VNIVERSITAT DE VALÈNCIA

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Programa de Doctorado en Biodiversidad y Biología Evolutiva

FUNCTIONAL AND TAXONOMIC DIVERSITY IN MARITIME ANTARCTIC LAKES

Diversidad funcional y taxonómica en lagos de la Antártida Marítima

Tesis Doctoral

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Valencia, 2015

Memoria titulada "**Functional and taxonomic diversity in Maritime Antarctic lakes**", presentada por el Licenciado **Juan Antonio Villaescusa Vinader** para optar al grado de Doctor en Biología.

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Fdo. Prof. Dr. Antonio Camacho González

A mis padres Teresa y Antonio

Agradecimientos

Parece que fuera ayer cuando me ofrecieron la oportunidad de realizar un trabajo de tesis doctoral en la Antártida. Os aseguro que la ilusión de visitar y explorar un lugar de dichas características es incomparable, sin embargo, he de admitir que nunca podría haber imaginado el enorme esfuerzo y trabajo que este proyecto y en especial mi trabajo de tesis ha conllevado. Durante este largo periodo ha habido momentos fáciles y difíciles, incluso momentos en los que uno se viene abajo y solo piensa en dejarlo y mirar hacia otro lado. Sin embargo, gracias a la ayuda y ánimo de muchas personas, entre ellos compañeros, familiares y amigos, el trabajo ha sido más llevadero y al final ha culminado en el presente manuscrito. Quiero agradecer el presente trabajo a todos los que me han apoyado y espero no dejarme a nadie. Gracias:

A mi director Antonio Camacho "Toni", por darme la oportunidad para entrar en el mundo de la investigación y apoyarme durante mi Tesis. Tengo que añadir que sin su inmensa ayuda, paciencia y confianza no habría finalizado este manuscrito.

A mis padres, por su apoyo y cariño incondicional que me ha permitido acometer el trabajo día a día.

A mi hermano, por estar siempre ahí, que más podría decir, espero que sigas siendo siempre así.

A Carlos Rochera y Antonio Picazo, por su ayuda incondicional el todo momento y por el gran ambiente que han promovido en el laboratorio durante todos estos años. "Siberia" no sería lo mismo sin vosotros.

A todos los compañeros del departamento, Maru Diazmacip, Sara Morata, Charo López, Nayeli Murueta, Olivier Schmit, Javi Marco, Carla Olmo, Andreu Escrivá, Nieves Sánchez, Lidia Romero, María Sahuquillo, Mari Loli Sendra y Carmen Ferriol.

A Eduardo Vicente y María Rosa Miracle por su excelente trabajo y apoyo en el departamento.

A todos los compañeros del proyecto Limnopolar, Antonio Quesada, Eduardo Fernández-Valiente, Manolo Toro, Eugenio Rico, Ana Justel, David Velázquez, Álvaro Chicote, Manolo Bañón, Javier Armengol, José Antonio Gil-Delgado.

A Emilio Ortega Casamayor del centro de estudios avanzados de Blanes (CEAB), por acogerme en su laboratorio y permitirme realizar parte de mi labor científica.

To Sven Erik Jørgensen (Department of Toxicology and Environmental Chemistry, University of Copenhagen) for his help in the development of the Lake Limnopolar ecological modeling.

Al personal de la Base Antártica Española Juan Carlos I y del buque Las Palmas A52, por su gran ayuda en la puesta a punto de las diversas campañas Antárticas y

garantizar en todo momento nuestra seguridad y comodidad. También agradecer al Comité Polar Español por permitir que nuestro trabajo fuera posible.

A todos los investigadores de diferentes países que han participado en las diversas campañas Antárticas y compartido conmigo su trabajo. En especial a la memoria de Luigi Michaud, por su gran camaradería en Byers y su posterior apoyo en mi trabajo. Descansa en paz amigo, siempre me acordaré de ti, gran compañero, gran persona.

Al Servei Central de Suport a la Investigació Experimental (SCSIE) de la Universidad de Valencia por su accesibilidad y profesionalidad.

A todos mis amigos, por todos los momentos buenos y malos pasados. Gracias por aguantarme todos estos años de ir y venir.

A la Universidad de Valencia, CICyT y distintos Ministerios, por conceder la financiación de los proyectos: REN2000-0435-ANT, CGL2005-06549-C02-02/ANT, POL2006-06635 y acciones especiales CGL2007-29841-E, CTM2008-05205-E, indispensables para el trabajo de mi grupo de investigación y para la elaboración de mi tesis doctoral.

¡Gracias a todos!

Abstract

Byers Peninsula (Livingston Island, Antarctica) is one of the largest ice-free areas in the maritime Antarctica, displaying an important number of ice-free water bodies during the austral summer. These features, as well as its status as Antarctic Special Protected Area (ASPA), featured Byers as a reference site for Limnological studies in maritime Antarctica. This thesis deals with the study of the lakes located in Byers Peninsula, focusing our attention in Lake Limnopolar, used as a model lake for limnological studies during the last two decades.

Primarily, our studies were focused on the environmental heterogeneity of the lakes and ponds of Byers Peninsula, showing a contrasting trophic status among these lakes, which was strongly determined by the external inputs of nutrients and the internal removal processes from the lake sediment. This environmental heterogeneity was also reflected in the composition of bacterial community, with lakes displaying higher trophic status showing the lower bacterioplankton diversity. Additionally, the study of the consumption of different carbon sources by bacterioplankton, evidenced differences among the studied lakes in their consumption profiles, that can be related to the origin of the organic matter and the relation of each lake with their catchment, flora and fauna. Our results revealed a pattern by which lakes with the highest trophic status also showed the highest diverse physiological activity, contrarily to the diversity of bacterial taxa that dropped as trophic status increased.

Continuous meteorological monitoring in the area and the study of Lake Limnopolar from 2001 to 2009 has revealed a high variability of meteorological features in this area of maritime Antarctica, with summers with different average temperatures that directly affect the ice melt and the length of the productive period. Accordingly, the bacterioplankton community of Lake Limnopolar shows changing dynamics between different summers. This feature could be directly related to the availability of organic carbon and the length of the productive period. The physiological studies on Lake Limnopolar also evidenced this changing pattern,

showing a statistically significant correlation between bacterioplankton abundance and the concentration of the different dissolved organic carbon compounds. Also, the bacterial community from in Lake Limnopolar shows a differential vertical distribution even though a physical and chemical vertical gradient did not appeared. The analysis of the bacterial sequences retrieved from the lake revealed that the origin, composition and abundance of the organic matter are directly affecting the bacterial composition in the lake and the appearance of this "biological stratification".

Phytoplankton and bacterioplankton production rates in Lake Limnopolar display a clear uncoupling. Allochthonous contributions of organic carbon from microbial mats, along with autochthonous contributions of organic carbon from benthic mosses, subsidize the planktonic bacterial production in Lake Limnopolar. Manipulative in-situ experiments performed in Lake Limnopolar have demonstrated the response of the planktonic community to fertilization processes and the strong relationship of phytoplankton growth and inorganic carbon fixation with light (including photoinhibition when excessive) and nutrient availability. Bacterioplankton, however, seems to be less responsive to glucose enrichment treatments, which can be related to the existence of specialist bacterial species. On the other hand, the study of the epibiosis on crustacean zooplankton in Lake Limnopolar has demonstrated the existence of a nutritional strategy of the algal epibionts, mainly composed by diatoms and euglenophytes, in response to extreme oligotrophic conditions. In this situation, epibiosis acts as efficient strategy when nutrient availability is low.

To summarize the obtained results and monitor Lake Limnopolar dynamics, an ecological model was developed. Our model clearly shows the control exerted by the temperature on the bacterioplankton dynamics and the availability of carbon sources, mainly dissolved organic carbon (DOC). However, the use and interpretation of the developed model should be taken with care due to the high interannual meteorological heterogeneity in Byers Peninsula.

Resumen

El área conocida como Península Byers (Isla Livingston, Antártida) se caracteriza por ser una de las zonas libres de hielo más grandes de la Antártida marítima, mostrando un importante número de cuerpos de agua libres de hielo durante el verano austral. Estas características y su estatus como zona Antártica de especial protección (ASPA) han convertido a la Península Byers en un sitio de referencia para estudios limnológicos en la Antártida marítima. La presente tesis trata sobre el estudio de los lagos ubicados en la Península Byers, centrándose en el lago Limnopolar, utilizado como modelo para estudios limnológicos durante las últimas décadas.

El estudio de la heterogeneidad ambiental en los lagos de la Península Byers ha mostrado la existencia de un fuerte contraste en el estado trófico de los lagos estudiados, claramente influenciado por los aportes externos de nutrientes y los procesos de remoción propios del sedimento. La heterogeneidad ambiental observada en los lagos de la Península Byers la podemos relacionar estrechamente con la composición bacteriana en estos sistemas, de esta forma lagos con mayor estado trófico muestran la menor diversidad de grupos bacterianos. Adicionalmente, el estudio de la actividad fisiológica sobre el aprovechamiento de carbono por parte del bacterioplancton en los lagos de la Península Byers, ha evidenciado diferencias claras en sus perfiles metabólicos. Estos pueden relacionarse con el origen de la materia orgánica y la relación de cada lago con su cuenca, flora y fauna. Nuestros resultados revelan un patrón mediante el cual los lagos con mayor estado trófico presentan patrones con mayor diversidad metabólica, y por el contrario una menor diversidad de taxones (OTUs) bacterianos

El seguimiento de la meteorología de la zona y de las características del Lago Limnopolar desde 2001 hasta 2009 ha revelado la existencia de una elevada variabilidad meteorológica en esta área de la Antártida marítima, con temperaturas muy dispares entre distintos veranos, lo cual afecta directamente al deshielo y la duración del periodo productivo en el lago. La abundancia del bacterioplancton

heterotrófico en el Lago Limnopolar presenta una dinámica cambiante entre los diversos años estudiados, lo cual puede estar directamente relacionado con la disponibilidad de carbono orgánico y la duración del periodo productivo. Los estudios sobre la actividad fisiológica en el Lago Limnopolar han mostrado esta variación, destacando la significativa correlación existente entre la abundancia del bacterioplankton y la concentración de los diferentes compuestos de carbono orgánico disuelto. Adicionalmente, la composición de la comunidad bacteriana en el Lago Limnopolar presenta una marcada heterogeneidad vertical a pesar de no existir una estratificación física. El análisis de las secuencias obtenidas en el Lago Limnopolar ha revelado que el origen, composición y abundancia de la materia orgánica afecta directamente a la composición de grupos bacterianos en el lago, lo cual se acaba traduciendo en una "estratificación biológica".

Las tasas de producción del fitoplancton y del bacterioplancton en el Lago Limnopolar muestran un claro desacoplamiento. De esta forma, los aportes externos de carbono orgánico por parte de los tapetes microbianos, junto con contribuciones de carbono orgánico por parte los musgos bentónicos contribuyen a la producción bacteriana en el lago y mantienen el desacoplamiento entre la producción primaria y bacteriana. Asi mismo, los experimentos de manipulación in-situ llevados a cabo en el lago Limnopolar han demostrado la rápida respuesta de la comunidad planctónica a la fertilización y la estrecha relación del fitoplancton con la disponibilidad de luz (incluido el exceso que provoca fotoinhibición) y de nutrientes durante el verano austral. Sin embargo, la respuesta de la comunidad de bacterioplancton en el aprovechamiento de glucosa parece no ser muy relevante, lo cual puede estar relacionado con la existencia de especies bacterianas especialistas. Por otro lado el estudio de la epibiosis sobre crustáceos planctónicos en el Lago Limnopolar ha demostrado la existencia de una estrategia nutricional por parte de las algas epibiontes, compuestas principalmente por diatomeas y euglenófitas, en respuesta a condiciones extremas de oligotrofia. En esta situación, parece claro que la epibiosis actúa como una estrategia eficiente cuando disminuye la disponibilidad de nutrientes.

A partir de los resultados obtenidos en el seguimiento del Lago Limnopolar y con objetivo de monitorizar la dinámica del mismo, se llevó a cabo un modelo ecológico. Nuestro modelo muestra el claro control que ejerce la temperatura sobre la dinámica del bacterioplancton y sobre la disponibilidad de fuentes de carbono, principalmente en la forma de carbono orgánico disuelto (DOC). Sin embargo el uso y la interpretación de los resultados obtenidos por el modelo deben ser tratados con cautela debido a la elevada heterogeneidad interanual en el clima de la Península Byers.

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1. Introducción general

1.1. Antecedentes

El desarrollo de esta tesis puede calificarse como una continuación de la labor científica desarrollada por parte del grupo de investigación Limnopolar, compuesto por un equipo multidisciplinario de investigadores de la Universidad de Valencia y de la Universidad Autónoma de Madrid. Este ambicioso programa de investigación antártica se ha desarrollado a lo largo de dos proyectos, LIMNOPOLAR I y LIMNOPOLAR II, financiados por parte del Programa de Investigación Español. Ambos se han caracterizado por presentar un carácter multidisciplinar centrado principalmente en el estudio de los sistemas acuáticos epicontinentales de la Antártida marítima, especialmente la Península Byers, y su respuesta frente al cambio climático.

La mayor parte del trabajo incluido en esta tesis trata de continuar la labor científica realizada durante la primera parte del programa Limnopolar, desarrollado durante varias campañas Antárticas a lo largo del verano austral del periodo 2002-2005. Durante este período se llevaron a cabo diversos trabajos de campo, incluyendo aquí muestreo y experimentación in-situ, y en laboratorio, teniendo en cuenta todas las medidas de seguridad y procedimientos a seguir en una zona de especial protección (ASPA) como lo es la Península Byers.

Las actividades llevadas a cabo durante el periodo comprendido entre 2002-2005 se centraron en tres objetivos específicos, los cuales pueden definirse como:

- Incrementar el conocimiento ecológico de la Península Byers, caracterizada como área de especial protección Antártica (ASPA), con el objetivo de poder llevar a cabo estudios comparativos con otras regiones Antárticas con figuras de protección menos restrictivas y por ende bajo una mayor influencia humana.
- Estudiar la ecología trófica de los sistemas acuáticos epicontinentales de la Antártida marítima, centrándose en el estudio de la transferencia de biomasa y energía en los mismos.

- Establecer la Península Byers como un punto de referencia internacional en la Antártida para monitorizar los efectos del cambio climático en sistemas acuáticos no marinos.

