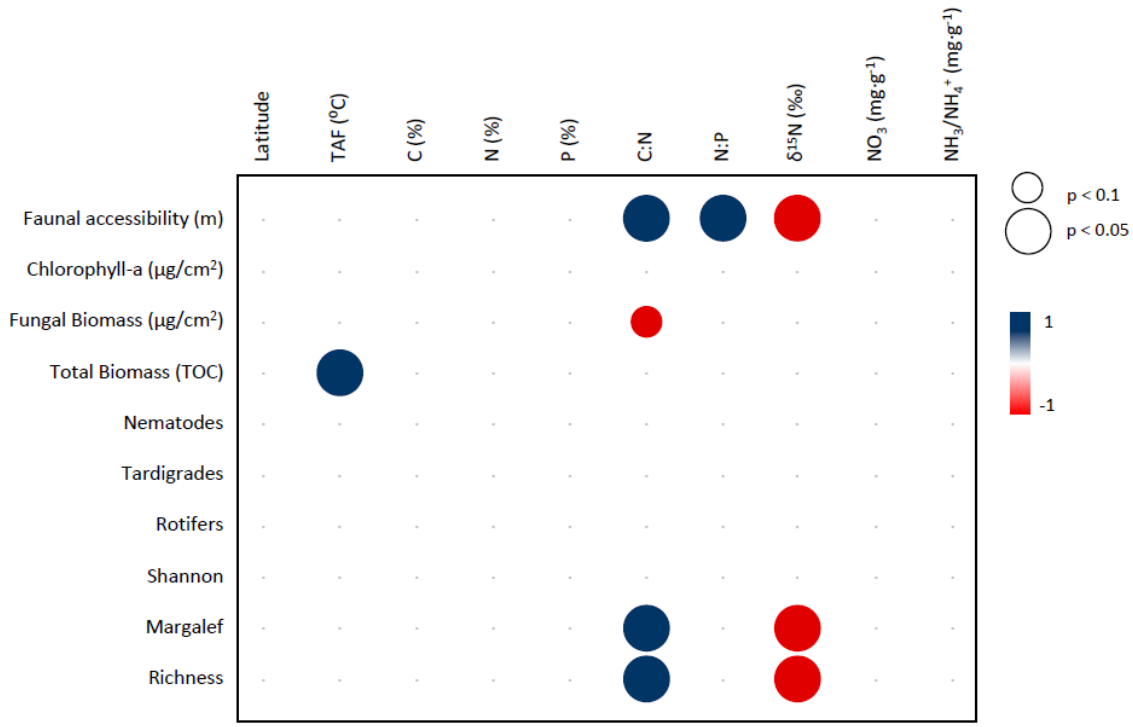


Figure 2. Correlogram for the independent variables identified as relevant to explain the differences in the microbial mats. Blue colour indicates strong positive correlation, while red colour indicates strong negative correlation. The different sizes of the spheres indicate different levels of correlation.

4.4.3 The meiofauna groups are heterogeneously distributed and without correlation with the nutrient availability

The meiofauna found in the studied microbial mats consisted of tardigrades *Hypsibiidae*, bdelloid rotifers, and *Plectidae* and *Monhysteridae* nematodes. Several tardigrades *Macrobiotidae* were found in the BC mat. None of the groups in any of the samples was clearly associated with a specific layer or portion of the microbial mats. The relative abundance of tardigrades, rotifers and nematodes (**Table 1**) was highly heterogeneous and not strongly correlated with nutrients availability (**Figure 2**). Nonetheless, tardigrades were more abundant in microbial mats with higher influence of local fauna,

as LI and BC (86 % and 87 % of total meiofaunal individuals captured per surface, respectively), counting hundreds of individuals per analysed sample (**Table 1**). Nematodes predominated in CP and BP microbial mats. The relative abundance of bdelloid rotifers was 49 % and 40 % of total meiofaunal individuals captured per surface in AI and CP, remaining below 15 % in the other microbial mats.

4.4.4 Trophic relationships change among the studied microbial mats

For all microbial mats, Cyanobacteria was the most abundant primary producer, and consequently a potential carbon source for the trophic community. Also, Bacillarophyta (BC microbial mat), *Chlamydomonadaceae* (Chlorophyta) for CP and LI mats, and *Prasiolaceae* (Chlorophyta) for AI mat and mosses (BP mat) were included in the food web study. In CP and BP, $\delta^{13}C$ of Cyanobacteria, POM and DOM (**Table S1**) showed signals similar to each other, while in the other mats the sources showed different values of both stable isotopes. $\delta^{13}C$ and $\delta^{15}N$ signals in the BP microbial mat were clearly different that the isotopic signals found in the other mats. While $^{15}N/^{14}N$ values of BP were negative in all separated groups (with the exception of nematodes where it was slightly positive), in all the other microbial mats ^{15}N was always clearly positive averaging from 8.8 to 22.3 ‰. The same pattern was observed in BP for $^{13}C/^{12}C$ values, showing less negative values in all the trophic groups studied compared to the other mats.

Consumer's category consisted for all samples of tardigrades *Hypsibiidae*, rotifers and nematodes. Tardigrades in LI (*Hypsibiidae*) and BC (*Macrobiotidae*) microbial mats were classified as top consumers from the marked differences in the ^{15}N signatures, showing $\delta^{15}N$ values that exceed those of nematodes by 2.8 and 2.0, respectively (**Table S1**). In

AI, CP and BP mats, nematodes were situated at the top of the food web, as assumed from the observed differences of 4.3, 2.5 and 3.5 $\delta^{15}N$ with tardigrades (*Hypsibiidae*), and differences of 2.5, 2.2 and 2.3 $\delta^{15}N$ with rotifers (**Figure 3B**). Enrichments in $\delta^{15}N$ showed between nematodes and rotifers for LI (0.8), and between rotifers and tardigrades for BC, AI, CP and BP (1.6, 1.9, 0.2 and 1.2) remained below typical 2-4 ‰ described for diet to consumer.

To determine carbon pathways, we plotted the credibility interval of the potential food sources contribution in each mat (**Figure 3A**). The Bayesian isotopic mixing model predicted the preferred C sources by consumers, showing DOM as the main food source in most of the microbial mats. Thus, the estimated dietary proportions for DOM in BC, AI, CP and BP were 57 %, 46 %, 29 % and 49 %, respectively. For LI mat, POM fraction appeared with the highest dietary proportion (24 %) of available sources. Cyanobacterial fractions show low proportions as a potential source in all microbial mats. Chlorophyta proportions from BC, AI and CP (29, 41 and 12 %, respectively) placed them as the second potential food source, as mosses did in BP (29 %).

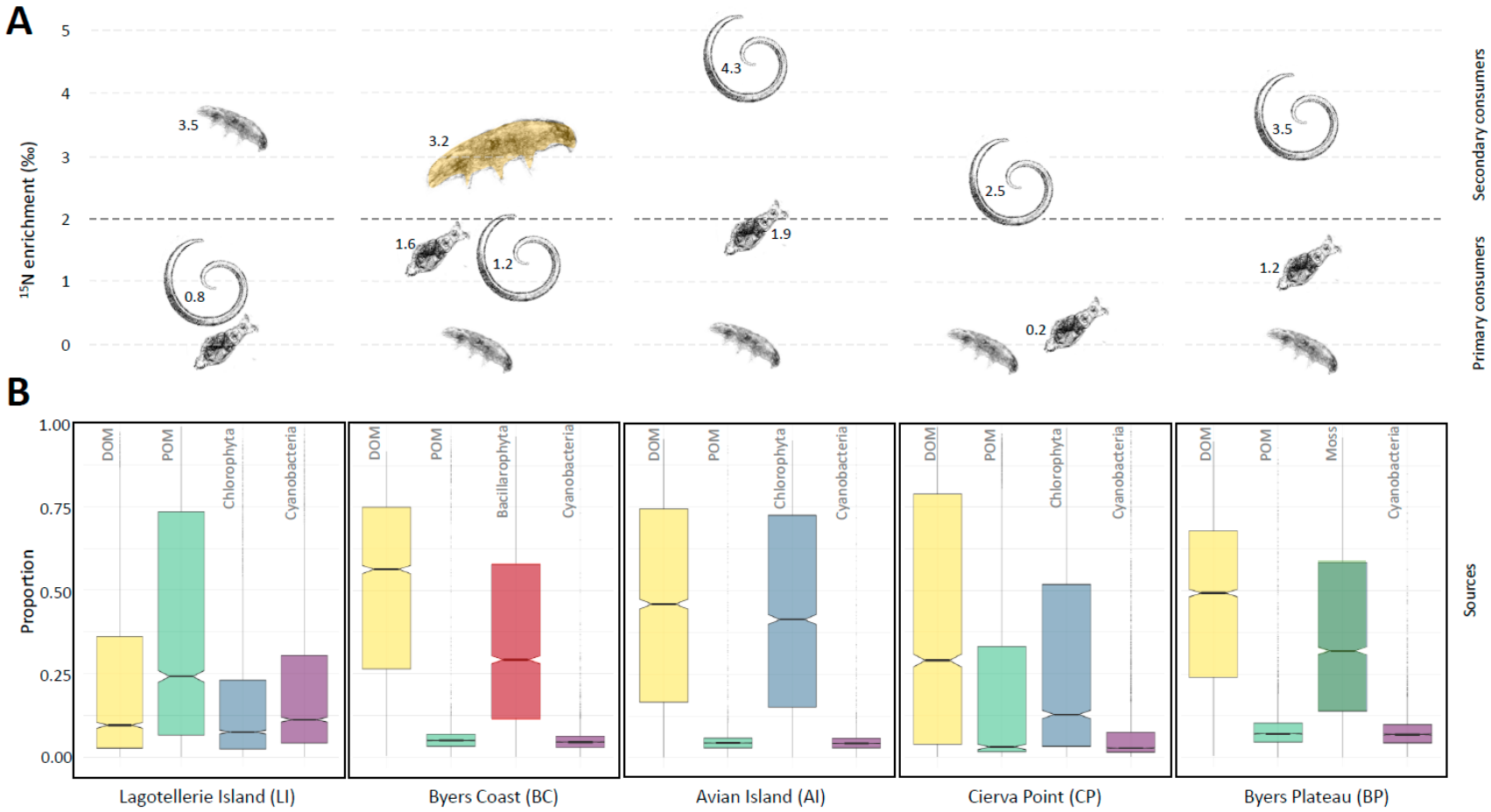


Figure 3. (A) Simmr credibility interval plot of the contribution of potential food sources of the studied microbial mats (boxes enclose the 50% credibility interval; lines within boxes represent median values). Cyanobacteria, dissolved organic matter (DOM) and particular organic matter (POM) were included in all the trophic webs studied, while Chlorophyta, Bacillarophyta and Moss groups were included according to their presence in the different microbial mats. (B) Enrichment in $\delta^{15}N$ of rotifers, nematodes, *Hypsibiidae* tardigrades (white) and *Macrobiotidae* tardigrades (orange). For estimating the differences between 'primary consumers' and 'secondary consumers', an enrichment of 2-4 ‰ was considered.

4.4.5 Bacterial richness and diversity decrease with local fauna

The total number of 16S rRNA gene sequences surveyed was 1,531,930. Total number of ASV for the 5 microbial mat samples was 3599. Analysis showed that bacterial communities (**Figure 4**) were dominated across all samples by sequences assigned to Proteobacteria (24-45 %) and Bacteroidetes (18-43 %). Cyanobacteria sequences ranked as the second phyla in BP with the highest number of assigned sequences (27.8 %), and third in the remaining samples (5.6-21.3 %). The orders that explained the highest proportion of the diversity were Burkholderiales, Leptolyngbyales and Flavobacteriales, followed by Sphingobacteriales, Chitinophagales and Sphingomonadales. Those taxa (order level) with relative abundances lower than 1 %, reached 13 % of the total community sequences in BP, while in the other microbial mats represented between 5-8 % of the total.

The lowest number of different ASVs was found in LI mat (449), as well as the lowest values for Shannon and Margalef indices (7.4 and 43.9, respectively). The highest number of different ASVs (581), as well as the highest values for Shannon and Margalef indices (7.9 and 56.6), was showed for BP microbial communities. Richness and Margalef index showed significant positive correlation with C/N ratio and significant negative correlation with $\delta^{15}N$ signatures (**Figure 2**). No significant differences were obtained for Shannon index and nutrients availability.

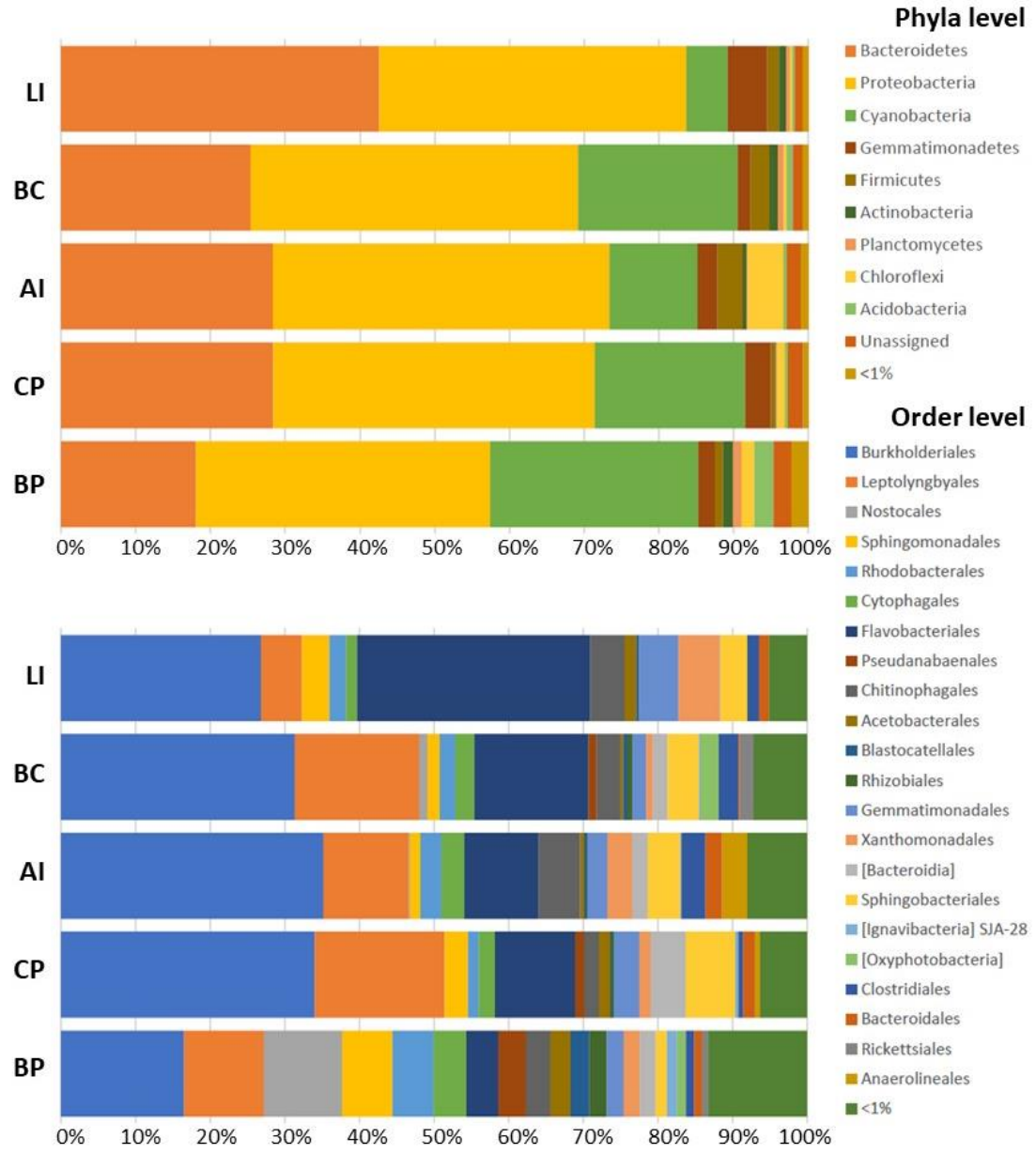


Figure 4. Relative abundance of 16S rRNA gene at different taxonomic resolution (Phyla vs Order) of the bacterial community composition of Lagotellerie Island (LI), Byers Peninsula Coast (BC), Avian Island (AI), Caleta Primavera (CP) and Byers Peninsula Plateau (BP) microbial mats.

In order to analyse the differences in the community structure among the samples of different locations, the taxonomic abundance profiles were used to compute a Bray–Curtis dissimilarity matrix, summarized in two dimensions by using NMDS (**Figure 5A**).