A partir de los trabajos realizados durante las campañas Antárticas incluidas entre los años 2002-2005 y las posteriores campañas enmarcadas en el Proyecto Limnopolar II, se han llevado a cabo diversas publicaciones y tesis doctorales de interés científico. La revisión realizada por Benayas et al. (2013) recopila los trabajos científicos realizados en las últimas décadas en la Península Byers y refleja el carácter multidisciplinar de la investigación realizada en la zona. Desde hace décadas, el área de la península Byers se ha caracterizado mayoritariamente por su gran interés científico debido a la importante presencia de poblaciones de elefantes marinos (*Arctrocephalus gazella*) y su relación con los cazadores de focas durante el siglo XVII, sin embargo no es hasta la década de los 60 cuando se llevan a cabo los primeros estudios científicos sobre la geología y estratigrafía en la zona. A partir de estos estudios, el interés científico se centró en investigar la historia climática y el proceso de desglaciación de la Península Byers. Sin embargo, tras el cambio en su figura de protección, pasando a ser zona de especial protección Antárctica (ASPA) en el año 2001, gran cantidad de estudios científicos de carácter multidisciplinar se llevaron a cabo en la zona por diferentes grupos de investigación. De esta forma y tras las investigaciones realizados durante el Año Polar Internacional (2007-2008) en la Península Byers, quedó reconocido su valor como sitio de referencia internacional para la investigación en sistemas terrestres, limnéticos y marinos (Quesada et al. 2009).

Dentro de la labor científica realizada durante la última década por el grupo de investigación Limnopolar en la Península Byers, podemos tomar como claro punto de referencia para la presente tesis doctoral el trabajo de investigación realizado por el Dr. Carlos Rochera Cordellat en su tesis titulada "**Functional ecology of microbial freshwater communities from Byers Peninsula**". Este trabajo trata de dar una visión descriptiva, estructural y funcional de las comunidades microbianas de los ecosistemas acuáticos de la Península Byers centrándose especialmente en el Lago

Limnopolar como un punto de referencia y modelo en el estudio de los sistemas acuáticos epicontinentales de la Antártida marítima. Adicionalmente, se aborda el estudio funcional y estructural de las comunidades microbianas bentónicas, centrando su atención en los tapetes microbianos y "biofilms" presentes en las áreas encharcables próximas al Lago Limnopolar.

De esta forma, la presente tesis parte de las bases implantadas en dicha tesis doctoral y aborda de forma más profunda los aspectos ecológicos de la comunidad microbiana del Lago Limnopolar con el objetivo de describir con mayor precisión el funcionamiento de la comunidad microbiana, su composición taxonómica y su respuesta frente a las condiciones ambientales. De esta forma se da una visión más profunda del funcionamiento del lago, abordando el estudio de procesos productivos y centrándose en el metabolismo del carbono para modelizar el funcionamiento del lago.

1.2. Introducción científica. La Península Byers como zona de especial interés limnológico

La región de la Península Antártica y las islas del Arco de Scotia engloban lo que comúnmente se conoce como Antártida marítima. Esta región presenta características únicas, con un clima menos severo que el de la Antártida continental que deriva en la aparición de ecosistemas de agua líquida durante el verano austral (Ellis-Evans 1996). En esta región se han llevado a cabo gran diversidad de estudios limnológicos durante las últimas décadas, centrándose en las características únicas de estos sistemas acuáticos y el impacto que el hombre ha ejercido y está ejerciendo sobre algunos de ellos. Una parte importante de trabajos los podemos situar en la Isla Rey Jorge, reconocida como zona Antártica de gestión especial (ASMA), con zonas de interés como la Península Fildes (62º12´S, 58º54´W), la Península Potter (62º14´S, 58º39W) y la Península Barton (62º13´S, 58º47´W). Otra zona de especial interés es la región de la Isla Signy (60º43´S, 45º38´W), la cual ha sido ampliamente estudiada por el Servicio Antártico Británico durante las últimas décadas (Heywood 1967, 1968,1970)

evidenciando la gran diversidad de lagos con características únicas, como la presencia de microcrustaceos planctívoros. Alguno de sus lagos, como es el caso del lago Heywood, representa un modelo de eutrofización natural debido a la estrecha relación con la fauna marina, en especial elefantes marinos (Butler 1999), dando lugar a un importante desarrollo de fitoplancton durante el verano austral.

No obstante, gran parte de los estudios limnológicos en la Antártida marítima durante los últimos años, se han centrado en los ecosistemas acuáticos de la Península Byers (Camacho 2006a, Fernández-Valiente et al. 2007, Toro et al. 2007), en los cuales se ha demostrado la estrecha relación entre las características ambientales de los sistemas acuáticos y su comunidad biológica. De esta forma, la gran abundancia de cuerpos de agua con características únicas y el estatus especial de protección de la península (ASPA No. 126), han hecho de Byers uno de los sitios de referencia para los estudios limnológicos en la zona de la Antártida marítima (Quesada et al. 2009).

Como característica general de los sistemas acuáticos de la Antártida marítima, podemos destacar la dominancia de los microorganismos como componente principal del plancton, incluyéndose aquí virus, bacteria, archaea, protistas heterotróficos, algas y metazooplancton (Ellis-Evans et al. 1998, Hansson et al. 1996, Izaguirre et al. 2003, Laybourn-Parry et al. 1995, 2001). Partiendo de este concepto, estudios recientes mediante el uso de técnicas moleculares independientes de cultivo (Pearce et al. 2003, 2007) han revelado que la diversidad de las comunidades bacterianas en estos sistemas acuáticos está estrechamente relacionada con la concentración de nutrientes en los mismos y por tanto con las características limnológicas del sistema donde se encuentran (Pearce 2005, Schiaffino et al. 2009). Así, el empleo de técnicas moleculares basadas en el uso de ARNr ha permitido describir con precisión la heterogeneidad ambiental de las comunidades microbianas presentes en los lagos de la Antártida marítima.

La producción primaria del plancton en los sistemas antárticos, está principalmente controlada por la luz incidente y la temperatura (Lewiss 2011). Debido a esto, los lagos situados en zonas polares sufren un fuerte impacto en sus tasas de

producción por la aparición de una capa de hielo permanente o temporal. Sin embargo, los lagos situados en la zona de la Antártida marítima presentan periodos libres de hielo durante el verano austral, fomentando el incremento en las tasas de producción. Además, estos periodos de deshielo favorecen la entrada al lago de nutrientes y materia orgánica por excorrentia, favoreciento el desarrollo del bacterioplancton heterotrófico. En relación con esta característica, algunos estudios en la zona de la Antártida marítima (Thingstad 2000) han relevado que estos aportes externos de materia orgánica pueden producir un desacoplamiento entre el la producción primaria y la producción bacteriana. De esta forma supondría una excepción al fenómeno de acoplamiento entre el fitoplancton y el bacterioplancton derivado de la materia orgánica procedente de la producción primaria (Kritzberg et al. 2004, Fouilland & Mostajir 2010).

Es importante destacar que la mayor parte de los estudios que se han llevado a cabo durante las últimas décadas en la Península Byers, han tratado directamente o indirectamente de hacer referencia o de tener en cuenta el calentamiento local que se está produciendo en el área de la Península Antártica y que afecta directamente a los ecosistemas de la Antártida marítima (Meredith & King 2005). De esta forma, los trabajos encaminados a estudiar o predecir los efectos del calentamiento van a suponer una importante herramienta ecológica para comprender la evolución de estos sistemas sometidos a una clara presión por el aumento de las temperaturas.

La Península Byers se encuentra situada en la isla Livingston (62º34´35´´S, 61º13´07´´O), la cual es una de islas del archipiélago de las Shetland del Sur. Esta área presenta una superficie de 60,6 km^2, se caracteriza por ser un terreno libre de hielo durante el verano con abundante presencia de masas de agua, situado al oeste de la margen occidental de la capa de hielo permanente de la Isla Livingston.

La abundante presencia de lagos, charcas y arroyos en la Península Byers la convierte en la zona de mayor interés limnológico dentro del archipiélago de las Shetland del Sur y una de las de mayor importancia en la Península Antárctica. Su importancia desde el punto de vista limnológico fue inicialmente reconocida tras su

establecimiento como zona antártica de especial protección (ASPA) y recogida en el Plan de Manejo para el Área de Especial Protección N126 (SCAR 2003).

Tras su designación como zona ASPA, la península Byers ha gozado de especial protección durante las últimas décadas, permitiendo un buen estado de conservación alejado de la actividad humana. Debido a las importantes medidas a tomar antes de visitar Byers, las actividades recientes en la zona se han limitado casi exclusivamente a la investigación científica y la mayoría de muestreos y estudio en la zona han estado supeditados a las condiciones enunciadas en el Plan de Manejo.

1.3. Proyecto Limnopolar II. Campañas Antárticas comprendidas entre 06-09

Tras la intensa labor científica desarrollada en el área de la Península Byers durante el período 2002-2006, el grupo multidisciplinar Limnopolar impulso un nuevo proyecto titulado "**Ecosistemas acuáticos no marinos de áreas polares. Tendencias ecológicas en un contexto de cambio climático global**" en el cual se marcó como objetivo global el estudio de la diversidad biológica de los ecosistemas acuáticos no marinos de la Antártida marítima y el estudio ecoclimático de los mismos.

En particular, si nos centramos en el estudio de los ecosistemas acuáticos no marinos de la Península Byers, podemos destacar tres campañas antárticas distribuidas a lo largo de tres años entre 2006-2009 donde se llevaron a cabo diversas actividades científicas en diversos lagos y sistemas acuáticos de la Península Byers. A continuación se detallarán las diversas campañas Antárticas y el trabajo elaborado durante las mismas.

La campaña antártica 2006/07 en la Península Byers se centró principalmente en el estudio de la variación estacional de los ecosistemas acuáticos a lo largo del verano austral. Durante este periodo se llevaron a cabo diversas actividades científicas por parte del grupo de investigación Limnopolar, repartidos en varios grupos de trabajo a

lo largo del verano austral. Si nos centramos en la labor científica llevada a cabo de manera compartida por el grupo de investigación de la Universidad de Valencia, podemos destacar diversos tipos de actividades:

- Seguimiento de las condiciones ambientales en el Lago Limnopolar a lo largo del verano austral.
- Muestreo de otros cuerpos de agua previamente muestreados durante otras campañas antárticas, incluyendo aquí lagos como Chica, Chester, Asa, Midge, Somero, Turbio, Las Palmas, Domo, Maderos, Cerro Negro y Refugio.
- Estudio del metabolismo de la comunidad planctónica en el Lago Limnopolar.
- Estudio de la depredación, los nutrientes orgánicos e inorgánicos y su reciclado en la estructuración de la comunidades planctónicas mediante experimentos de manipulación in-situ.
- Estudio de los anostráceos como consumidores sobre las comunidades planctónica y bénticas en los lagos Limnopolar y Somero.
- Evaluación de la epibiosis en el Lago Limnopolar bajo distintas concentraciones de nutrientes.

La campaña llevada a cabo durante el verano austral de 2007/08 sirvió mayoritariamente para la instalación de una sonda permanente para la medición de variables físico-químicas, equipada con un sistema de toma de muestras. Adicionalmente se instaló una cámara especial para seguir la dinámica de hielo y deshielo en el lago. Por parte del grupo de la Universidad de Valencia se llevaron a cabo diversas actividades centradas en la puesta a punto de metodología para posteriores campañas, así como toma de muestras para análisis moleculares de los diversos lagos estudiados durante la campaña anterior.

Además se realizó la campaña antártica 2008/09, enmarcada dentro del año polar internacional (nombrada como IPY Byers). Dentro de este marco de trabajo, diversos investigadores de varios países se unieron al grupo de investigación Limnopolar para

aumentar el conocimiento de los distintos compartimentos biológicos de la Península Byers. Por parte del grupo de investigación de la Universidad de Valencia se establecieron diversas actividades las cuales se elaboraron principalmente entre los meses de Diciembre a Febrero. A continuación se destacan las actividades llevadas a cabo:

- Cuantificación de las entradas y salidas de carbono orgánico y particulado en el Lago Limnopolar a lo largo del verano austral.
- Estudio del deshielo, balance hídrico y variaciones de volumen en el Lago Limnopolar.
- Estudio de las características limnológicas en el Lago Limnopolar.
- Estudio de la variación estacional de la dieta y del tamaño de la puesta en anostraceos.
- Estudio de la cobertura de musgos y la producción primaria en el Lago Limnopolar
- Experimentos in-situ de manipulación de nutrientes y luz para estudiar la respuesta de las comunidades microbianas planctónicas.
- Estudio de la elongación celular bacteriana en el Lago Limnopolar
- Estudio de la red trófica del Lago Somero mediante isótopos estables.
- Estudio de la eclosión y viabilidad de huevos de *Branchinecta gaini*
- Estudio de la dispersión del zooplancton
- Pigmentación en copépodos
- Selección de dieta en *Branchinecta gaini*

Como probablemente se verá, bastantes de estos estudios se recogerán en la presente Tesis.

1.4. Objetivos de la tesis doctoral

En este apartado se van a resumir brevemente los objetivos a tratar dentro del presente trabajo de tesis doctoral, los cuales se desarrollaran con mayor precisión en cada uno de los capítulos del mismo. Partiendo del trabajo realizado durante las campañas

antárticas anteriormente mencionadas, este trabajo de tesis doctoral se presenta dividido en varios apartados, cada uno dirigido a estudiar un aspecto singular de los lagos de la Península Byers. A continuación aparecen de forma breve los distintos objetivos que se van a tratar en cada capítulo del presente trabajo de tesis doctoral y las hipótesis que se plantean para cada uno de ellos.

- Estudio de la relación existente entre la composición de la comunidad microbiana y la gran heterogeneidad ambiental entre los lagos de la Península Byers. Este objetivo queda englobado dentro del Capítulo 3 y pretende responder a la hipótesis de si existe una relación directa entre la diversidad ecológica y la diversidad biológica en los lagos de la Antártida marítima.
- Estudio de la dinámica estival del Lago Limnopolar a lo largo de las campañas 2006/07 y 2008/09. El Capítulo 4 engloba este objetivo el cual pretende realizar un estudio comparativo de la dinámica del plancton durante distintos años.
- Estudio de la producción primaria y bacteriana en el Lago Limnopolar y su relación con la dinámica del carbono en el lago y su cuenca. Este objetivo queda recogido dentro del Capítulo 5 y pretende responder a la cuestión de si existe un desacoplamiento entre la producción primaria y la producción bacteriana en el Lago Limnopolar.
- Estudio del aprovechamiento de diferentes tipos de fuentes de carbono por parte de las poblaciones microbianas de los lagos de la península Byers, mediante el uso de placas multipocillo Biolog. Este estudio queda recogido en el Capítulo 6 donde se pretende estudiar si existe una relación entre la diversidad fisiológica planctónica y el origen de la materia orgánica en cada uno de los lagos de la Península Byers.
- Descripción de la diversidad molecular de las poblaciones de bacterioplancton en el lago Limnopolar mediante la construcción de librerías clónicas. Con este objetivo, recogido en el Capítulo 7, se pretende responder a la cuestión de si la diversidad y composición bacteriana del Lago

Limnopolar es homogénea a lo largo de la columna de agua y durante el verano austral.

- Estudio de la respuesta ecológicas de las poblaciones microbianas frente a la manipulación en la disponibilidad de luz y nutrientes orgánicos e inorgánicos. El Capítulo 8 recoge el trabajo de manipulación experimental donde se estudia la respuesta de las poblaciones planctónicas del Lago Limnopolar frente a la limitación por nutrientes y luz.
- Estudio de la epibiosis como respuesta a la oligotrofia en el lago Limnopolar. Este objetivo queda recogido en el Capítulo 9 y pretende evaluar la aparición de epibiontes algales sobre el zooplancton como respuesta a la limitación extrema de nutrientes.
- Modelización de la dinámica del carbono en el Lago Limnopolar, partiendo de los datos recogidos durante los años 2006/07 y 2008/09. Este objetivo queda plasmado en el Capítulo 10 donde se ha llevado a cabo un modelo ecológico del Lago Limnopolar con la intención de estudiar la respuesta de la comunidad planctónica a los cambios de temperatura y la disponibilidad de luz y nutrientes.

1.5. Estructura de la tesis doctoral

La presente tesis comprende un total de 11 capítulos, entre los cuales a excepción de la introducción general, material & métodos y discusión general, están desarrollados con un formato propio de un artículo científico. El **Capítulo 1** presenta una introducción general del presente trabajo, remarcando el estado actual de la investigación en la Península Byers, así como las hipótesis y objetivos a abordar. En el **Capítulo 2** se recogen los métodos instrumentales y analíticos usados en la tesis, así como la descripción detallada de la zona de estudio y su estatus especial como área antártica de especial protección (ASPA). El **Capítulo 3** se centra en el estudio de la heterogeneidad ambiental de los lagos presentes en la Península Byers mediante el estudio comparativo de las características morfológicas y ecológicas de cada uno de los lagos y la diversidad de grupos bacterianos presentes. Tras dar una visión global

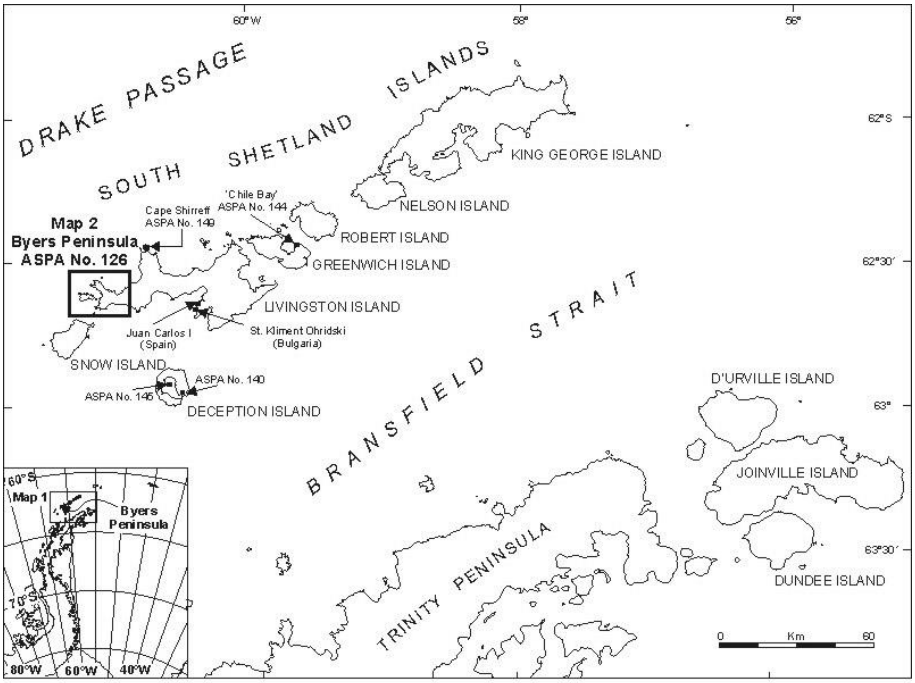
de los lagos presentes en la Península Byers, en el **Capítulo 4** se aborda el estudio de la dinámica de la comunidad planctónica en el Lago Limnopolar durante dos campañas Antárticas comprendidas entre los veranos de 2006-2007 y 2008-09. Atendiendo a los datos recogidos durante las mencionadas campañas Antárticas (2006-2007, 2008-2009), el **Capítulo 5** se centra en el estudio de las tasas de producción del fitoplancton y del bacterioplancton y la relación existente entre ambas, mostrando el desacoplamiento existente en el lago debido a los importantes aportes alóctonos de nutrientes y materia orgánica desde la cuenca. En el **Capítulo 6** se describe la composición del bacterioplancton en el Lago Limnopolar, dando una descripción detallada de los taxones presentes a lo largo de la columna de agua, demostrando la existencia de distribución heterogénea de grupos bacterianos a pesar de la no estratificación del lago. A diferencia de los capítulos previos donde se centraba la atención en el Lago Limnopolar, en el **Capítulo 7** se vuelven a estudiar varios lagos de los ya tratados en el Capítulo 3, con el objetivo de conocer la diversidad fisiológica del bacterioplankton y su relación con la materia orgánica autóctona y alóctona. Tras esto, en el **Capítulo 8** se aborda un estudio experimental en el Lago Limnopolar mediante el uso de recipientes de plástico de volumen conocido donde se manipularon las concentraciones naturales de nutrientes y luz, con el objetivo de estudiar la respuesta de la comunidad planctónica a la disponibilidad de recursos. Por otro lado, el **Capítulo 9** se centra en el estudio de la epibiosis por parte de algas sobre el zooplankton como respuesta a las condiciones de oligotrofia en el Lago Limnopolar. El **Capítulo 10** agrupa toda la información recogida en las campañas Antárticas 2006/07 y 2008/09 con el objetivo de elaborar un modelo ecológico donde se describe la dinámica del carbono en la comunidad planctónica, relacionándola estrechamente con las variaciones de temperatura, disponibilidad de luz y aportes de nutrientes y materia orgánica al lago. Para finalizar, el **Capítulo 11** ofrece una discusión general de los aspectos mostrados en la tesis así como las conclusiones generales. Para acabar, se muestra una compilación de toda la bibliografía utilizada en la presente tesis.

2. Área de estudio. Metodología

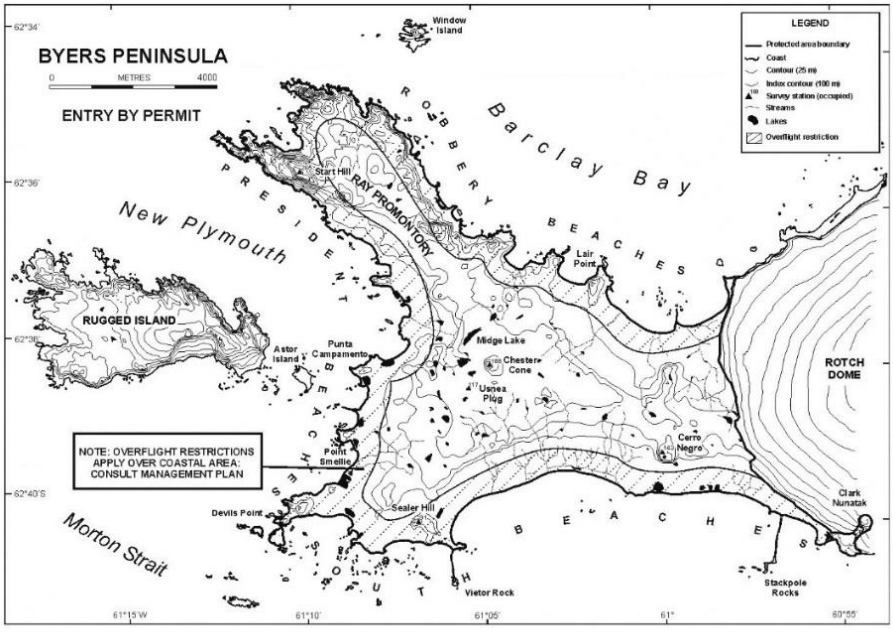
2.1. Área de estudio

2.1.1. Situación geográfica. Características geológicas

La Península Byers se encuentra situada en el extremo oeste de la Isla Livingston, en el Archipiélago de las Islas Shetland del Sur (latitud 62°34'35" S, longitud 61°13'07" W), Antártida (Figura 2.1). Este archipiélago pertenece a la zona conocida como Antártida marítima, que comprende tanto las costas de la Península Antártica como las islas circundantes. La Península Byers cubre una superficie de 62,6 km^2, extendiéndose 9 km de oeste a este y sobre 18,2 km de noroeste a suroeste. La zona interior de la península está dominada por extensas mesetas con suaves ondulaciones de hasta 105m sobre el nivel del mar, interrumpidas por formaciones volcánicas aisladas entre las que podemos destacar Chester Cone (188m) o Cerro Negro (143m) (Thomson & López-Martínez 1996). La formación montañosa de mayor altitud es Cerro Start (265m), situado al noroeste de la península. Las formaciones geológicas del Jurásico Superior y Cretácico inferior de tipo marino sedimentario y volcánico abundan en la zona, caracterizadas por presentar marcados signos de erosión glacial y periglaciar (Thomson & López-Martínez 1996). Los suelos de la península, presentan una capa de tipo litosol con piedras fragmentadas que se extiende aprox. 50-70cm sobre el permafrost. Esto permite un flujo subterráneo de agua sobre esta capa helada (Serrano et al. 1996). Además de estas características, Byers es una de las mayores zonas libres de hielo en la Antártida marítima, y la segunda más extensa en el archipiélago de las Shetlands del Sur (SCAR 2003). La península permanece cubierta por nieve durante 7-8 meses al año, manteniendo pequeñas acumulaciones de nieve durante el verano austral. Estas características hacen de Byers un sitio de referencia para estudios ecológicos tanto costeros, terrestres como limnológicos (Quesada et al. 2009, 2013).



Map 1. Byers Peninsula, ASPA No. 126, Livingston Island, South Shetland Islands, location map.
Inset: location of Byers Peninsula on the Antarctic Peninsula



Map 2. Byers Peninsula, ASPA No. 126, topographic map.

Figura 2.1. Situación geográfica de la Península Byers en la Antártida marítima (arriba). Península Byers como se muestra en el ASPA nº 128 (abajo).

2.1.2. Características limnológicas

La Península Byers presenta un elevado interés limnológico debido a la abundancia de masas de agua epicontinentales que cubren parte de su superficie. Estos lagos y zonas encharcables se formaron como consecuencia de la retirada del glaciar que actualmente cubre el resto de la isla. El mayor interés biológico en estos lagos recae en la existencia de comunidades microbianas planctónicas y bentónicas sencillas, que desarrollan un fuerte crecimiento durante el verano austral (Toro et al. 2007, Villaescusa et al. 2010, Villaescusa et al. 2013a,b). Este fenómeno se produce tras la rotura de la estratificación inversa invernal debido a la desaparición de la capa de hielo, permitiendo la transición hacia un lago completamente mezclado por efecto del sol y del viento. Además, la existencia de comunidades complejas de tapetes microbianos sobre la cuenca de estos lagos (Fernández-Valiente et al. 2007, Rochera et al. 2013) juega un papel muy relevante en los aportes de nutrientes y materia orgánica durante el período libre de hielo.

Los lagos de la Península Byers presentan características morfológicas y ecológicas muy diversas, reflejando la existencia de una elevada heterogeneidad ambiental en la zona. La mayor parte de los lagos estudiados por parte del grupo Limnopolar se encuentran en la zona de la meseta central de la Península Byers (Toro et al. 2007, Villaescusa et al. 2010). Los lagos de la meseta central se caracterizan por presentar mayor profundidad, entre 4 y 8 m, y un marcado carácter de oligotrofia. Sin embargo, en la meseta también se pueden encontrar algunos lagos de escasa profundidad (ej. Lago Somero) con características propias. Algunas de las características físico-químicas más relevantes de los mismos aparecen en la Tabla 2.1. Estos lagos presentan un buen sistema de drenaje, con pequeños ríos que desembocan en las costas del sur o suroeste de la Península, formando arroyos de gran interés biológico (Rodríguez & Rico 2008). Es aquí en la costa donde aparecen lagos con características limnológicas muy diferentes a los anteriores. Estos lagos se caracterizan por presentar escasa profundidad (0.5m) y un marcado carácter de eutrofia. La actividad de aves y mamíferos marinos en estos lagos es muy relevante (Gil-Delgado et al. 2013a,b), suponiendo el principal aporte de nutrientes orgánicos e

inorgánicos en estos sistemas. De entre los citados, los lagos Refugio y Maderos (Tabla 2.1) reflejan las características propias de este tipo de lagos.