Samples were grouped according to site, showing greater dissimilarities among samples than among replicates within the same sample. A PERMANOVA test corroborated this result ($f = 28.7$, $p < 0.00$), showing that bacterial communities were not homogeneous among microbial mats. The segregation of bacterial taxa (order level) among samples is shown in the hierachical cluster dendogram (**Figure 5B**). The analysis revealed four main clusters according to differences in community composition, where similarity between AI and CP microbial mats was 77 %. BP showed the community with the lowest percentage of similarity (~50 %) with the other microbial mats. These results also highlighted heterogeneity among replicates within each microbial mat. The most important factors within the physicochemical environment that directly or indirectly affected the bacterial community composition of microbial mats, based on the variance partitioning with redundancy analysis (RDA), were NO_3^- ($R^2 = 0.45$, $p = 0.01$), N/P ($R^2 = 0.41$, $p = 0.01$), NH_4^+ ($R^2 = 0.22$, $p = 0.02$) and % C ($R^2 = 0.24$, $p = 0.02$). These factors together contributed significantly to the variance (~90 %, $p < 0.01$). Relative abundance of sequences assigned to Acidobacteria, Deinococcus-Thermus, Fibrobacteres and Nitrospirae were positively correlated with N/P, while Bacteroidetes and Gemmatimonadetes were correlated with NO_3^- (**Table S2**). Planctomycetes and Deinococcus-Thermus were negatively correlated with NH_4^+, showing lower relative abundances in those mats from areas with a higher presence of marine vertebrates.

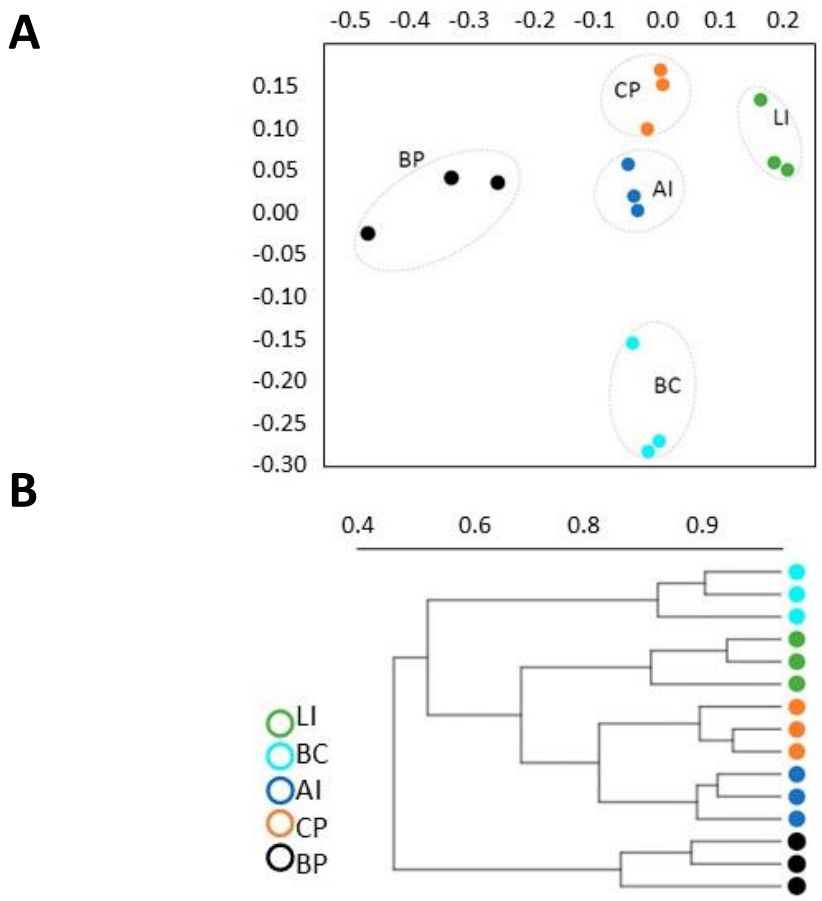


Figure 5. (**A**) Non-metric multidimensional scaling (NMDS) of the bacterial community composition of Lagotellerie Island (LI), Byers Peninsula Coast (BC), Avian Island (AI), Caleta Primavera (CP) and Byers Peninsula Plateau (BP) microbial mats. The multivariate analysis is based on Bray-Curtis dissimilarity matrices between the microbiome profiles at family level. Distances between symbols on the ordination plot reflect relative dissimilarities in community structures. (**B**) Hierarchical cluster dendrogram of the bacterial community (order level) within the microbial mats included in this study. The relative abundances were used to evaluate the relationships between bacterial communities, using weighted pair clustering based on Barry-Curtis measurements.

4.5 DISCUSSION

Our main results focus on the influence of nutrient availability derived from marine vertebrates on the structure and trophic relationships of the microbial mat communities from terrestrial aquatic ecosystem. The trophic characterization of each sample, based on nutrients availability in the ecosystem, its correlation with bacterial and meiofaunal

relative abundances, and the studied trophic relationships, were useful in detecting patterns among the microbial mat communities. These patterns are in turn correlated with the presence of terrestrial macrofauna in the Antarctic environment. Hence, these changes are relevant in the panorama of global change, since the distribution of marine vertebrates will be affected as Antarctic temperatures continue to rise (Clucas *et al.,* 2014).

It is already known that vertebrate wildlife from marine ecosystem has direct consequences on the Antarctic terrestrial ecosystem (Jakubas *et al.,* 2008; Zmudczyńska *et al.,* 2012; Ball *et al.,* 2015; Zawierucha *et al.,* 2016; Zawierucha *et al.,* 2019; Bokhorst *et al.,* 2019; Wang *et al.,* 2020), but their consequences in cyanobacterial-based microbial mats remain understudied. According to our results, microbial mats growing at areas where marine vertebrates were present, showed the highest values of nitrate and ammonium. However, absolute values do not provide enough information related to the trophic status or potential limitations in nutrients availability of these microecosystems. The ecological stoichiometry referred to in ratios (e.g. C/N or N/P) focuses on the relationships between organism, ecosystem structure and function with the environment (Elser *et al.,* 2000). These elemental ratios can be associated with important ecological features such as ecosystem-specific composition and diversity (Venterink *et al.,* 2003), processes of organic matter decomposition (Güsewell and Gessner, 2009), and with the ability of organisms to adapt to environmental stresses (Woods *et al.,* 2003). For the five studied microbial mats, C/N ratio becomes an indicator of their trophic status, being nitrogen considered as a limiting actor in oligotrophic ecosystems (Turner *et al.,* 1994). The faunal accessibility variable allows us to study the sampling areas according to the influence of marine vertebrates, since we assumed that

the distance of the studied microbial mat to the coast, in areas with high concentrations of marine vertebrates, can be used as a proxy for their presence. Our results, based on the Redfield ratio (C/N/P), showed a relationship between microbial mats elemental content and the presence of local macrofauna. Microbial mats closer to the coast and with greater influence of marine vertebrates (LI and AI) showed a eutrophic status, while BP, grown in an area far from the coast and geographically isolated for marine vertebrates, showed the lowest values of N and P availability. Thus, BP was associated with the most oligotrophic environment. Considering the ultraoligotrophic characteristics of freshwaters far from animal colonies (Davey, 1993), and the role that microbial mats play in ecosystem processes such as carbon and nitrogen cycles (Alcántara-Hernández *et al.,* 2014), these results would have major implications for the Antarctic terrestrial ecosystem.

Our results have shown that Antarctic cyanobacterial-based microbial mats are nitrogen enriched according to the presence of local macrofauna. It was evidenced that ^{15}N values were higher following the increase of local fauna influence, which may be related with increases in marine N source due to a high trophic position of seabirds (Cherel *et al.,* 2007) and preferential volatilization of ^{14}N from guano (Mizutani *et al.,* 1985). These results are consistent with previous studies (Otero *et al.,* 2018), probing that penguin guano and the faeces of other marine vertebrates transform the environmental conditions of the sites where they establish their breeding colonies, and become especially relevant in the Antarctic ecosystem (Wang *et al.,* 2007; Smykla *et al.,* 2015; Smykla *et al.,* 2018; Bokhorst *et al.,* 2019). Those microbial mats grown in environments close to penguin colonies and elephant seal aggregations also presented higher values of ^{15}N in their food webs, as reported by Wang *et al.* (2020) when studying the impact

of penguin guano on terrestrial and aquatic nitrogen cycles in Victoria Land (Antarctica). Therefore, N from marine vertebrates would flow through the trophic community of microbial mats, and their major elements could be directly linked to the marine-derived $\delta^{15}N$ signature (Bokhorst *et al.,* 2019). However, and considering that guano contains ~15-20 % N and 10 % P (Lorrain *et al.,* 2017), our results have only shown a correlation between %P of microbial mats and the presence of penguins in the surroundings, but not with %N. These differences could be associated with the higher accumulation rates of P in soils (Simas *et al.,* 2007), and with the presence of other marine vertebrates, such as seals and other sea birds, that would also act as biological nutrients pumps between marine and terrestrial ecosystems.