Tabla 2.1. Características morfométricas y físico-químicas de algunos de los lagos estudiados en la Península Byers y sus cuencas

Lagos	Tamaño de la cuenca (km^2)	Profundidad máxima (m)	Conductividad (μScm^{-1} 25ºC)	pH
Lagos de interior				
Limnopolar	0.58	5.5	51-80	6.54-7.76
Somero	0.06	0.5	35-105	6.6-7.82
Midge	0.27	8.2	63-73	6.58-7.28
Chester	0.09	5	36-68	6.69-7.03
Chica	0.01	4	20-60	6.84-7.10
Turbio	0.58	7.8	35-81	6.04-7.23
Escondido	0.08	4.5	50-69	6.31-7.00
Lagos costeros				
Refugio	0.12	0.5	128-134	7.60-7.70
Maderos	2.41	0.5	189	7.18

2.1.3. La Península Byers como zona ASPA

La Península Byers fue designada como Zona Antártica de Especial Protección (ASPA) atendiéndose a la Recomendación IV-10 del Tratado Antártico en el año 1966. Este área protegida incluía tanto las zonas libres de hielo en la zona oeste del glaciar permanente de la Isla Livingston (Domo Rotch) como la Isla Window, situada a unos 500 m hacia el noroeste de las costas de Byers además de otras cinco pequeñas zonas libres de hielo permanente situadas en la costa, justo hacia el este de la Península. Esta declaración se realizó debido al gran interés científico en cuanto a su diversidad de plantas y animales, así como de invertebrados, en una zona de tan pequeño tamaño. Parte del interés se centró en la importancia de las poblaciones de elefantes marinos (*Mirounga leonina*) (Gil-Delgado et al. 2013b) y de pequeñas colonias de lobos marinos (*Arctocephalus gazella*). Más tarde la Península Byers sufrió algunos cambios en cuanto a su figura de protección, pasando de ser una ASPA, a un sitio de especial interés científico (SSSI), debido a la recomendación VIII-2 y VIII-4 del Tratado Antártico en el año 1975. Posteriormente, debido al gran interés

geológico, arqueológico y biológico en la zona, Chile y Reino Unido propusieron la necesidad de volver a establecer la figura de protección de ASPA No.126 (Recomendación XVI-5 del Tratado Antártico, 1991), pero en este caso únicamente incorporando la zona libre de hielo de la Península Byers así como la zona litoral de la misma, pero no la Isla Window ni las zonas cercanas. El conjunto de valores biológicos reconocidos sobre esta zona es de gran importancia, quedando recogidos en Plan de Manejo para el Área de Especial Protección N126 (SCAR 2003).

Dentro de estas recomendaciones, se da gran importancia a la diversidad de plantas vasculares autóctonas como *Deschampsia antárctica* y *Colobanthus quitensis*. Los lagos costeros e interiores cobran gran importancia debido a su población de musgos acuáticos y su relación con la mosca enana *Parochlus steinenii*, siendo este el único insecto alado de la Antártida. Adicionalmente, se menciona la importancia de otro díptero antártico, la mosca enana sin alas *Belgica antartica*, localizada en los arroyos cercanos al Cerro Negro.

Por otro lado se denota el valor arqueológico de la península, siendo esta la zona con mayor concentración de sitios históricos de la Antártida, con abundante presencia de refugios, aparejos y restos de navíos de las expediciones de caza de focas durante principios del siglo XIX. De esta forma se reafirman los valores de protección que ya aparecían en los planes de gestión originales de la Península Byers.

Adicionalmente se incluyen otros puntos de interés para conferir una protección especial a esta zona:

- Presencia de huesos de ballena subfosilizados en las terrazas costeras. De gran importancia para la datación de terrazas por métodos de radiocarbono.
- Gran diversidad de flora y fauna terrestre bien caracterizada.
- Importante presencia de lagos, charcas de agua dulce y arroyos de extensión variable. Esta característica, califica a Byers como el área limnológica de mayor importancia en las islas Shetland del Sur y una de los más importantes de la Península Antártica.

- Reconoce la importancia de los sedimentos lacustres como un importante archivo para los estudios paleoclimáticos del holoceno en la Península Antártica.
- Denota la importancia de *Parochlus steinenii* y *Belgica antárctica* por su abundancia en varios lagos y charcas de la Península Byers.
- Gran importancia de tapetes microbianos en la parte central de la meseta, compuestos mayoritariamente por cianobacterias del género Phormidium.
- Importancia sobre la avifauna de la zona, incluyendo dos especies de pingüinos (*Pygoscelis antárctica* y *P. papua*), golondrinas antárticas (*Sterna vittata*), diversas especies de petreles (*Oceanites oceanicus*, *Daption capense* y *Macronectes giganteus*, *Freggeta tropica*), skúas pardas (*Catharacta ioennbergi*) y otras especies de aves antárticas.

Gracias a la designación como ASPA y a la importante labor científica en la zona durante la era moderna, Byers ha gozado de una especial protección y atención durante las últimas décadas. La mayor parte de las actividades humanas realizadas en la zona ha estado relacionada casi exclusivamente a la investigación científica, caracterizando a la Península Byers como una zona prístina con una influencia reducida por parte del ser humano.

2.2. Registro meteorológico

El clima en la Península Byers está fuertemente influenciado por la cercanía a la costa, siendo el característico de la Antártida marítima. Debido a esto el clima es de carácter menos severo que el que se presenta en la zona de la Antártida continental. Con veranos suaves donde la temperatura media se establece en torno a 1-3ºC, con máximos diarios que no superan los 10ºC. En invierno las temperaturas mínimas raras veces bajan de los -35ºC con máximos de alrededor de 0ºC. Las precipitaciones en la Península son más elevadas que las de la zona continental de la Antártida con valores medios anuales de en torno a 700-1000 mm (Bañón 2001) debido a la influencia marítima.

Para llevar a cabo el registro meteorológico se recurrió principalmente a la estación que ya había sido previamente instalada en la zona por parte de del proyecto Limnopolar. La estación meteorológica está situada en la zona central de la península, entre dos lagos (Limnopolar y Somero) en los cuales se desarrollan gran parte de los estudios sobre los que se trabajó. La estación está equipada con termómetros para registrar la temperatura del aire y con un sensor de temperatura colocado dentro del lago Somero. También está dotado de una sonda para cuantificar la humedad del aire y un anemómetro para medir la velocidad del viento. Los datos referentes a la radiación luminosa incidente en la zona fueron tomados por una fotocélula instalada en la estación meteorológica, de esta forma se cuantificó la irradiancia total y la fracción correspondiente a la radiación fotosintéticamente activa (PAR). La estación meteorológica está alimentada por un pequeño generador eólico y por una placa de fotovoltaica. Las precipitaciones medias durante el verano austral se midieron empleando un pluviómetro instalado en el campamento base en las cercanías de la playa, con registro diario de la misma.

2.3. Registro de variables físico-químicas in-situ

Como paso previo, en cada uno de los muestreos que se llevaron a cabo en los lagos de la Península Byers se procedió al registro "in-situ" de las variables físico-químicas de cada uno de los lagos. Podemos clasificar las variables en tres tipos. Por un lado variables de tipo morfométrico, englobándose aquí parámetros tales como profundidad, área del lago, área de la cuenca, etc... Por otro lado variables lumínicas, se midió la extinción de la luz en el lago, concretamente de la radiación fotosintéticamente activa (PAR) en el perfil vertical. Adicionalmente, se cuantificaron variables de tipo químico como pH, concentración de oxígeno disuelto, temperatura del agua y conductividad.

2.3.1. Variables morfométricas

El área del lago y el área de la cuenca de los lagos estudiados se tomó de Toro et al. (2007) tal como se refleja en la Tabla 2.1. La profundidad máxima de cada uno de los lagos se midió mediante el uso de una ecosonda de mano digital (Hondex PS-7). Para el caso del Lago Limnopolar se emplearon los datos del perfil batimétrico realizado durante el verano austral 2002-2003 usando un GPS y una sonda de profundidad. Se realizaron un total de 10 transectos para dibujar el contorno del lago y resaltar las características principales del mismo. Con estos datos se creó un mapa de isolíneas a intervalos de 1 metro para definir la morfología del lago (Figura 2.2).

Tras realizar las medidas de profundidad de cada lago, se llevó a cabo la toma de muestras de agua y organismos junto con la medida de las variables físico-químicas sobre el punto de máxima profundidad de los mismos.

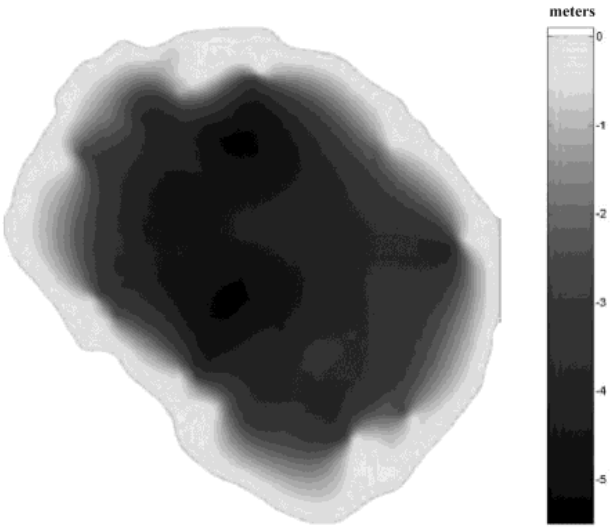


Figura 2.2. Mapa del contorno batimétrico del Lago Limnopolar obtenido durante Febrero de 2003. La escala de grises muestra la profundidad en metros.

2.3.2. Medida de la luz (PAR)

La fracción fotosintéticamente activa (PAR) de la radiación incidente fue medida empleando un luxómetro (Li-cor L1-1000) equipado con dos fotocélulas planas funcionando en continuo (sensor de irradiancia escalar 2π, Li 192SA), una para medir la radiación externa incidente sobre el lago y otra para realizar un perfil de luz en profundidad. La utilidad de emplear dos fotocélulas en continuo es la de corregir el efecto que supone la elevada variabilidad ambiental de la luz incidente que se produce en estos sistemas debido a la meteorología altamente cambiante. Las dos fotocélulas estaban conectadas a un "datalogger", recogiendo de forma continua los valores de luz registrados en superficie y para cada profundidad, dando valores instantáneos corregidos para la radiación incidente en superficie. Adicionalmente, se empleó el método clásico del disco de Secchi para medir la transparencia del agua.

2.3.3. Otras variables físico-químicas medidas in-situ

Se empleó una sonda multiparamétrica (6920 YSI) con tres dispositivos dedicados a medir concentración de oxígeno disuelto, temperatura y conductividad eléctrica. El pH fue medido y anotado, empleando un pH-metro de campo (Eutech Instrumets) a partir de agua recogida de cada una de las profundidades muestreadas.

2.4. Toma de muestras, procesado y almacenamiento

Una vez registradas las diferentes variables en cada uno de los lagos se procedió a la toma de muestras de agua, biota o sedimento, que serían empleadas para realizar análisis químicos y biológicos. El agua para cada una de las profundidades de muestreo se recogió empleando una botella hidrográfica de tipo Ruttner. Se emplearon recipientes de plástico de cinco y quince litros para transportar el agua hasta el laboratorio situado en el campamento, una vez allí, las muestras fueron procesadas en el mismo día y almacenadas en diversos recipientes de pequeño tamaño para su

transporte y conservación. Tanto los recipientes de plástico de cinco y quince litros como los recipientes de pequeño tamaño fueron previamente lavados con ácido sulfúrico 1N y después con agua mili-Q para eliminar los posibles restos de ácido. Adicionalmente, los recipientes se lavaron con agua de la muestra previamente a su llenado. En cada uno de los recipientes se anotaba un código descriptivo de la muestra, así como la fecha y el lugar de muestreo. Esto se realizó también para los filtros envueltos en papel de aluminio. Las botellas y recipientes almacenados a -20ºC no se llenaron completamente para evitar posibles roturas por la congelación.

A continuación se detalla el procedimiento de recogida y almacenamiento de las muestras empleadas para medir diferentes variables químicas y biológicas en los lagos:

- **Nitrógeno y fósforo total**. Las muestras se recogieron mediante botellas de polietileno de 125 ml y se almacenaron a -20ºC.

- **Nutrientes inorgánicos**. Parte de la muestra de agua extraída del lago fue filtrada in-situ empleando filtros GF/F de fibra de vidrio (Whatman). A partir del filtrado, se recogieron muestras en botellas de polietileno de 125 ml. Las botellas fueron almacenadas a -20ºC anotando el volumen filtrado.

- **Aniones**: Las muestras se recogieron en botellas de polietileno de 50 ml y se almacenaron a -20ºC.

- **Cationes**: Las muestras se recogieron en botellas de polietileno de 50 ml y se fijaron con 0,5 ml de ácido nítrico (HNO_3) 60%, almacenándose posteriormente a 4ºC.

- **Carbono, nitrógeno y fósforo particulado**. Se filtró la mayor cantidad de muestra posible a través de filtros GF/F de fibra de vidrio de 47 mm (Whatman). Los filtros se almacenaron a -20ºC plegados sobre sí mismos y envueltos con papel de aluminio.

- **Picoplancton y virioplancton**. Se recogieron 10 ml de muestra en tubos Falcon de 15 ml, los cuales se fijaron con un volumen de glutaraldehído de manera que este quedara a una concentración final de 1%. Se dejó actuar el fijador durante 30 minutos y posteriormente los tubos se almacenaron a -20ºC.

- **Nanoplancton**. Se recogieron 100 ml de muestra en botellas de polietileno de 125 ml y se fijaron con un volumen de formaldehído tamponado de manera que este quedara a una concentración final de 2%. Posteriormente las botellas se almacenaron a 4ºC.

- **Fitoplancton**. Se recogieron 200 ml de muestra en botellas de vidrio actínico de 250ml y se fijaron con un volumen de lugol de manera que este quedara a una concentración final de 1%. Posteriormente, las botellas se almacenaron a 4ºC.

- **Zooplancton**. Un volumen conocido de muestra se filtró a través de una malla de nytal de 30 µm de poro con la ayuda de un embudo de plástico, (aproximadamente entre 10 y 15 litros, aumentándose el volumen en caso de que la muestra obtenida fuera escasa). El filtro se guardó en un vial de 20ml conteniendo una solución de formol al 4%. Los viales se almacenaron a 4ºC.

- **Pigmentos fotosintéticos**. Se filtró in-situ un volumen de agua de entre 1-2 litros a través de un filtro GF/F de fibra de vidrio de 47 mm. Los filtros GF/F se almacenaron a -20ºC plegados sobre sí mismos y envueltos con papel de aluminio.

- **Carbono orgánico disuelto.** Previamente a su almacenamiento, se filtró la muestra mediante filtros de nitrato de celulosa de 0.2 µm de poro. El filtrado se recogió en botellas de vidrio actínico de 30 ml. Las muestras fueron fijadas con un volumen de HCl 2N de tal manera que el pH final de la muestra quedara por debajo del punto de equilibrio del CO_2 para liberar el carbono inorgánico. Las botellas fueron almacenadas en 4ºC en oscuridad.

- **ADN**. Se filtraron entre 1-2 litros de muestra mediante filtros de membrana de 3 µm de poro y 47 mm de diámetro. A partir del agua filtrada, se realizó un segundo proceso de filtrado empleando filtros de policarbonato de 0,2 µm de poro y 47 mm de diámetro. Los filtros se guardaron por separado en crioviales conteniendo 1ml de tampón de lisis (40 mM EDTA, 50 mM Tris pH=8.3, 0.75 M de sacarosa). Los crioviales se almacenaron a -20ºC.

- **Hibridación in situ fluorescente (FISH)**. Se fijó un volumen de muestra con paraformaldehído, de manera que el fijador quedara a una concentración final de 2%. Previamente a esto fue necesario descongelar y filtrar el paraformaldehído mediante filtros estériles de acetato de celulosa de 0.2 µm de poro para eliminar las posibles impurezas que pudiera contener. La mezcla muestra:paraformaldehído se mantuvo durante 2-4 horas para dejar actuar al fijador. Una vez transcurrido este tiempo se filtraron 10 ml de muestra a través de filtros de policarbonato blanco de 25 mm y 0.2 µm de poro, excepto para el caso de lagos eutróficos donde no se filtró más de 1 ml. Una vez finalizado el filtrado y sin retirar el filtro de la torre de filtración, se lavó con 10 ml de agua Milli-Q. Posteriormente se dejó secar el filtro, asegurándose que este quedaba completamente seco, para garantizar el posterior tratamiento de hibridación en el laboratorio. Los filtros se guardaron en crioviales de modo que la cara filtrada quedase dispuesta hacia el interior del tubo y no en contacto con la pared de este. Los crioviales se almacenaron a -20ºC.

2.5. Análisis de variables químicas

2.5.1. N y P total, particulado y formas disueltas

2.5.1.1. Análisis de nitrógeno

Dentro de este apartado se hace referencia a las técnicas empleadas para la cuantificación de las diferentes formas de nitrógeno (solubles y particuladas) presentes en los lagos. Se presenta de forma que sea explicado el principio del método así como los reactivos utilizados, seguido posteriormente por un desarrollo del procedimiento.

a) Nitrato

El nitrato puede ser reducido a nitrito de manera cuantitativa al atravesar una columna de cadmio y cobre, estando en solución alcalina tamponada a pH 8. El nitrito resultante se estima como tal. Añadiendo una amina aromática (sulfanilamida) se forma una sal diazónica que se aparea cuantitativamente con una amina aromática (naftiletilendiamida), dando un color rosa fucsia cuya concentración se mide espectrofotométricamente a una longitud de onda de 543 nm. La determinación se basa en la reacción de Griess (APHA 1992) según el método 4500-NO_3^--E: reducción por cadmio.

Reactivos

- Agua Milli-Q.
- Solución de cloruro amónico. Se disuelven100 g de NH_4Cl en agua Milli-Q. Se añaden 20 g de tetraborato sódico y 1 g de sal disódica etilendiamino tetraacético (EDTA). Se completa hasta 500 ml.
- Solución diluida de cloruro amónico. Se diluyen 10 ml de la solución anterior en agua Milli-Q hasta 400 ml.

- Solución de sulfato de cobre. Se pesan 5 g de sulfato cúprico pentahidratado en 250 ml de agua Milli-Q.
- Sulfanilamida. Se disuelven 5 g de sulfanilamida en una mezcla de 50 ml de HCl concentrado y 300 ml de agua Milli-Q. Se completa con agua Milli-Q hasta 500 ml.
- N-(1-naftil)-etilendiamida, dihidrocloruro (NNED). Se pesan 0.5 g y se disuelven en agua Milli-Q hasta 500 ml. Se guarda en frasco oscuro.
- Solución patrón de nitrito. Se deseca $NaNO_2$ anhidro a 110ºC durante una hora. Se pesan 6,90 g y se disuelven en agua Milli-Q hasta alcanzar el volumen de 1 litro. Se añade 1 ml de cloroformo y se guarda la solución en frasco oscuro. Es estable uno o dos meses. Esta solución presenta una concentración de 0.1 M de nitrito. Cada vez se prepara una dilución intermedia 1:100. A partir de la dilución intermedia se preparan aforados para 3 concentraciones diferentes. (5, 10 y 20 µM de NO_2^-).
- Columna reductora de cadmio. Para elaborar la columna se emplea un tubo de vidrio de 10 mm de diámetro y unos 30 cm de longitud, ensanchado en su parte superior con un volumen aproximado de unos 100 ml. La parte inferior comunica con un tubo capilar en forma de cuello de cisne por medio de un tubo de goma de silicona, con una pinza para regular el flujo. Se toman limaduras de cadmio en cantidad suficiente para llenar unos 15 cm de la columna. Se colocan en un vaso de precipitado y se añade un volumen de solución de sulfato de cobre, aproximadamente 10 ml de solución por cada 5 g de limaduras, agitando hasta que desaparezca el color azul de la disolución. Se decanta varias veces hasta que no queden partículas finas. Se pone un tapón de fibra de vidrio en la base y se llena la columna de solución diluida de cloruro amónico. Se añaden las limaduras tratadas de modo que no queden atrapadas burbujas de aire en el interior de la columna. Posteriormente se lava la columna y se mantiene llena de solución de lavado. Es conveniente que la columna no llegue nunca a secarse. Cuando la columna pierde poder reductor o se seca, se debe sacar las limaduras y tratarlas con ácido clorhídrico al 5%. Se lavan con agua destilada hasta que no quede ácido y se vuelven a tratar con el sulfato de cobre.

Procedimiento

Se tomaron 100 ml de muestra y se le añadieron 2 ml de solución de cloruro amónico concentrado en una probeta. Se agitó la muestra y se vertió en la columna reductora regulándose el flujo de salida. Se desecharon los primeros 70 ml, recogiéndose los 25 siguientes en una probeta. Se desechó el resto de muestra hasta terminar. Previamente a comenzar el análisis y tras finalizar el mismo, se hicieron pasar por la columna patrones de concentración conocida y dos blancos con agua Milli-Q. Al terminar el trabajo, la columna se rellenó con solución diluida de cloruro amónico. A partir de los 25 ml de muestra recogidos se procedió a realizar el análisis, para ello se añadieron 0,5 ml de solución de sulfanilamida y se mantuvo la muestra de reacción en oscuridad entre dos y ocho minutos. Una vez transcurridos se añadieron 0,5ml de NNED y se esperó entre diez minutos a dos horas para que se produjera la reacción. Una vez finalizada la reacción se midió la absorbancia de la muestra a 543 nm frente a un blanco preparado con agua mili-Q y frente a los patrones correspondientes.

b) Nitrógeno total

El nitrógeno total engloba al amonio, nitrato, nitrito y nitrógeno orgánico disuelto y particulado. El nitrógeno total se oxida a nitrato mediante una digestión alcalina con persulfato. Tras una neutralización, el nitrato se reduce a nitrito empleando una columna de cadmio. Por último, la concentración de nitrito es determinada espectrofotométricamente tal como se describe en el apartado a) según APHA (1992) método 4500-NO_3^--E: reducción por cadmio. En el caso de tener concentraciones superiores a 10-20 µM y baja materia orgánica disuelta, se puede recurrir a un método alternativo basado en la cuantificación por UV. El método empleado se basa en las técnicas referenciadas en APHA (1992), 4500-NO_3^--C: método espectrofotométrico de la segunda derivada en UV.

Reactivos

- Solución de oxidación. Se disuelven 6 g de NaOH en 100 ml de agua destilada. Se añaden 6 g de $K_2S_2O_8$ y se disuelven. Se prepara diariamente.
- Solución 1M HCl. Esta solución es estable durante semanas.
- Solución tampón. Se disuelven 75 g NH_4Cl en 400 ml de agua. El pH se ajusta a 8.5 con una solución de amonio, ajustándose el volumen a 500ml. Esta solución es estable durante semanas.

Procedimiento

Se colocaron 20 ml de muestra no filtrada en tubos de centrífuga de 50 ml resistentes a la digestión y el autoclavado (PYREX). Se añadieron 3 ml de la solución de oxidación a cada uno de los tubos. Los tapones se recubrieron con papel de aluminio y posteriormente fueron autoclavados durante 1-2 horas a 2 atm de presión. Una vez enfriados los tubos, se añadió un volumen de HCl o NaOH para neutralizar las muestras, empleando fenoftaleína como indicador de pH. Posteriormente se llevó a cabo el análisis de nitrato tal como se explica en el apartado a) para Nitrato.

Para aquellas muestras donde la concentración de nitrógeno podía ser elevada y por tanto el nitrato resultante podía ser medido por espectrometría UV se llevó a cabo el método alternativo. A partir de las muestras autoclavadas y neutralizadas se llevó a cabo una cuantificación mediante un espectrofotómetro (Beckman, DU-7). Para ello se realizó un barrido en la zona del ultravioleta, entre 194 y 254 nm a una velocidad de 120nm/min tomándose el valor de absorbancia de la 2ª derivada a 224 nm.

c) Nitrógeno particulado

Procedimiento

Los filtros previamente almacenados fueron troceados e introducidos en tubos de centrífuga de 50 ml. A cada tubo se añadieron 20 ml de agua Milli-Q y 3 ml de la solución de oxidación tal como se describe en el apartado b) para el Nitrógeno Total. Los tapones se cubrieron con papel de aluminio y los tubos fueron autoclavados durante 1-2 horas a 2 atm de presión. Los tubos se dejaron enfriar y la muestra fue neutralizada mediante un volumen de HCl o NaOH. Posteriormente se llevó a cabo el procedimiento de análisis de nitrato descrito en el apartado a) para Nitrato según APHA (1992) método 4500-NO_3^--E: reducción por cadmio

d) Amonio

- *c.1. (Procedimiento I)*

En esta reacción, el amonio reacciona con el fenol y el hipoclorito en solución alcalina mediante catálisis con nitroprusiato para formar azul de indofenol (APHA, 1992).

Reactivos

- Reactivo Fenol-Nitroprusiato. Se disuelven 15 g de fenol y 0.015 g de nitroprusiato sódico (añadido como 1ml de solución acuosa 1.5% p/v recién preparada, 1.5 g en 100 ml) en 500 ml de agua. La solución se almacena a 4ºC.
- Reactivo hipoclorito alcalino. Se disuelven 10 g de NaOH en 400 ml de agua y se enfría la solución. Se añade un volumen de solución de hipoclorito sin diluir conteniendo 0.265 g de clorína. Se diluye hasta 500 ml, se mezcla intensamente y se guarda en el refrigerador.

- Solución patrón de NH_4Cl. Se disuelven 3.281 g de NH_4Cl en agua destilada hasta 1 l. La solución de trabajo (10 µg ml^{-1}) se prepara con 1 ml de la solución "stock" diluida en 100 ml de agua destilada.

Procedimiento

Se emplearon 5 ml de muestra filtrada recogidos en tubos de ensayo de vidrio. A cada tubo se añadieron 2 ml de fenol-nitroprusiato. Los tubos se agitaron y posteriormente se añadieron 2 ml de hipoclorito alcalino, volviéndose a agitar suavemente. Las muestras se colocaron a una temperatura estable (aprox. 25ºC) protegiéndose de la incidencia de la luz. Transcurrida una hora se cuantificó la absorbancia de la muestra a 635 nm empleando agua Mili-Q como blanco. La concentración de las muestras se estimó frente a patrones de concentración conocida preparados en el momento de realizar el análisis.

- *c.2. (Procedimiento II)*

Reemplazar el hipoclorito sódico por dicloroisocianurato sódico (DIC) supone un método mucho más estable frente a los cambios de temperatura y luz (Verdouw et al. 1978). Debido a las ventajas en cuanto a estabilidad de este método, se empleó para realizar el análisis de las muestras de la última campaña (08-09).

Reactivos

- Solución de Salicilato sódico 40% en agua. Esta solución no puede almacenarse y debe ser preparada diariamente.
- Se disuelve 1 g de Na_2 $\{Fe(NO)(CN)_5\}.2H_2O$ (nitroprusiato sódico) en 100 ml de agua destilada. El color habitual es "naranja-marrón" y tiende a tornarse verde cuando se deteriora.
- Se diluye 1 ml de la solución de nitroprusiato sódico en 10 ml de agua destilada a la cual se añaden 100 mg de dicloro-isocianurato sódico

($Cl_2Na(CON)_3.2H_2O$). Esta solución no puede almacenarse y debe ser preparada diariamente.

Procedimiento

Para realizar el análisis se colocó 1 ml de muestra en un tubo de ensayo de 5 ml. Se añadieron 0.625 ml de solución de salicilato al 40%. Se dejó reaccionar durante 30 minutos. Transcurrido este tiempo, se añadieron 0.125 ml de NaOH 10 M se mezclaron bien y posteriormente añadieron 0.25 ml de la solución de isocianurato. Transcurrida una hora se midió la absorbancia a 650 nm empleando agua Mili-Q como blanco. La concentración de las muestras se estimó frente a patrones de concentración conocida preparados en el momento de realizar el análisis.

2.5.1.2. Análisis de fósforo

Dentro de este sub-apartado se va a hacer referencia a las técnicas empleadas para la cuantificación de diferentes formas de fósforo (solubles y particuladas) presentes en el agua de los lagos.

a) Fósforo total

El fósforo se puede presentar en combinación con materia orgánica, por lo que se puede determinar el fósforo total por medio de una digestión previa de la muestra dándose como resultado fósforo en forma de ortofosfato libre. El ortofosfato reacciona con el molibdato amónico y el tartrato antimonílico potásico, en medio ácido, para formar un heteropoliácido de color amarillo pálido, el ácido fosfomolíbdico, que se reduce a azul de molibdeno, de color intenso, por el ácido ascórbico. El método empleado se basa en las técnicas referenciadas en APHA (1992), *4500-P Phosphorus (*método H *para* digestión manual de fósforo total y E para método del ácido ascórbico).