The consequences of natural fertilization should become more evident in primary producers than in consumers, since typically nitrogen and phosphorus limit primary production in marine and terrestrial environments (Chadwick *et al.,* 1999; Mills *et al.,* 2004), and subsequently on the complete food web (Zawierucha *et al.*, 2019). Allochthonous nutrients enrichment of Antarctic lakes by local fauna has been documented, reporting increases in chlorophyll concentrations due to these processes (Hawes, 1990; Vinocur and Unrein, 2000; Izaguirre *et al.*, 2001; Rochera, 2012). In the studied cyanobacterial microbial mats, chlorophyll concentrations varied along the samples within the values reported in previous studies from Antarctica (Hawes *et al.,* 2019), with more than a 2-fold increase of chlorophyll concentration between AI and BP. Despite this, a significant correlation with trophic status was not found. Thus, nutrient availability may influence primary producers activity and growth in these forced ecosystems, but most likely other variables, such as the length of the liquid water period,

could be relevant limiting the growth as suggested by Billy and Potts (2002) even in nutrient replete ecosystems.

Understanding the structure of the food web is necessary to comprise how organisms and the environment interact and therefore the functioning of the ecosystem (Perkins *et al.,* 2014). According to our results, the trophic structures of the microbial mats were different among samples, with consumers appearing in different abundances and trophic positions within the studied food webs. Our data indicated that tardigrades appeared by hundreds in samples related to eutrophic status while nematodes proportionally dominated microbial mats related to oligotrophic status. It has been suggested that primary producers communities affect the abundance and diversity of higher trophic levels (Bokhorst *et al.,* 2015), which in turn represent an important driver of its populations by grazing and nutrient recycling (Zawierucha *et al.,* 2018). The most widespread tardigrades genera in Antarctica (*Acutuncus, Hypsibius* and *Isohypsibius*) are defined as grazers (Nelson *et al.,* 2018). Therefore, higher densities of grazers at those areas with higher nutrient availability, may be explained by the availability of other resources such as green algae (Vonnahme *et al.,* 2016), as suggested by increases in chlorophyll a concentration, and probably higher abundances of fungi compared to areas less influenced by local fauna (Zwolicki *et al.,* 2016; Zawierucha *et al.,* 2019), as suggested by increases in ergosterol concentration.

Based on the potential food sources analysis model and the feeding ecological strategies of consumers, our results suggest that the differences in $\delta^{15}N$ may be related to an enrichment of $\delta^{15}N$ for rotifers intakes caused by preferential consumption of dissolved organic matter (DOM) and/or bacteria, and for tardigrades caused by particulate organic

matter (POM) and algae, as previously suggested in microbial mats from Antarctica (Velázquez *et al.,* 2017; Almela *et al.,* 2019a). Data on feeding preferences of Antarctic rotifers (bdelloids), which are filter feeders, suggested that the diet consisted of dead organic matter and unicellular algae (Iakovenko *et al.,* 2015), although they could probably feed on bacteria (Jaromerska *et al.,* 2021). In cyanobacterial-based microbial mats, POM fraction would be composed of EPS, bacteria and ciliates, but we also found abundant unicellular cyanobacteria (~1.5 µm in diameter) forming this matrix. As nitrogen-fixing organisms, such as cyanobacteria, have high content of proteins and high ^{15}N values (Kohler *et al.,* 2018), tardigrades from LI mat would consequently present higher ^{15}N compared with rotifers, as it has been suggested for tardigrades in Arctic cryoconite holes (Jaromerska *et al.,* 2021). Fungi should also be considered as a potential food source for these herbivores, although their role within the trophic relationships could not be included in this study. In BP mat, tardigrades probably feed on POM, which would contain mostly moss fragments, present in the sampling area surrounding the microbial mat, but not coccoid cyanobacteria neither such amount of fungi. Bacterivorous nematodes are common in Antarctic soils (Nielsen *et al.,* 2011; Caruso *et al.,* 2019), but due to their versatility as omnivores, they would probably feed on other consumers, as suggested in Almela *et al.* (2019a) for microbial mats. Therefore, the shift in POM composition would explain the different trophic positions of tardigrades and rotifers, which in turn would be related to the availability of nutrients and the presence of different primary producers densities in the studied microbial mats. Furthermore, the differences shown in $\delta^{15}N$ among consumers suggest that nematodes positioned at the top of the trophic web in those cyanobacterial-based mats with less nutrients availability, with a typical enrichment of 2-4 ‰ from diet to consumer (Post, 2002;

Vanderklift and Ponsard, 2003; Caut *et al.,* 2009). These data indicate the likely existence of a tertiary trophic level, as shown for Antarctic soils (Shaw *et al.,* 2018). The differences in ^{15}N shown by tardigrades could also suggest a different trophic level in LI for *Hypsibiidae* specimens, and in BC for the omnivorous tardigrade *Macrobiotidae*, where could be feeding on small nematodes (Nelson *et al.,* 2018), among other small invertebrates. However, the carbon sources of consumers will probably vary throughout the summer, as primary producers do (Velázquez *et al.,* 2011), and this could modify ^{15} N ratios within the consumers community.

Our analysis revealed that inorganic nitrogen (NH_4^+ and NO_3^-), %C and N/P ratio, were key factors in driving bacterial community composition, which was similar to the previously described (Varin *et al.* 2010; Kleinteich *et al.,* 2017; Valdespino-Castillo *et al.,* 2018; Dillon *et al.,* 2020; Jackson *et al.,* 2021). A similar pattern was determined for Antarctic soils (Smith *et al.,* 2010), where ammonia, nitrate, and total N, among other physicochemical parameters, were driving the microbial community composition. It is well known that N is a key nutrient in ecosystems, and its availability is correlated with the presence of marine vertebrates (Bokhorst *et al.,* 2019; Wang *et al.,* 2020). Therefore, this correlation between nutrient availability and the presence of local fauna could explain the greater similarity showed between AI and CP microbial communities, since the presence of penguins in their surroundings was greater than in the other mats, due to the proximity of large breeding areas. Consequently, LI would show a greater similarity with this cluster. BC was related to the presence of elephant seals with very occasional penguins visits. Bacteria belonging to the order Clostridiales and Bacillales, reported higher relative abundances in those microbial mats influenced by marine vertebrates, as was reported in soils influenced by penguins (Santamans *et al.,* 2017)

and lakes frequented by elephant seals (Picazo *et al.,* 2019). However, *Clostridiaceae* predominated in BC, while *Christensenellaceae, Lachnospiraceae, Family XI* and *Family XIII* did so in AI and CP microbial mats. Therefore, this would indicate that some specific groups of local fauna could influence differently the composition of the Antarctic terrestrial ecosystem.

Cyanobacteria ranked as the second phyla with the highest number of assigned sequences only in oligotrophic BP, showing relative abundances significantly lower in samples with higher inputs of allochthonous nutrients due to local fauna. A similar pattern was observed when comparing oligotrophic to nutrient-rich lakes in Antarctica, suggesting than high nitrogen concentration associated with vertebrates enrichment would tend to favour non-cyanobacterial taxa (Pearce *et al.,* 2005). Sequences analysis among the samples indicated an irregular pattern of filamentous Cyanobacteria assigned to Leptolyngbyales*,* and an increase in reads related to the nitrogen cycle taxa, such as Pseudanabaenales and Nostocales, in non-enriched mats. *Pseudanabaena* and *Geitlerinema* potentially fix nitrogen (N_2) into ammonium (Bergman *et al.,* 1997; Fernández-Valiente *et al.,* 2007; Grim and Dick, 2016), and nitrifying bacteria, such as *Nitrospira* found in BP, oxidizes it into nitrite, increasing the availability of nitrogen to the community. Marine vertebrates breeding areas, especially penguins rookeries, are considered nutrient hotspots, since a large proportion of the excreted N and P is present in highly bioavailable forms (Otero *et al.,* 2018). The uric acid contained in the faeces of these seabirds serves as a substrate for its mineralization in different forms of N through ammonification and nitrification processes. Considering that N would be widely available in areas with local fauna, diazotrophs such as Nostocales, whose activity may constitute up to 23 % of the assimilated nitrogen by mats community (Fernández-

Valiente *et al.,* 2007), may be less competitive in these communities, and consequently their relative abundance would decrease. Despite the amounts of ammonia reported in the samples most influenced by the local fauna, no anammox bacteria were found, which in turn could be associated with the negative correlation showed between Planctomyces and the availability of ammonia.