Reactivos

- Reactivo de digestión. Se disuelven 6 g $K_2S_2O_8$ en 80 ml de agua Milli-Q, se añaden 10 ml de H_2SO_4 10N y agua Milli-Q hasta 100 ml.
- Molibdato amónico. Se disuelven 15 g de $Mo_7(NH_4)_6.4H_2O_4$ en 500 ml de agua Milli-Q.
- Ácido sulfúrico diluido. Se mezclan 140 ml de H_2SO_4 concentrado en 900 ml de agua Milli-Q.
- Ácido ascórbico. Se disuelven 3.86 g en 100 ml de agua destilada.
- Tartrato de antimonio y potasio. Se disuelven 0.34 g de $C_8H_4K_2O_{12}Sb_2.3H_2O$ en 250 ml de agua Milli-Q.
- Reactivo mixto. Se añaden en el orden indicado 100 ml de solución de molibdato amónico, 250 ml de la solución de sulfúrico, 100 ml de la solución de ascórbico y 50 ml de la de tartrato. Se prepara la cantidad necesaria y se agita enérgicamente.
- Solución patrón. Se pesan 0.817 g de KH_2PO_4, previamente desecado, y se disuelven en agua destilada saturada de cloroformo completándose hasta 1 litro. Esta solución contiene 6mM de fosfato conservándose como patrón concentrado a 4ºC. Se prepara una dilución intermedia 1:100 con una concentración 60 µM. Las diluciones de trabajo son de 3, 6 y 12 µM.

Procedimiento

Se colocaron 25 ml de agua de muestra no filtrada en tubos de ensayo de vidrio y se añadieron 5 ml del reactivo de digestión. Se cubrieron los tapones con papel de aluminio y se introdujeron en un autoclave durante 30 minutos a 2 atm de presión. Después del autoclavado, se dejaron enfriar a temperatura ambiente y posteriormente se neutralizó el pH empleando NaOH y HCl. A partir de la muestra digerida y neutralizada, se tomaron 5ml en tubos de ensayo de vidrio y se añadió 1 ml de reactivo mixto. Tras esperar entre cinco minutos y antes de dos horas, se midió la absorbancia a 882 nm. Los blancos se hicieron con agua

Milli-Q. La concentración de fósforo se estimó frente a patrones de concentración conocida preparados durante el análisis.

b) Fósforo particulado

Se sigue el mismo principio que para el caso del fósforo total con la única diferencia de que en este caso se cuantificó la fracción particulada recogida in-situ en filtros.

Reactivos

Se emplearon los mismos reactivos que para el análisis de fósforo total.

Procedimiento

Los filtros previamente almacenados fueron troceados y colocados en tubos de centrífuga de 50 ml. Se añadieron 25 ml de agua y 5 ml de solución de oxidación para llevar a cabo la digestión de la muestra. Se cubrieron los tapones con papel de aluminio y se introdujeron en un autoclave durante 30 minutos a 2 atm de presión. Tras el proceso de digestión, se dejaron enfriar los tubos y se neutralizaron las muestras mediante NaOH y HCl. Se tomaron 5 ml de la muestra digerida y neutralizada y se colocaron en tubos de ensayo de vidrio, añadiéndose posteriormente 1 ml de reactivo mixto. Tras esperar entre cinco minutos y antes de dos horas, se midió la absorbancia a 882 nm. Se realizaron blancos con agua Milli-Q. La concentración de las muestras se estimó frente a patrones de concentración conocida.

c) Fósforo reactivo soluble

En este caso únicamente se cuantificó la fracción soluble del fósforo previa filtración in-situ de la muestra, por lo tanto no se requiere de un proceso de digestión previo. El principio del método de cuantificación es el mismo que para

el caso del fósforo total. El método empleado se basa en las técnicas referenciadas en APHA (1992), 4500-P Phosphorus *(*E-método de ácido ascórbico).

Reactivos

Los mismos empleados para él análisis del fósforo total excepto que no se requiere de reactivo de digestión para el paso previo inicial.

Procedimiento

A partir de la muestra de agua previamente filtrada, se tomaron 5 ml y se colocaron en tubos de ensayo de vidrio. A cada tubo se le añadió 1 ml de reactivo mixto. Tras esperar entre cinco minutos y antes de dos horas se midió la absorbancia a 882 nm. Se realizaron blancos con agua Milli-Q. La concentración de las muestras se estimó frente a patrones de concentración conocida.

2.5.2. Análisis de silicato

a) Silicato reactivo soluble

El ácido silícico y algunos derivados reactivos, en solución ácida con el molibdato, forman ácido molibdosilícico el cual es reducido a su vez hasta un compuesto de silico-molibdeno. El método empleado se basa en las técnicas referenciadas en APHA (1992), 4500-SiO_2-B: método de molibdosilicato.

Reactivos

- Solución de ácido molibdato amónico. Se mezclaron 10 g de molibdato amónico con un volumen de 350 ml de agua Milli-Q a la cual se le añadieron 30 ml de HCl completándose con Milli-Q hasta un volumen de 500 ml.

- Solución de metol-sulfito. Se pesaron 6 g de sulfito sódico anhidro y 10 g de metol y se disolvieron en 500 ml de agua Milli-Q. La solución fue filtrada (filtros GVWP de 0,22 μm) para eliminar impurezas y se guardó en oscuridad.
- Solución de ácido oxálico. Se pesaron 10 g de ácido oxálico dihidrato y se disolvieron en 100 ml de agua Milli-Q. Para favorecer la disolución se aplicó calor.
- Solución de ácido sulfúrico. Se preparó una solución de ácido sulfúrico 25% (vol/vol) en agua Milli-Q.
- Solución reductora. Se mezclaron 100 ml de solución de metol-sulfito con 60 ml de solución de ácido oxálico en un vaso de precipitados. Tras colocar el vaso de precipitados en hielo, se añadieron cuidadosamente 120 ml de la solución de ácido sulfúrico. El volumen se llevó hasta 300 ml mediante agua Milli-Q.
- Solución patrón. Se disolvieron 0.6714 g Na_2SiF_6 en 400 ml de agua Milli-Q. La mezcla se calentó para favorecer la disolución y se dejó enfriar añadiéndose posteriormente agua Milli-Q hasta un volumen final de 1 litro. La solución debe quedar de tal manera que contenga 100 mg de SiO_3^- por mililitro. Previamente a realizar esta solución, el fluorosilicato debe ser secado a 80ºC durante 30 minutos y guardado en desecador de vacío durante los 10 minutos previos a pesar una cantidad específica.

Procedimiento

Se emplearon 20 ml de muestra en tubos de ensayo de 50 ml. Se le añadieron 3 ml de solución de molibdato, tapándose y mezclandose varias veces durante 10 minutos. Tras realizar esto, se añadieron 15 ml de solución reductora llevándose hasta un volumen 50 ml con agua Milli-Q. Tras esperar tres horas, se midió la absorbancia a 812 nm frente a blancos de agua Milli-Q. La concentración de las muestras se estimó frente a patrones de concentración conocida.

2.5.3. Análisis de cationes y aniones

La concentración de cationes y aniones mayoritarios (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, SO_4^{2-}, HCO_3^{-}, Cl^{-}) fueron determinadas empleando un analizador iónico capilar (Waters), en el servicio de análisis de la Universidad Autónoma de Madrid. El fundamento de la separación de este método se basa en el uso de resinas de intercambio iónico. Cuando la muestra a analizar atraviesa las columnas del sistema de intercambio, los iones se separan debido a las diferentes retenciones que sufren durante el paso por la fase estacionaria de las columnas. Una vez separada, la muestra pasa a través de un detector de conductividad donde se registra la señal obtenida respecto a su tiempo de retención.

La alcalinidad de las muestras se cuantificó en el laboratorio empleando el método de valoración mediante HCl, como viene definido en Standard Methods APHA (1992) 2320 alcalinidad B: método del tritatión. La alcalinidad del agua la podemos definir como su capacidad para neutralizar ácidos o aceptar protones, entendiéndose de esta forma como la capacidad para tamponar el pH. Esta representa la suma de las bases que pueden ser tituladas en una muestra de agua. En aguas naturales, la alcalinidad se debe principalmente a la presencia de ciertas sales de ácidos débiles, aunque también puede contribuir la presencia de bases débiles y fuertes. En general, son los bicarbonatos los compuestos que más contribuyen ya que se forman fácilmente a partir del dióxido de carbono atmosférico (Manahan 2000).

2.6. Análisis de carbono y materia orgánica

2.6.1. Análisis de la fracción fluorescente de la materia orgánica disuelta (CDOM)

Las características fluorescentes de la materia orgánica, especialmente del material húmico, permiten su caracterización mediante el uso de un fluorímetro (F7000 Hitachi). Esta característica permite identificar el origen de la materia orgánica

disuelta, ya sea de origen terrestre o de origen proteico. Se basa en la cuantificación mediante espectros de fluorescencia (barrido de un rango de longitudes de onda de excitación y de emisión), lo que permite identificar de forma detallada el tipo de materia orgánica disuelta.

Procedimiento

Se realizaron medidas de emisión/excitación de cada muestra, empleando para ello un fluorímetro F7000 Hitachi. La medida se llevó a cabo empleando los siguientes parámetros: Excitación 240-450 nm con un intervalo de 5nm, Emisión 240-600nm con un intervalo de 2nm. El salto de excitación y de emisión fue de 5nm en ambos casos. La velocidad del barrido se mantuvo en 12000 nm/mín empleando un voltaje de 700V.

A cada una de las matrices de excitación-emisión obtenidas se le restó una matriz obtenida a partir de una muestra de agua destilada ultra pura (Mili-Q), eliminándose así el efecto de dispersión o "raman" que produce el agua. Una vez realizado este paso para cada una de las muestras, se identificaron los distintos grupos de materia orgánica disuelta cromófora (CDOM) presentes tal y como describe Stedmond (2005). Se diferenciaron dos tipos mayoritarios de compuestos orgánicos de carbono con capacidad cromófora (CDOM), dentro de los cuales se pueden separar dos subtipos. Por un lado el CDOM de tipo proteico o autóctono, donde se puede diferenciar la fracción similar a Tirosina (Tyrosin-like) y la fracción parecida a Triptófano (Tryptophan-like). Por otro lado, el grupo de CDOM de tipo húmico o alóctono, donde podemos separar la fracción de los ácidos húmicos de origen terrestre (AHT) y la fracción de ácidos húmicos debidos a fertilización animal (AHF).

Una vez determinada la fluorescencia máxima para cada una de las diferentes fracciones de CDOM, se estimaron las concentraciones refiriéndolas a unidades de sulfato de quinina (CDOM de tipo húmico) y seroalbúmina bovina "BSA" (CDOM de tipo proteico), empleando para ello patrones de concentración conocida.

2.6.2. Análisis del carbono orgánico total

La muestra de agua se inyecta en una cámara de reacción a 680°C junto con un catalizador oxidante. El agua se vaporiza y el carbono (orgánico e inorgánico) se oxida a CO_2. Este CO_2 se transporta en corriente de aire y se mide en un analizador de infrarrojos no dispersivo. Dado que con el procedimiento anteriormente descrito se determina carbono total (TC), se debe medir también el carbono inorgánico (IC), para obtener el TOC por diferencia. La fracción de IC se mide inyectando la muestra en una cámara de reacción distinta, donde se le añade un volumen de HCl. Bajo condiciones ácidas todo el IC se convierte en CO_2 pasando a medirse en el analizador de infrarrojos. En estas condiciones el carbono orgánico no se oxida por lo que sólo se determina el IC.

En la mayoría de las muestras de agua la fracción de IC es muy superior a la fracción de TOC por lo que en numerosas ocasiones se elimina previamente el IC. Para ello se acidifican las muestras a pH $\leq$2 (a fin de convertir el IC en CO_2) y a continuación se purga la muestra con un gas puro (para eliminar el CO_2), esta determinación se denomina carbono orgánico no purgable (NPOC). Es de señalar que en los análisis de NPOC, se produce la pérdida de sustancias orgánicas volátiles.

Reactivos

- Botellas de vidrio opacas previamente lavadas con ácido
- Agua destilada Mili-Q
- Ácido clorhídrico 1N
- Solución patrón de carbono total de 1000 mg C/l. Se disolvieron 2,125 g de Ftalato de hidrógeno de potasio ($C_8H_5KO_8$) en agua exenta de carbono, añadir H_2SO_4 hasta pH $\leq$2 y enrasar a 1000 ml. A partir de esta solución, se prepararon las diluciones apropiadas.
- Solución patrón de carbono inorgánico de 1000 mg C/l. Se disolvieron, en agua Mili-Q, 4,4122 g de carbonato de sodio anhidro (CO_3Na_2), previamente calentado a 180°C durante 30 minutos y enfriado en

desecador, se añadieron 3,497 g de bicarbonato de sodio anhidro (CO_3HNa) enrasándose a 1000 ml. A partir de esta solución, se prepararon las diluciones apropiadas.

- Analizador TOC (TOC-V csn SHIMADZU)

Procedimiento

Se filtró la muestra con filtros de nitrato de celulosa de 0.2 µm y se conservaron en botellas de vidrio actínico de 30 ml previamente lavadas con ácido. Se fijaron las muestras con un volumen de HCl 2N para reducir el pH por debajo del punto de equilibrio del CO2. Se conservaron en oscuridad a 4ºC.

Previamente al análisis se llevan a cabo dos curvas de calibrado. La primera para el carbono total, empleando el patrón de ftalato de hidrógeno de potasio. La segunda curva se realizó para el carbono inorgánico, empleando para ello el patrón de carbonato de sodio. Una vez realizadas ambas curvas, se llevó a cabo la cuantificación del carbono orgánico total.

2.7. Variables bioquímicas

2.7.1. Análisis de los pigmentos fotosintéticos del plancton mediante HPLC

El análisis de pigmentos fotosintéticos mediante cromatografía líquida de alta presión se basa en la separación diferencial de compuestos sobre una matriz compactada previa disolución en una mezcla de solventes orgánicos. Tras la separación, los compuestos fueron identificados teniendo en cuenta su espectro de absorbancia y su tiempo de retención sobre la matriz.

Reactivos

- Tubos de vidrio de 50 ml
- Balanza
- Acetona para análisis
- Acetona, metanol, acetato amónico y acetonitrilo para cromatografía
- Filtros de 0.45 µm de Nylon
- Jeringas de 5 ml
- Rotavapor
- Microviales de vidrio de 200 µl
- HPLC (Waters 715 Ultra Wisp) "Millipore"

Procedimiento

a) (Extracción de pigmentos fotosintéticos)

Se sacaron los filtros guardados en papel de aluminio del congelador y se colocaron en tubos de vidrio. A cada uno de los tubos se le añadió un volumen de acetona de manera que los filtros quedaran cubiertos completamente (debido al posterior proceso de concentración, no fue necesario anotar el volumen de acetona añadido). Los tubos se dejaron en un baño sonicador durante unos minutos y a una temperatura de -20ºC durante toda una noche, para que se llevara a cabo la extracción de pigmentos. Se centrifugaron los tubos a 2900 rpm durante 10´ a 4ºC y se recogió el sobrenadante (este paso se repitió varias veces hasta que el sobrenadante quedó transparente). Los nuevos volúmenes extraídos se añadieron al primero.

b) (Concentración de pigmentos y cuantificación mediante HPLC)

Las muestras se concentraron en un volumen final de 1ml mediante el uso de un rotavapor (Laborota 4000, Heidolph Instruments). Se colocaron 200 µl de muestra concentrada en microviales para HPLC, añadiéndose a cada uno un volumen de acetato amónico concentrado de manera que la concentración final en el inserto fuera

0,1M. Se empleó una carrera cromatográfica de 100 minutos con un gradiente binario no linear, según el método de Pickney (adaptado de Van Heukelem et al. 1992) (Tabla 2.2.).