The predominance of Proteobacteria and Bacteroidetes in freshwater bacterial communities seems to be a general trend in Antarctica (Valdespino-Castillo *et al.,* 2018). These groups have been classified as heterotrophic microbes and are major players in microbial communities around the globe, due to its high metabolic versatility. Bacteroidetes, a highly abundant group in aquatic ecosystems, appeared correlated to ^{15}N and NO_3- concentrations. Flavobacterium was associated to microbial mats highly influenced by local fauna, due to *Flavobacteriaceae* was tenfold more abundant in LI than in BP, and *Weeksellaceae* was found in all samples except in BP. Therefore, these heterotrophic bacteria may have a specialized role in those microbial mats related to an enriched environment (Kirchman, 2002). Burkholderiales (Betaproteobacteriales) and Flavobacteriales species have been recognised as initial metabolizers of labile carbon inputs in soils (Padmanabhan *et al.*, 2003) during initial stages of the microbial loop. Nutrient recycling by bacteria might be extremely relevant for microbial mat survival and regeneration, in an ecosystem were the input of allochthonous organic C is limited due the unproductive surrounding catchment (Lyons *et al.* 2013). According to our results, nutrient recycling processes could be accelerated by the concurrence of fungal community, especially in those microbial mats associated with eutrophic states.

Our data showed a consistent pattern of richness and diversity of bacterial communities with the trophic status of the studied microbial mats, showing a greater number of species and its abundance in those environments where the availability of allochthonous nutrients was smaller. These results are consistent with Pearce *et al.,* (2005), were nutrient-enriched lake due to seal activity showed reduced species richness in comparison to more oligotrophic lakes. This reduction in bacterial species richness was also accompanied by an increase in evenness among key groups. In proportion to the overall population size, Burkholderiales, Flavobacteriales and Leptolyngbyales sequences explained 35 % of the bacterial community in BP, while in BC and LI mats ranged between 62-77 %. Also, while in the other mats, the taxa (order level) with relative abundances lower than 1 % represented between 5-8 % of the total, in BP reached 12 %. Therefore, there is an apparent change in dominance with the trophic state gradient, which together with species richness, induces oligotrophic systems into communities previously associated with low resistance to environmental change (Pearce *et al.,* 2005).

4.6 CONCLUSIONS

The availability of nutrients in the terrestrial Antarctic ecosystem derived from the transfer of nitrogen and phosphorus from sea to land of marine vertebrates influence the diversity and distribution of bacterial communities, and trophic interactions of communities within microbial mats from the Antarctic Peninsula. Our results have suggested relevant changes, not only in the diversity and richness of microbial communities, but also in the structure of bacterial communities, with nutrient enriched

mat communities appearing as less complex. Furthermore, the cyanobacterial populations, responsible for the structure of these microecosystems, are altered, as are the relative abundances of the biological components at higher trophic levels, such as the meiofauna and the fungal community. These results show the influence of marine vertebrates on biocomplexity of one of the most diverse ecosystems in non-marine Antarctica. These community shifts are also relevant in the panorama of global change since nutrients availability in the ecosystem could change and alter the metabolic equilibrium of the ecosystem, due to alterations in the local fauna because of Antarctic temperatures continue to rise.

4.7 SUPPLEMENTARY MATERIAL

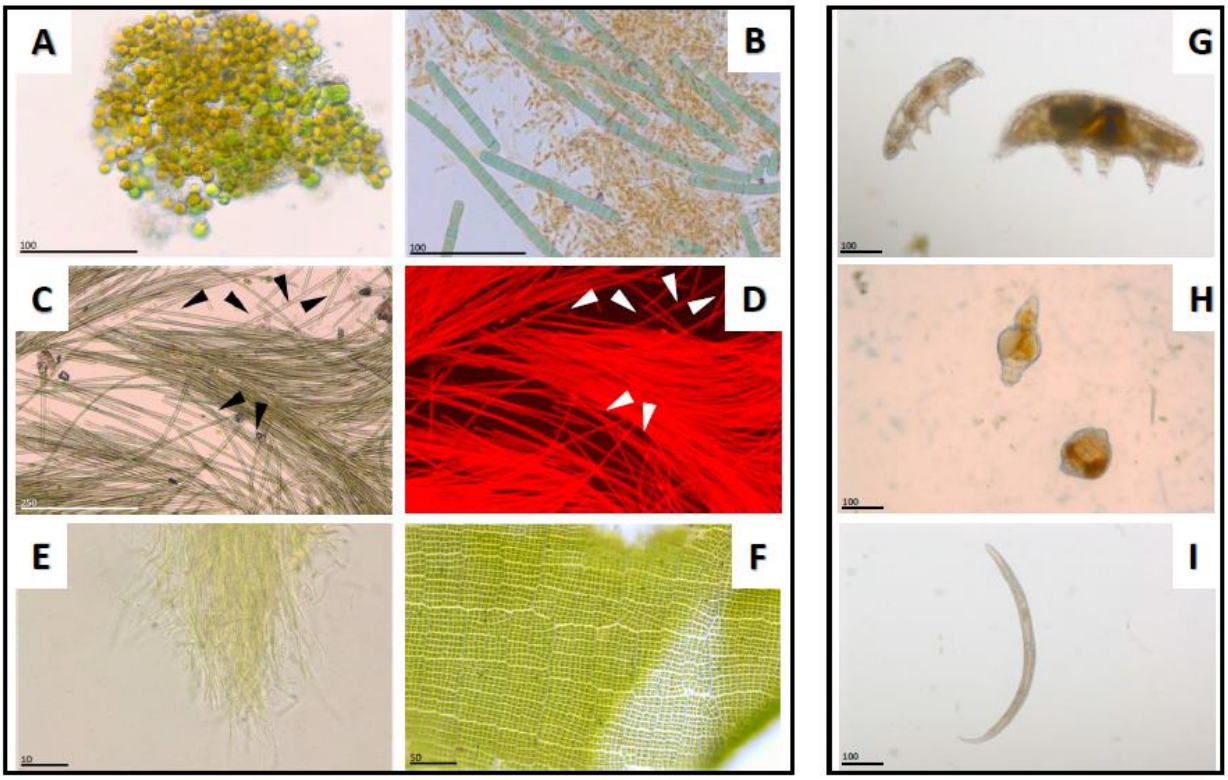
Table S1. Description of samples and isotopic values ($\delta^{13}C$ and $\delta^{15}N$) of sources and consumers included in trophic web analysis. Isotopic values are presented in per mille (‰) and related to the international standards of the Pee Dee Belemnite for carbon and atmospheric N_2 for nitrogen. Standard deviation (SD) is shown between parentheses.

	Lagotellerie island		**Byers Coast**		**Avian Island**		**Cierva Point**		**Byers Plateau**	
	$\delta^{13}C$ (SD)	$\delta^{15}N$ (SD)	$\delta^{13}C$ (SD)	$\delta^{15}N$ (SD)	$\delta^{13}C$ (SD)	$\delta^{15}N$ (SD)	$\delta^{13}C$ (SD)	$\delta^{15}N$ (SD)	$\delta^{13}C$ (SD)	$\delta^{15}N$ (SD)
Sources										
Cyanobacteria (filamentous)	-21.94 (0.8)	25.09 (2.4)	-17.3 (0.1)	11.75 (0.6)	-23.06 (0.2)	10.42 (0.43)	-26.73 (0.1)	9.22 (0.16)	-13.91 (0.5)	-4.91 (0.6)
Chlorophyta	-24.00 (0.5)	24.81 (0.5)	_	_	-25.74 (0.5)	10.9 (0.3)	-28.96 (0.6)	6.45 (1.2)	_	_
Bacillariophyta	_	_	-21.84 (0.2)	11.76 (0.02)	_	_	_	_	_	_
Moss	_	_	_	_	_	_	_	_	-20.54 (0.0)	-2.67 (0.7)
POM	-18.21 (0.1)	22.75 (0.1)	-20.52 (0.1)	10.44 (0.1)	-26.64 (0.1)	12.23 (0.1)	-26.19 (0.0)	8.64 (0.2)	-14.39 (0.1)	-3.37 (0.1)
DOM	-20.63 (0.1)	24.36 (0.2)	-18.47 (0.0)	12.54 (0.1)	-26.01 (0.4)	12.25 (0.1)	-25.05 (0.5)	8.94 (0.1)	-13.70 (0.1)	-2.56 (0.2)
Consumers										
Tardigrades (*Hypsibiidae*)	-23.58 (0.1)	28.34 (1.9)	-19.35 (0.4)	12.07 (0.3)	-27.77 (0.0)	12.21 (0.5)	-27.36 (0.2)	11.46 (0.3)	-15.26 (0.4)	-1.31 (0.4)
Tardigrade (*Macrobiotidae*)	_	_	-21.33 (0.3)	15.22 (0.6)	_	_	_	_	_	_
Rotifers	-23.10 (0.8)	24.83 (1.1)	-20.42 (0.2)	13.71 (0.3)	-28.46 (0.4)	14.08 (0.6)	-26.29 (0.2)	11.70 (0.1)	-17.18 (0.5)	-0.08 (0.4)
Nematodes	-24.09 (0.5)	25.59 (1.4)	-22.10 (0.4)	13.25 (0.3)	-25.31 (0.2)	16.53 (0.8)	-26.52 (0.4)	13.96 (0.9)	-13.94 (0.3)	2.23 (0.7)

Table S2. Pearson correlations of bacterial community and physicochemical parameters. Bold letters were used to highlight significative correlations (≥0.9; $p \leq 0.05$) of those parameters that contributed significantly to the variance of the community.

	C	N	P	C/N	N/P	^{15}N	NO_3^-	NH_4^+
Bacteroidetes	-0.63	-0.12	0.52	-0.81	-0.80	0.90	**0.90**	0.34
Proteobacteria	0.57	0.90	0.50	-0.61	-0.62	0.43	-0.15	0.85
Cyanobacteria	0.32	-0.19	-0.80	0.83	0.87	-0.87	-0.72	-0.48
Gemmatimonadetes	-0.76	-0.46	0.36	-0.46	-0.59	0.60	**0.97**	0.13
Firmicutes	0.62	0.88	0.66	-0.46	-0.32	0.36	-0.37	0.39
Actinobacteria	-0.16	-0.32	-0.36	0.34	0.69	-0.23	-0.37	-0.89
Planctomycetes	-0.12	-0.45	-0.63	0.60	0.89	-0.50	-0.48	**-0.96**
Chloroflexi	0.82	0.73	0.69	0.09	-0.18	-0.20	-0.38	0.47
Acidobacteria	0.16	-0.39	-0.56	0.92	**0.96**	-0.86	-0.60	-0.82
Patescibacteria	0.65	0.37	0.51	0.42	0.07	-0.47	-0.32	0.17
FBP	-0.83	-0.89	-0.18	0.15	0.03	0.03	0.72	-0.40
Deinococcus-Thermus	-0.04	-0.52	-0.65	0.83	**0.96**	-0.74	-0.50	**-0.93**
Epsilonbacteraeota	-0.65	-0.27	0.51	-0.58	-0.54	0.72	0.79	0.00
Armatimonadetes	0.13	-0.45	-0.50	0.95	0.70	-0.93	-0.31	-0.43
Dependentiae	-0.89	-0.46	0.00	-0.65	-0.50	0.76	0.86	0.03
BRC1	0.38	0.44	0.86	-0.13	-0.31	0.13	0.01	0.23
Cloacimonetes	0.75	0.85	0.84	-0.22	-0.41	0.10	-0.26	0.61
Elusimicrobia	-0.12	-0.18	-0.27	0.05	-0.24	-0.11	0.32	0.45
Fibrobacteres	0.09	-0.46	-0.50	0.93	**0.91**	-0.86	-0.48	-0.80
Nitrospirae	0.14	-0.22	-0.72	0.62	**0.92**	-0.60	-0.73	-0.80
Omnitrophicaeota	-0.12	-0.18	-0.27	0.05	-0.24	-0.11	0.32	0.45
Spirochaetes	0.3	-0.3	-0.4	0.9	0.6	0.9	-0.3	-0.2
Synergistetes	0.8	0.8	0.8	-0.2	-0.4	0.1	-0.3	0.6
Verrucomicrobia	0.11	0.27	-0.39	-0.26	0.18	0.19	-0.44	-0.12

Figure S1. Light microscopy and fluorescence micrographs of the main primary produces and consumers within the food webs. **(A)** Chlamydomonadaceae (green algae) visualized in LI. **(B)** Diatoms (Bacillarophyceae, green algae) and Oscillatoria filaments found in BC. Oscillatoria filaments (cyanobacteria) visualized in BP with light microscopy **(C)** and fluorescence microscopy **(D)**. Black and white triangles indicate the presence of *Leptolyngbya sp.* filaments (cyanobacteria). **(E)** *Leptolyngbya sp.* filaments (cyanobacteria) in BP. **(F)** Prasiola sp. (green algae) visualized in AI. **(G)** *Hypsibiidae* (right) and *Macrobiotidae sp.* (left) tardigrades in BC. **(H)** Rotifers and **(I)** nematode found in CP.



“No person who has not spent a period of his life in those 'stark and sullen solitudes, that sentinel, the Pole' will understand fully what trees and flowers, sun-flecked turf and running streams mean to the soul of a man.”

Ernest Shackleton.

DISCUSION GENERAL

Las regiones polares intervienen de forma muy relevante en el contexto actual de calentamiento global, siendo ecosistemas especialmente sensibles al aumento de las temperaturas. La Península Antártica es una de las zonas de la Tierra donde se ha registrado un incremento mayor de las temperaturas en los últimos cincuenta años. Estudiar los ecosistemas de esta región es necesario para conocer y comprender cómo funcionan, monitorear los cambios que se están produciendo en esta región, y predecir situaciones futuras que podrían tener importantes repercusiones locales y globales.

En este trabajo se aborda el estudio de los tapetes microbianos de cianobacterias, desde los procesos de sucesión primaria de las cianobacterias en suelos recientemente deglaciados, hasta las interacciones tróficas que tienen lugar en los tapetes microbianos. Las cianobacterias constituyen el grupo fotosintético procariota más importante del ecosistema Antártico, y su papel en las áreas de retroceso glaciar podría ser determinante para el desarrollo del resto de la comunidad. Por otro lado, los tapetes microbianos acumulan grandes cantidades de carbono orgánico para el ecosistema, altas diversidades de organismos procariotas y eucariotas, y son considerados sistemas de alta sensibilidad útiles en el monitoreo de los cambios ambientales. La búsqueda de una relación entre las comunidades de cianobacterias del suelo y los tapetes microbianos, además del estudio en detalle de ambos sistemas, permitirá entender algunos de los aspectos relacionados con el establecimiento y desarrollo de una de las comunidades de mayor complejidad del ecosistema Antártico.

Cianobacterias: organismos clave del ecosistema Antártico

El estudio simultáneo realizado en esta Tesis Doctoral de tres glaciares en retroceso en áreas situadas a lo largo de la Península Antártica, junto con el análisis metagenómico de procariotas y de la comunidad de cianobacterias, nos ha proporcionado una visión más completa del papel de este grupo sobre las dinámicas de sucesión en el ecosistema antártico. Las cianobacterias han aparecido con mayores abundancias en los suelos más próximos al frente glaciar, actuando como organismos pioneros en suelos recientemente deglaciados, tal y como había sido determinado en otras regiones del planeta (Fernández-Martínez *et al.,* 2017; Pessi *et al.,* 2019; Pushkareva *et al.,* 2019; Knelman *et al.,* 2021). Además, gracias al uso de cebadores específicos de cianobacterias, que hasta la fecha no habían sido empleados para el estudio de zonas de retroceso glaciar en la Antártida, hemos podido resolver la composición de la comunidad al nivel taxonómico de género. A partir de estos resultados, hemos comprobado en el Capítulo 2 como no aparece un patrón común en la dinámica de sus poblaciones a lo largo de los suelos estudiados, lo que podría estar relacionado con las interacciones con el resto de la comunidad procariota y/o eucariota. Estos suelos recientemente expuestos tras la desaparición de la cubierta de hielo se caracterizan por presentar un escaso grado de desarrollo y una baja disponibilidad de nutrientes (Vega-García *et al.,* 2021), además de por una falta de cubierta vegetal. Por tanto, la actividad microbiana será responsable del desarrollo que puedan tener estos suelos (Boy *et al.,* 2016), sin olvidar el resto de los factores bióticos y abióticos que pudieran estar implicados en dichos procesos edafológicos. Así, la capacidad de las cianobacterias de incorporar carbono y nitrógeno en las capas superficiales del suelo a través de sus actividades fototróficas y eventualmente diazotróficas y, en consecuencia, facilitar la

colonización posterior de otros organismos, es clave para el establecimiento de ecosistemas de mayor complejidad, como los tapetes microbianos.