Tabla 2.1. Condiciones de cromatografía para la separación de pigmentos mediante HPLC. Aparece reflejado el flujo y el % empleado de cada uno de los solventes en la fase móvil.

Tiempo (min)	Flujo ($nl \cdot min^{-1}$)	% CH_3OH	% CH_3COOHN_4	% $CH_3(CO)CH_3$
0	0.80	80	20	0
5	0.80	80	10	10
45	1.25	80	5	15
50	1.50	80	0	20
65	0.80	80	0	20
67	0.80	80	20	0
95	0.80	80	20	0

A partir de los cromatogramas generados, se identificaron y se cuantificaron los pigmentos fotosintéticos mayoritarios. Se empleó el tiempo de retención de cada pigmento junto a su espectro característico para su identificación, empleando como referencia resultados de patrones de concentración conocida. La concentración de cada pigmento se cuantificó mediante la medida del área del pico correspondiente en el cromatograma.

2.8. Determinación de la producción primaria del plancton, tapetes microbianos y musgos bentónicos a partir de la asimilación de carbono (^{13}C)

La incubación de muestras con isótopos de carbono inorgánico no radioactivos puede emplearse para determinar las tasas de incorporación de carbono por parte de los organismos. Estas técnicas son empleadas como una alternativa a las técnicas habituales que emplean isótopos radioactivos (Laybourn-Parry et al., 2004,2006). Sin embargo, esta alternativa presenta limitaciones en cuanto a los límites de detección, siendo algo superiores a los que se alcanzan con las técnicas de isótopos radioactivos. Por el contrario, no genera contaminantes peligrosos ni supone un riesgo para el usuario. Debido a las difíciles condiciones de trabajo de campo y a los peligros de uso de métodos radioactivos en zonas protegidas (ASPA, Antarctic Special Protected Area), se llevaron a cabo todos los análisis de producción primaria empleando isotopos de ^{13}C.

Reactivos

- Botellas de incubación de 250 ml
- Bolsas de incubación Whirlpack
- Botella hidrográfica Ruttner
- Boya y soporte para incubación
- Solución stock de $NaH_{13}CO_3$
- HCl 2 N
- Equipo de filtración para filtros de 25 mm de diámetro
- Bomba de vacío
- Filtros de fibra de vidrio Whatman GF/F de 25 mm de diámetro

Procedimiento

Para llevar a cabo el análisis se añadió el isotopo de carbono a cada una de las muestras en concentraciones bajas (aprox 10% del carbono inorgánico total) de forma que no representara una interferencia con la incorporación natural de carbono. La tasa de incorporación se calculó como el porcentaje del total de carbono incorporado, llevándose a cabo la metodología empleada por Wetzel y Likens (1991).

Se emplearon dos botellas de incubación transparentes de aproximadamente 250 ml y una tercera oscura (tapada con cinta opaca de manera que quedase aislada de la luz) que se empleó como blanco. Para el caso del Lago Limnopolar, este patrón se repitió para cada una de las profundidades ensayadas (0.5, 2, 4m).

Cada una de las botellas se llenó con agua de la profundidad correspondiente evitando en lo posible la incidencia directa de la luz. Como ya se indicó con anterioridad, antes de llevar a cabo la incubación se añadió a cada una de las botellas un volumen de $H^{13}CO_3$, de manera que el ^{13}C fuera aproximadamente un 10% de la concentración total de carbono inorgánico disuelto. Las incubaciones se llevaron a cabo in-situ empleando para ello un sistema de soportes metálicos de manera que cada botella quedara situada a la profundidad correspondiente al agua de muestra que contenía. La incubación se mantuvo durante 2-3 horas dentro de un periodo de luz comprendido entre las 9:00 y 15:00 horas.

Una vez finalizada la incubación, se recuperaron las botellas y se guardaron en completa oscuridad para su posterior transporte hasta el laboratorio. Una vez en el laboratorio y habiendo transcurrido menos de 2 horas, las muestras fueron filtradas empleando filtros de fibra de vidrio GF/F (Whatman) de 25mm de diámetro previamente precombustionados a 450 ºC durante una hora y media. Los filtros se dejaron secar y se guardaron doblados sobre sí mismos, envueltos en papel de aluminio y a una temperatura de -20ºC. Los posteriores cálculos de incorporación de carbono se realizaron previa cuantificación de la abundancia natural del isotopo pesado de carbono en el lago. Para ello, se filtró un volumen de agua del lago en filtros

de fibra de vidrio GF/F (Whatman) de 25 mm de diámetro para cada una de las profundidades, siguiendo el mismo protocolo descrito anteriormente.

Una vez las muestras congeladas se llevaron al laboratorio en nuestro centro de investigación en Valencia fueron preparadas para su análisis. Para ello, los filtros se descongelaron y secaron a 105 ºC durante 3 horas y se almacenaron en eppendorf individuales. Se introdujeron en bolsas de cierre hermético y se enviaron al servicio de análisis elemental de la Universidad Autónoma de Madrid (SIDI). Él análisis se llevó a cabo a partir de una porción del filtro empleando un analizador elemental conectado a un espectrómetro de masas (GCT Agilent Technologies 6890N, Waters) mediante una porción del filtro para el análisis.

Para los cálculos se requiere conocer la cantidad disponible de carbono orgánico disuelto (CID). Para ello, previamente se realizó un análisis de alcalinidad tal como se explica en el apartado sobre análisis de cationes y aniones. Tras recibir los resultados de cada muestra como la proporción de $^{13}C/^{12}C$, se cuantifico la tasa de incorporación de Carbono como se indica a continuación:

$$\boldsymbol{T_i}(h^{-1}) = \%C_m - \left(\frac{AN}{\left(\left(\frac{C_a}{100\ (C_a + CID)} \right) - AN \right) t} \right)$$

$$\boldsymbol{TIC}\ (\mu gC\ l^{-1}h^{-1}) = \boldsymbol{T_i}\ (h^{-1}) \times \boldsymbol{COP}\ (\mu gC\ l^{-1})$$

T_i: tasa de incorporación (h^{-1})

$\%C_m$: % de carbono (^{13}C) en la muestra

AN: abundancia natural de carbono (^{13}C) en la muestra

C_a: carbono (^{13}C) añadido

CID: carbono inorgánico disuelto

t : tiempo en (h^{-1})

COP: carbono orgánico particulado (µgC l^{-1})

TIC: tasa de incorporación de carbono (µgC l^{-1} h^{-1})

La incorporación de carbono en *tapetes microbianos* y en *musgos bentónicos* puede ser medida empleando una metodología similar. Para ello se emplearon pequeñas porciones de superficie conocida (5-8 mm de diámetro) para el caso de tapetes o una porción de peso conocido para el caso de musgos. Cada una de las muestras se incubaron en el interior de bolsas de incubación (Whirlpack) añadiendo aproximadamente unos 10 ml de agua del lago junto con el isótopo de carbono ^{13}C, siguiendo el mismo procedimiento indicado para el caso de muestras líquidas. Cada muestra fue incubada manteniendo las condiciones de luminosidad de origen (superficie para los tapetes y fondo del lago para los musgos). Para el caso de los musgos bentónicos se empleó el mismo soporte utilizado para la incubación de las muestras de producción primaria del plancton. De la misma forma, la incubación se llevó a cabo durante un tiempo de 2-3 horas dentro del periodo de máxima iluminación (9:00-15:00).

Para detener la fijación fotosintética, las bolsas de incubación fueron introducidas en un recipiente opaco bajo condiciones de oscuridad para evitar la incidencia directa de la luz. Una vez en el laboratorio de campo, las muestras fueron extraídas de las bolsas mediante el uso de pinzas y se dejaron secar sobre papel absorbente, almacenándose posteriormente en bolsitas individuales a -20ºC. Posteriormente en el centro de investigación, las muestras se secaron a 105ºC y se homogeneizaron mediante un mortero para obtener un material fino. Cada porción del homogenizado se introdujo en tubos eppendorf de 2ml, guardándose todos ellos en bolsas de cierre hermético. Las muestras fueron enviadas al servicio de análisis elemental de la Universidad Autónoma de Madrid (SIDI). El procedimiento de análisis y cálculo de las tasas de fijación de carbono se realizó de la misma forma que para el caso del plancton aunque en este caso los resultados fueron referidos por unidad de biomasa (µgC/biomasa.h) en lugar de por unidad de volumen de agua.

2.9. Determinación de la producción bacteriana

Al igual que para el caso del análisis de producción primaria, se midió la producción bacteriana mediante el uso de isótopos de carbono, en este caso orgánico, no radiactivo. Se empleó como marcador moléculas de leucina en las cuales uno de sus carbonos era un átomo pesado de carbono ^{13}C. Esto nos permitió cuantificar las tasas de incorporación de carbono orgánico por parte del bacterioplancton en los lagos.

Reactivos

- Botellas de incubación de 500 ml
- Bolsas de incubación Whirlpack
- Botella hidrográfica tipo Ruttner
- Boya y soporte para incubación
- Solución de leucina ^{13}C
- Solución de ácido tricloroacético (TCA) 50% w/v
- Solución de Etanol 80% v/v
- Filtros de fibra de vidrio GF/F (47mm) precombustionados
- Equipo de filtración
- Bomba de vacío

Procedimiento

Se utilizó la misma metodología que empleamos para la cuantificación de la producción primaria, sin embargo se aplicaron algunas modificaciones. Para la incubación se utilizaron dos botellas de incubación transparentes (por duplicado) de aproximadamente 500 ml para cada una de las profundidades. Se tomaron muestras de tres profundidades (superficie, profundidad media y fondo) para llenar las botellas de incubación. A cada una de las muestras se le añadió un volumen de ^{13}C-Leucina hasta saturación, alcanzando una concentración final de 10mM. Las incubaciones se llevaron a cabo durante un período de 2-3 horas. Tras finalizar la incubación las muestras fueron filtradas empleando filtros de fibra de vidrio GF/F

precombustionados y la actividad bacteriana se detuvo mediante la adicción de un pequeño volumen de TCA (50%) el cual actúa como agente precipitante, tras el cual las muestras se lavaron con un volumen de TCA (5%). Posteriormente los filtros fueron lavados con etanol (50%) guardándose plegados sobre sí mismos a -20ºC y envueltos en papel de aluminio.

Una vez las muestras llegaron a nuestro centro de investigación en Valencia, los filtros fueron descongelados y desecados a 105ºC durante 3 horas y posteriormente almacenados en eppendorf individuales. Las muestras fueron enviadas al servicio de análisis de la Universidad Autónoma de Madrid (SIDI) para cuantificar la proporción relativa de isótopo pesado en las muestras. Este análisis se llevó a cabo con el mismo analizador elemental que para la cuantificación de la producción primaria.

Para llevar a cabo los cálculos de incorporación debemos conocer la cantidad de ^{13}C-Leu añadida y su abundancia natural en la muestra, que en este caso supondremos que es muy baja o nula, por lo que la despreciamos. Sera necesario conocer la concentración de carbono orgánico particulado (COP). La fórmula empleada para los cálculos es la siguiente:

$$\boldsymbol{T_i}(h^{-1}) = \%C_m - \left(\frac{AN}{\left(\left(\frac{C_a}{100\ (C_a + CID)} \right) - AN \right) t} \right)$$

$$\boldsymbol{TIC_o}\ (\mu gC\ l^{-1}h^{-1}) = \boldsymbol{T_i}\ (h^{-1}) \times \boldsymbol{COP}\ (\mu gC\ l^{-1})$$

T_i: tasa de incorporación (h^{-1})

$\%C_m$: % de carbono (^{13}C) en la muestra

AN: abundancia natural de leucina (^{13}C) en la muestra

C_a: leucina (^{13}C) añadida

CID: carbono inorgánico disuelto

t : tiempo en (h^{-1})

COP: carbono orgánico particulado (µgC l^{-1})

TIC_o: tasa de incorporación de carbono orgánico (µgC l^{-1} h^{-1})

Una vez calculada la incorporación de carbono en forma de Leucina, se estimó la cantidad total de carbono asimilada por los organismos. Para ello empleamos un coeficiente que establece la proporción de carbono total incorporado en función de la cantidad de Leucina incorporada. El valor empleado fue de 1,5 Kg de carbono por mol de Leucina incorporada (Simon y Azam 1989).

2.10. Análisis metabólico de la utilización de diferentes fuentes de carbono (Biolog)

El aprovechamiento de las diferentes fuentes de carbono por las comunidades microbianas puede emplearse como una herramienta útil para estudiar la biodiversidad metabólica presente en los sistemas acuáticos. Para cuantificar la actividad metabólica de aprovechamiento de las fuentes de carbono se empleó el sistema de placas Biolog Ecoplate™. Este sistema fue creado específicamente para análisis de la comunidad microbiana y estudios en ecología microbiana. El análisis de la comunidad microbiana empleando microplacas Biolog fue originalmente descrito por Garland y Mills (1991). Este grupo de investigadores descubrió que la inoculación de cultivos mixtos de microorganismos en estos sistemas de placas multipocillo, y la

A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine	A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine	A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine
B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine	B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine	B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine
C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine	C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine	C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine
D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine	D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine	D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine
E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine	E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine	E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine
F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid	F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid	F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid
G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine	G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine	G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine
H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine	H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine	H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine

Figura 2.3. Fuentes de carbono de uso frecuente de las placas Ecoplate. Obsérvese la distribución por triplicado de cada una de las fuentes de carbono.

cuantificación de la actividad metabólica de las diferentes fuentes de carbono a lo largo del tiempo, podía ser empleada como una herramienta útil para estudiar la estructura de las comunidades microbianas. Esta aproximación fisiológica se ha demostrado que puede llegar a ser muy efectiva a la hora de distinguir cambios espaciales y temporales en las comunidades microbianas. Las placas Ecoplate de Biolog, se caracterizan por presentar 31 tipos diferentes de recursos de carbono de uso frecuente (Figura 2.3). Cada una de las fuentes de carbono se encuentra en forma tres pocillos que representan tres réplicas, para poder dar mayor solidez a los resultados.