Las superficies recientemente deglaciadas son pobladas rápidamente, aunque hay dudas acerca del origen de estos organismos pioneros. No sabemos con certeza si estos grupos presentes en las primeras etapas de sucesión ya estaban en los suelos antes de la retirada del hielo, si son atrapados por la nieve y alcanzan estas zonas por el agua de deshielo, o si son directamente transportados por el aire hasta estas zonas o depositados por la lluvia y/o la niebla (Foght *et al.,* 2004; Kastovská *et al.,* 2005; Hodson *et al.,* 2008; Takeuchin 2011; Evans *et al.,* 2019). De hecho, la situación más probable es que la llegada de organismos pioneros tenga lugar por varias de estas vías descritas, siendo por tanto un proceso complejo de estudiar y delimitar. De esta forma, no sólo se desarrollarán aquellos organismos cuyo metabolismo les permite establecerse en condiciones oligotróficas, sino aquellos organismos adaptados que primero alcancen dichos espacios por cualquiera de las vías de entrada descritas, añadiendo una variable de 'casualidad' a los procesos de establecimiento y sucesión primaria. Este planteamiento de la composición de las comunidades, donde 'no todo está en todas partes, sino solo aquello que ha conseguido llegar y establecerse', encajaría con la heterogeneidad de las comunidades procariotas descrita en el Capítulo 1, aun cuando las condiciones fisicoquímicas y ambientales de las localizaciones estudiadas son muy similares entre sí. La presencia de cianobacterias ha sido constatada tanto en aire, como en niebla, lluvia, dentro de las rocas, en la superficie de los glaciares o atrapadas en el propio hielo (Humbert y Fastner, 2017; Casero *et al.,* 2019; Evans *et al.,* 2019; Galbán *et al.,* 2021). Esta capacidad de habitar distintos biotopos estaría a su vez relacionada con la variedad de estrategias desarrolladas para soportar las condiciones ambientales más

extremas, a destacar la producción de EPS (Chrismas *et al.,* 2016), la síntesis de carotenoides (Vincent y Quesada, 2012), los mecanismos de reparación de ADN (Vincent, 2007; Zakhia *et al.,* 2008), o la acumulación preferente de ciertos ácidos grasos en sus membranas (D'Amico *et al.,* 2006; Zakhia *et al.,* 2008), entre otras estrategias. Por tanto, considerando su distribución en las fuentes potenciales de entrada, y su resistencia y versatilidad, las probabilidades de éxito de las cianobacterias en zonas recientemente deglaciadas se maximizan.

Pero no todas las cianobacterias han mostrado la misma abundancia relativa. Los géneros de cianobacterias que conforman la 'Soil Cyanobacterial Core Community' descrita en el Capítulo 2, con un peso relativo grande dentro de la comunidad total, probablemente constituyan el grupo de cianobacterias mejor adaptado a las condiciones ambientales de la Antártida. La presencia de estos mismos géneros en dos tapetes microbianos separados por más de 400 km a lo largo de la Península Antártica, a partir del análisis comparativo de secuencias parciales del gen ARNr 16S, permite además entender su distribución en los ecosistemas antárticos no marinos, y probablemente la alta movilidad de estos genotipos entre los diferentes lugares. Pero la distribución de estas secuencias comunes no se limita a la Antártida, ni siquiera a regiones criosféricas del planeta, como hemos podido comprobar a partir de los datos aquí presentados. A nivel biogeográfico, estos resultados sugieren la alta capacidad de movimiento de ciertos microorganismos a través de la atmósfera, cuestionado el efecto de la conectividad limitada de los ecosistemas polares con el resto del planeta (Pearce *et al.,* 2009; Bottos *et al.,* 2014; Archer *et al.,* 2019).

Tapetes microbianos: complejos microecosistemas en la Antártida

Las pocas áreas libres de hielo que aparecen salpicadas a lo largo del continente antártico, en su mayoría concentradas en la Península Antártica, están compuestas principalmente por suelos oligotróficos pedregosos y faltos de cubierta vegetal. No es de extrañar que la presencia de tapetes microbianos en las zonas encharcadas de esta región, de colores anaranjados, verdosos y negros, llamase la atención de los primeros exploradores y científicos que arribaron a esta región a principios del siglo XX (Murray, 1910). Desde entonces, estas comunidades microbianas han sido objeto de estudio, al considerarse una de las fracciones principales de biomasa no marina y acumular una gran biodiversidad dentro del ecosistema terrestre antártico.

En esta Tesis Doctoral hemos podido constatar como la gran mayoría de biomasa que conforma los tapetes microbianos no participa en primer término en la red trófica. Sabemos que esta biomasa está compuesta por capas de polímeros extracelulares, constituidos principalmente por derivados de carbohidratos, aunque también por otros compuestos como proteínas y lípidos (Vincent *et al.,* 1993; De los Ríos *et al.,* 2004; Rochera *et al.,* 2013). Estos derivados de carbohidratos son producidos principalmente por microorganismos fotosintéticos, en su mayoría cianobacterias, que aprovechan los fotones solares para producir energía y fijar carbono y nitrógeno. La acumulación de toda esta materia orgánica es fundamental para que se establezca el resto de la comunidad (De los Ríos *et al.,* 2014; Chown *et al.,* 2015), que encuentra el soporte estructural, además de unas condiciones favorables que permiten una mayor retención del agua, una disminución del punto de congelación y un aumento en la concentración de los nutrientes. Por tanto, la complejidad de estos sistemas depende de las

cianobacterias, que gastarán grandes cantidades de energía en la producción de exopolisacáridos. Se considera que la integridad estructural y funcional de estos sistemas se mantiene por la baja bioturbación y depredación de la meiofauna (Fenchel, 1998). Por tanto, pequeñas variaciones en las condiciones ambientales o en las abundancias de su comunidad podrían tener importantes repercusiones. Pero estos microecosistemas también deben ser considerados como sistemas de alta versatilidad. El estudio de cinco tapetes microbianos afectados de forma distinta por la macrofauna local nos ha permitido conocer cómo las comunidades que los conforman se ven alteradas significativamente por los niveles de nutrientes, que a su vez definen el estado trófico de dichos sistemas. La presencia de vertebrados marinos en las áreas de estudio no es casual, ya que son zonas habituales de cría de mamíferos pinnípedos y aves marinas, por lo que asumimos que las comunidades que conforman los tapetes microbianos están habituadas a dichos ambientes. Nuestros resultados han mostrado como la abundancia relativa de cianobacterias se reduce en aquellos tapetes microbianos crecidos en áreas con altas concentraciones de vertebrados, probablemente en favor de organismos fotosintéticos eucariotas, que encontrarían unas condiciones más favorables para el crecimiento en aquellos ambientes con mayor carga de nutrientes. Sin embargo, considerando a las cianobacterias como las arquitectas de los tapetes microbianos, y teniendo en cuenta la reducción de su abundancia relativa en las muestras relacionadas con ambientes más eutróficos, no hemos detectado cambios aparentes en la cohesión y/o estructura de las muestras analizadas, ni siquiera como consecuencia de un aumento de los procesos de bioturbación por parte de la macrofauna local. Sabemos que ciertos grupos de cianobacterias producen exopolisacáridos en abundancia, y que por tanto son clave para

la formación de estos microecosistemas (De los Ríos *et al.,* 2004; Zakhia *et al.,* 2008; De los Ríos *et al.,* 2015; Chrismas *et al.,* 2016). Por todo ello, resulta necesario estudiar en detalle el efecto de las interacciones de la macrofauna local con estas comunidades concretas de cianobacterias, para predecir qué escenarios serían incompatibles con la viabilidad de los tapetes microbianos.

El estudio de las redes tróficas llevado a cabo en los Capítulos 3 y 4 de esta Tesis Doctoral, ha permitido conocer con más detalle la complejidad que estos microecosistemas albergan en apenas unos milímetros de espesor. Gracias al uso, por primera vez en estas comunidades, de carbono-13 como trazador de las relaciones tróficas, hemos determinado la presencia de al menos 4 niveles tróficos. La entrada de materia y energía se diversifica a través de distintos productores primarios fotosintéticos. Así, el nivel trófico de las fuentes presenta organismos con óptimos metabólicos distintos (Velázquez y Quesada*,* 2011), asegurando la actividad de estos sistemas en un rango más amplio de condiciones ambientales. Los consumidores conformarían dos niveles tróficos distintos, con filtradores y herbívoros en un nivel inferior, y con aquellos grupos omnívoros en un segundo nivel trófico. Las abundancias de consumidores herbívoros han aparecido relacionadas con los nutrientes disponibles, que a su vez se relacionan con la biomasa de los organismos fotosintéticos. Estos resultados sugieren que los tapetes microbianos podrían ser ecosistemas con un control *bottom-up,* donde la entrada de nutrientes, la productividad y las poblaciones de productores primarios controlarían la estructura del ecosistema. Sin embargo, deberíamos considerar las diferencias en la abundancia de invertebrados a lo largo de los años, que podrían alterar el flujo de energía a través de la red trófica (Andriuzzi *et al.,* 2018). En el Capítulo 4, los nematodos han demostrado un papel ecológico clave

dentro de la comunidad, conectando las distintas entradas de carbono orgánico descritas, y relacionándose tróficamente con los consumidores primarios. La presencia de tardígrados *Macrobiotidae* en un tapete microbiano estudiado en el Capítulo 5, también sugiere que estos grupos podrían conformar un nivel trófico superior en la comunidad. En base a estos resultados, y teniendo en cuenta que una parte considerable de la comunidad trófica no ha podido ser incluida en este estudio por incompatibilidad con la metodología empleada, podemos asegurar que la complejidad de estos microecosistemas es elevada. Sin embargo, a partir de los experimentos de enriquecimiento hemos visto como existe un acoplamiento de su comunidad que permite la transferencia de carbono y energía desde las fuentes a los consumidores en poco tiempo, adaptándose así a los cortos periodos de actividad durante el verano austral. Por tanto, serán necesarios más estudios para llegar a entender con detalle cómo y a través de quién tienen lugar los flujos de energía de los tapetes microbianos de la Antártida.

Los tapetes microbianos de cianobacterias son *hotspots* de diversidad para el ecosistema Antártico. Nuestros resultados han mostrado como aquellos microecosistemas crecidos en áreas con presencia de vertebrados marinos tienen comunidades menos ricas en especies y menos diversas, donde unos pocos taxones dominan. Los eventos extremos influyen en la dinámica de las poblaciones de especies y su biogeografía (de Pol *et al.,* 2017; Jenouvrier *et al.,* 2015). Sabemos que las poblaciones de macrofauna local están variando en la Antártida, como consecuencia del incremento de las temperaturas y el cambio asociado a la disponibilidad de alimento en los océanos. Se ha comprobado que las distintas poblaciones de vertebrados condicionan la composición y abundancia de las comunidades de lagos (Picazo *et al.,*

2019), suelos (Santamans *et al.,* 2017; Guo *et al.,* 2018; Ramírez-Fernández *et al.,* 2021), musgos y líquenes (Bokhorst *et al.,* 2019), y tapetes microbianos. Por tanto, un cambio significativo en las poblaciones de vertebrados marinos, como se ha sugerido, por ejemplo, con el aumento de las poblaciones de pingüino Papua (*Pygoscelis papua*), podría llevar asociado un cambio sustancial de la diversidad del ecosistema terrestre antártico. En este contexto, no menos importante es la presencia de especies invasoras, con enorme potencial para desplazar a las especies autóctonas y alterar la biodiversidad antártica (Hughes y Worland, 2010; Chown *et al.,* 2012; Hughes *et al.,* 2015). Si además tenemos en cuenta otros factores de carácter antrópico, como la presencia de plásticos en las costas (Almela y González, 2020) y aguas continentales (Pleiter *et al.,* 2021), así como el aumento de las actividades marítimas, como la actividad turística, que en los dos últimos años ha aumentado un 43 % (IAATO, 2020), los riesgos que amenazan la sostenibilidad del ecosistema antártico son considerables. El estudio de las comunidades microbianas y las redes tróficas, consideradas de gran sensibilidad frente a cambios ambientales (Vincent, 2010; Camacho *et al.,* 2012), se convierte así en una herramienta clave para el monitoreo de los efectos del cambio global sobre el ecosistema de la Antártida.

CONCLUSIONES

1. Las comunidades procariotas de suelos desnudos en la Antártida muestran un alto grado de heterogeneidad, con una proporción elevada de ASV únicas en cada una de las áreas de estudio, no compartidas con otras zonas. Si bien la heterogeneidad alcanza altos niveles dentro de los perfiles verticales de aquellos suelos que quedaron libres de hielo hace cientos de años, esta disparidad de las comunidades es más clara a escala geográfica entre las distintas localizaciones estudiadas a lo largo de la Península Antártica.

2. La presencia de ASVs compartidas entre las muestras de suelos analizadas ha permitido definir una comunidad central de bacterias en los suelos estudiados. Teniendo en cuenta las características espaciales y temporales de las diferentes ubicaciones estudiadas, los taxones que conforman dicha comunidad se relacionarían con grupos altamente versátiles, a nivel metabólico, y transportables (muy probablemente por el viento). Por tanto, esta comunidad común tendría un papel clave en la colonización de los suelos oligotróficos recientemente deglaciados de la Antártida.

3. La presencia de cianobacterias en las tres áreas de retroceso glaciar confirma el papel de este phylum como organismos pioneros y fotótrofos esenciales en suelos recientemente deglaciados. Sin embargo, las diferencias en la abundancia relativa observadas entre las áreas de retroceso glaciar sugieren que estos organismos, no solo podrían desempeñar distintas funciones en los procesos de sucesión primaria, sino que lo hacen según los requerimientos de la comunidad.

4. A pesar de mostrarse una homogeneidad general a niveles taxonómicos altos, se observan claras diferencias en la composición de la comunidad de cianobacterias a lo largo de las zonas de retroceso glaciar estudiadas, con cambios en los géneros presentes, así como en sus abundancias relativas. Por tanto, nuestros resultados no muestran una relación consistente entre la estructura de la comunidad de cianobacterias y la edad del suelo.

5. Considerando que no aparece un desarrollo edáfico significativo a lo largo de las tres cronosecuencias para las variables fisicoquímicas estudiadas, nuestros resultados muestran que las interacciones bióticas con el resto de la comunidad procariota del suelo pueden ser determinantes para explicar las dinámicas de las poblaciones de cianobacterias, en los estados primarios de pedogénesis y las primeras fases de sucesión primaria.

6. La presencia de una comunidad común de cianobacterias en todos los suelos muestreados y en todos los lugares de muestreo, ha permitido definir una comunidad central de cianobacterias (Soil Cyanobacterial Core Community). Esta comunidad está compuesta por 14 ASVs adscritas a los géneros *Chroococcidiopsis sp., Pseudanabaena sp., Phormidesmis sp., Tychonema sp., Phormidium sp., Microcoleus sp., Nodularia sp.* y *Nostoc sp.*

7. La comunidad común de cianobacterias (Soil Cyanobacterial Core Community), con una elevada abundancia relativa sobre el total de secuencias analizadas, confirma la distribución de estos géneros en ecosistemas antárticos no marinos, y probablemente la alta movilidad de estos genotipos entre las diferentes localizaciones. La aparición de estas mismas ASVs en los tapetes microbianos analizados, sugiere que esta comunidad común de cianobacterias se asociaría con aquellos organismos que se adaptan mejor a las condiciones ambientales antárticas, adquiriendo un papel clave en estos ecosistemas.

8. Los tapetes microbianos de cianobacterias de la Península de Byers acumulan grandes cantidades de carbono. Según nuestros resultados, casi el 90 % del carbono orgánico acumulado, en su mayoría EPS, no formaría parte de ninguno de los niveles tróficos estudiados, y se relacionaría con la porción estructural de estos microecosistemas, dando así soporte físico a las comunidades procariotas y eucariotas que los conforman.

9. La composición isotópica de la comunidad del tapete microbiano de la Península de Byers ha revelado una red trófica más compleja de lo que se consideraba hasta la fecha, con al menos cuatro niveles tróficos: un nivel basal de productores primarios (formado por diatomeas y cianobacterias como diferentes entradas de carbono orgánico), un nivel secundario de consumidores (compuesto por rotíferos y tardígrados), un nivel secundario superior (compuesto por nematodos), y un cuarto

nivel donde los hongos y bacterias actuarían como parte de la comunidad de descomponedores.

10. Considerando la posición trófica dentro de la comunidad del tapete microbiano de la Península de Byers, el enriquecimiento en ^{13}C mostrado, y los hábitos alimenticios descritos, los nematodos juegan un papel clave como principales consumidores de la comunidad, conectando las dos entradas de carbono descritas en el ecosistema.

11. El tránsito de carbono desde los productores primarios hasta los consumidores se ha producido en menos de 24 h, llegando a los consumidores secundarios en menos de 11 días. Dadas las condiciones ambientales extremas del ecosistema antártico, los resultados sugieren que existe un fino acoplamiento temporal entre los organismos de la comunidad del tapete microbiano, minimizando la redundancia en el desempeño funcional entre los niveles tróficos.

12. Los tapetes microbianos de cianobacterias influenciadas por la macrofauna local muestran un enriquecimiento en ^{15}N, en todos los niveles tróficos estudiados. Por tanto, el N de los vertebrados marinos fluye desde los productores primarios de los tapetes hasta sus consumidores, conectando los ecosistemas terrestre y marino de la Antártida.

13. La estructura trófica de los tapetes microbianos resulta afectada por la presencia de fauna local, así como la abundancia relativa y la posición trófica de la meiofauna estudiada (nematodos, rotíferos y tardígrados).

14. La presencia de vertebrados marinos afecta a las comunidades bacterianas de los tapetes microbianos estudiados, apareciendo una relación entre la riqueza y diversidad de las comunidades bacterianas y el estado trófico de los microecosistemas. De esta forma, las comunidades procariotas de los tapetes microbianos relacionados con estados eutróficos muestran un número menor de especies, y están dominadas por menos taxones, en comparación con las comunidades relacionadas con estados oligotróficos.

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