Procedimiento

Para la cuantificación de la actividad metabólica, se inocularon todos los pocillos de diferentes placas "Ecoplate" con agua de cada uno de los lagos objeto de estudio (Limnopolar, Somero, Chica, Refugio, Maderos), o en el caso del Lago Limnopolar, de las distintas profundidades ensayadas. Tras la inoculación, las muestras se incubaron durante 12-13 días a temperatura ambiente, recogiendo una foto de las placas cada 24 horas. La detección de la actividad metabólica se efectuó mediante la medida de la aparición de color en cada uno de los pocillos. La aparición de color purpura en los pocillos ocurre cuando los microorganismos son capaces de degradar la fuente de carbono. La respiración de las células en la comunidad reduce la molécula cromófora de tetrazolio, que está incluida junto con el recurso de carbono. Habitualmente la reacción es detectada y analizada empleando un analizador de placas, pero debido a la imposibilidad de llevar uno hasta el campamento de la Península Byers, se optó por captar las imágenes de las placas cada 24 horas mediante una cámara (Canon EOS 350D, EF-S 18-55 mm, 1:3.5-5.6 DC). Tras esto, las imágenes fueron analizadas y procesadas mediante análisis de imagen (empleando el programa ImageJ) con el objetivo de estudiar la similaridad de los patrones de desarrollo de los pocillos, así como del grado de color que está relacionado de forma directa con la intensidad de la actividad metabólica.

2.11. Recuentos de organismos

2.11.1. Recuento de organismos planctónicos mediante citometría de flujo

Reactivos

- Glutaraldehído
- Tubos Falcon 15 ml
- Citómetro Beckman Coulter (5 canales) "Cytomics FC 500 MPL"
- Isotón
- Tubos de ensayo de plástico (especiales para citómetro)
- "Bolas" fluorescentes de 0.5 y 1 µm
- SYBR-Green (10x)

Procedimiento

Mediante este método se ha cuantificado el bacterioplancton y el picoplancton autótrofo de los sistemas acuáticos estudiados. A partir de la muestra de agua previamente almacenada y conservada, se llevó a cabo un recuento mediante citometría de flujo empleando para ello un Citómetro (Beckman Coulter "Cytomics FC 500MPL") con un láser de 488 nm y 5 canales de fluorescencia. Para llevar a cabo el análisis se empleó el método indicado por Gasol y del Giorgio 2000.

Para el caso del recuento del *bacterioplancton*, se preparó un volumen de 500 µl diluyendo la muestra original 1/5 (400ul Isotón Coulter ® + 100ul muestra) en tubos de ensayo de plástico tiñéndose con SYBR-Green 1x durante 30-60 minutos en oscuridad. El ratio de recuento se mantuvo en todo momento por debajo de 500 cel s^{-1}. El volumen de la muestra analizada se determinó haciendo pasar la muestra durante 60-300 segundos y aplicando posteriormente coeficientes estimados mediante

calibraciones previas. La fluorescencia del SYBR-Green se detectó en el canal verde (FL2). Se empleó el canal SSC "Side-Angle Light Scater" como proxy para el tamaño bacteriano (Trousselier et al. 1999). A cada una de las muestras se añadió 5µl de bolas de látex de 0,5 y 1 µm como marcadores de tamaño. La abundancia de bacterioplancton se determinó mediante el recuento de los grupos detectados en los histogramas entre el canal FL2 y SSC. Adicionalmente, la abundancia relativa del grupo de bacterias con alto contenido en ADN (HDNA) y del grupo de bacterias con bajo contenido en ADN (LDNA) fue estimada a partir de los histogramas según describe Li et al. (1995).

Para el caso del *picoplancton autótrofo*, se tomó un volumen de muestra en tubos de ensayo de plástico, a los cuales se añadieron 5 µl de bolas de látex fluorescentes de 0,5 y 1 µm respectivamente. Las poblaciones de picoplancton autótrofo se detectaron por autofluorescencia no siendo necesario la utilización de fluoróforos externos. En función de su tamaño y la relación entre las fluorescencias de los canales FL2 y FL3 se discriminaron las distintas poblaciones observadas.

2.11.2. Recuento de virioplancton mediante microscopía de fluorescencia

Reactivos

- Filtros Anodisc de 25 mm y tamaño de poro de 0.02 µm (Whatman)
- Filtros de membrana de 25 mm y tamaño de poro de 0.8 µm (Millipore)
- Solución de SYBR-Green I a una concentración de 2,5%.
- Solución anti-apagado. Vectashield ®
- Equipo de filtración
- Aceite de inmersión
- Porta y cubre-objetos
- Microscopio de fluorescencia (ZEISS III RS)

Procedimiento

A partir de muestras fijadas con formaldehido 1% se siguió el protocolo descrito por Noble y Fuhrman (1998). Se colocó un filtro Anodisc sobre un pre-filtro de membrana de 0.8 µm (reutilizable varias veces) en la torre de filtración. Se filtraron aproximadamente entre 0.5-1ml de muestra, aplicando una presión aproximada de 20 kPa. Sin reducir la presión, se retiró la torre de filtración con cuidado. Se redujo la presión y se extrajo el filtro Anodisc, tras lo que se dejó secar cuidadosamente por la parte de abajo del filtro mediante papel absorbente de forma que el filtro quedase bien seco. Es conveniente dejar secar completamente los filtros durante 2 o 3 horas empleando para ello algún tipo de sistema de desecación, como por ejemplo el uso de una campana de vacío con gel de sílice.

Para preparar las muestras para el recuento, se colocaron varias gotas de 100 µl de la solución de trabajo de SYBR-Green I sobre una placa petri recubierta con parafilm. Este soporte se empleó como superficie de tinción para los filtros. Una vez secos, los filtros se colocaron cada uno sobre una gota de SYBR I y se mantuvieron en oscuridad para su tinción durante unos 10-15 min. Una vez teñidos, se secaron cuidadosamente por la parte de abajo hasta retirar totalmente los restos de SYBR-Green I que pudieran quedar (es importante evitar en lo posible tocar con el papel secante la parte de arriba del filtro). Tras dejar secar los filtros (en oscuridad) durante unos minutos, se vertió sobre un portaobjetos de vidrio una gota de aprox 30 µl de solución anti-apagado colocándose sobre ella el filtro. Se puso otra gota de solución anti-apagado sobre el filtro y se cubrió mediante un vidrio cubre. Se eliminaron las burbujas de aire entre el filtro y el vidrio y posteriormente se pasó a envolver la preparación con papel de aluminio evitándose de esta forma la incidencia de la luz. Se anotó correctamente sobre el papel de aluminio (y previamente sobre el porta) todos los datos de la muestra, guardándose posteriormente en oscuridad y a -20ºC hasta su recuento.

Para llevar a cabo el recuento se extrajeron las preparaciones del congelador dejándose a temperatura ambiente unos minutos para que se descongelara y mediante

un papel secante se eliminó el exceso de agua sobre la preparación. Cada uno de los filtros se cubrió con 1 gota de aceite de inmersión y se observó directamente mediante microscopio de fluorescencia (ZEISS III RS) con el objetivo de 100X. Para la cuantificación, se contaron todos las partículas fluorescentes de pequeño tamaño en aprox. unos 50-100 campos en cuadriculas de 71.4 x 71.4 µm.

2.11.3. Recuento de nanoplancton mediante microscopía de fluorescencia

Reactivos

- Filtros de policarbonato de 25 mm y tamaño de poro de 0.8 µm (Whatman)
- Solución de DAPI. Se emplea una solución de trabajo de 50 µg/ml
- Equipo de filtración
- Aceite de inmersión
- Porta y cubre-objetos
- Microscopio de fluorescencia (ZEISS III RS)

Procedimiento

El procedimiento se realizó a partir de muestras de agua fijadas in-situ con formaldehido 1%. Se colocó un filtro de policarbonato de 0.8 µm sobre el equipo de filtración de forma que la torre de filtración quedase sobre el filtro, fijándose posteriormente mediante una pinza. Se colocaron aprox. unos 30 ml de muestra fijada con formaldehido 1% sobre la torre de filtración, aplicándose una presión de 20 KPa y moviendo cuidadosamente la torre para asegurar una filtración homogénea. Sobre los últimos 3 ml de volumen de muestra, previa detención del proceso de filtración, se añadieron 0,3 ml de solución de trabajo de DAPI sobre la propia torre de filtración y se dejó teñir durante unos 5-10 minutos en condiciones de oscuridad. Una vez

finalizada la tinción, se filtró el resto de muestra lavándose posteriormente con un volumen de unos 10 ml de agua mili-Q. Se retiró el filtro del soporte y se dejó secar en oscuridad durante unos minutos. Tras esto, los filtros fueron montados sobre porta-objetos usando aceite de inmersión. Cada una de las preparaciones fueron guardadas en oscuridad y a -20ºC hasta su recuento.

Para llevar a cabo el recuento se extrajeron las preparaciones del congelador dejándose a temperatura ambiente durante unos minutos para su descongelación retirando mediante papel secante el exceso de agua que pueda quedar sobre la preparación. Se cubrió cada uno de los filtros con 1 gota de aceite de inmersión y se observó directamente sobre un microscopio de fluorescencia (ZEISS III RS) con objetivo de 100X. Se contabilizaron todos nanoflagelados presenten en 100 campos.

2.12. Análisis moleculares

2.12.1. Estudio de la composición de las poblaciones bacterianas mediante hibridación fluorescente in situ (FISH)

Esta técnica es una excelente herramienta para conocer la estructura de la comunidad microbiana in situ, mediante el uso de sondas fluorescentes de oligonucleótidos (Pearce et al. 2003). Las sondas se diseñan para detectar diferentes niveles taxonómicos (pudiendo hacer objetivo a todo el dominio Bacteria, o centrarse específicamente en un phylum, género o especie). Cuando una sonda es empleada en una muestra de agua, esta se asociara con el organismo objetivo y el resultado será una señal fluorescente que marca las células diana. Cada una de las sondas se encuentra unida covalentemente al extremo 5´ de la secuencia de ADN específica por una molécula fluorescente, en este caso la molécula foto-estable indocarbocianina (CY3). Es posible el empleo de diferentes sondas con fluorescencia a diferentes longitudes de onda para detectar diferentes grupos de organismos, empleando filtros selectivos en microscopía de fluorescencia.

Procedimiento

A partir de los filtros previamente procesados y almacenándose realizó el procedimiento de hibridación. Para ello, se cortaron porciones de cada uno de los filtros, una para cada una de las sondas empleadas. Se empleó una sonda general para eubacterias y 4 sondas específicas para los grupos bacterianos mayoritarios presentes en los lagos antárticos (Cottrel & Kirchman 2003, Pearce 2003). Las sondas empleadas fueron: para el dominio Bacteria Eub338 complementaria a la región "338-355" del ARNr 16S (5´-GCTGCCTCCCGTAGGAGT-3´), α-Proteobacterias Alf1b complementaria de la región "19-35" del ARNr 16S (5´-CGTTCGYTCTGAGCCAG-3´), β-Proteobacterias Bet42a complementaria a la región "1027-1043" del ARNr 23S (5´-GCCTTCCCACTTCGTTT-3´), γ-Proteobacterias Gam42a complementaria de la región "1027-1043" del ARNr 23S (5´-GCCTTCCCACATCGTTT-3´) y para Cytophaga-Flavobacterium "Bacteroidetes" CF319a complementaria de la región del ARNr 16S "319-336" (5´-TGGTCCGTGTCTCAGTAC-3´). Cada una de las porciones de los filtros, se montó sobre portas previamente recubiertos con parafilm. Durante cada proceso de hibridación, es importante no mezclar sondas fluorescentes para evitar contaminaciones cruzadas entre muestras.

Se preparó una solución de 2ml de tampón de hibridación para cada uno de los portas. Se cubrió con la solución de hibridación cada una de las porciones de los filtros, vertiendo el resto del tampón de hibridación sobre las placas absorbentes que incorpora la cámara de hibridación "Thermomixer Confort" (Eppendorf), para crear una atmósfera de saturación. Se mantuvieron los filtros a 46ºC durante 2 horas. Las soluciones de hibridación se prepararon con 0,9 M NaCl, 20mM Tris-Hl (pH 7,4), 0,01%SDS y 50 ng/ml de cada una de las sondas. Además, se añadió formamida a cada una de las soluciones de hibridación, variándose la concentración de la misma según la sonda empleada: EUB 338: 30%, ALF 1b:20%, BET 42a: 35%, GAM 42a: 35%, CF 319a: 15%. La formamida tiene la función de incrementar la permeabilidad de las células durante el tratamiento. Una vez transcurrido el periodo de hibridación, cada uno de los filtros se sometió a un proceso de lavado usando para ello un tampón de lavado previamente calentado a 48ºC. El tampón de lavado se preparó con 20mM

Tris-HCl (pH7.4), 5mM EDTA, 0,01% SDS y con concentraciones variantes de NaCl, en función de las concentraciones de formamida empleadas en el proceso de hibridación. La concentración de NaCl empleada fue: 0,9M para EUB 338, 0,225M para ALF 1b, 80mM para BET 42a, 80 mM para GAM 42a y 80 mM para CF 319a. Cada uno de los filtros se introdujo dentro de su tampón de lavado durante 10 minutos a 48ºC. Una vez finalizado este paso, los restos del tampón se eliminaron sumergiendo las porciones de filtro dentro de agua destilada. Tras esto y para eliminar el agua residual, se sometieron a un lavado con etanol absoluto. Se dejaron secar los filtros protegiéndolos en todo momento del efecto de la luz. Cada una de las porciones de filtro se sometió a un segundo proceso de tinción mediante un fluorocromo que se liga al ADN, el 4',6-diamidino-2-phenylindole (DAPI) a una concentración de trabajo de 50 ng/ml. Se cubrieron los filtros con esta solución de trabajo y se dejaron hibridar durante 10 minutos. Tras este paso, se lavaron cada uno de los filtros con agua destilada y con etanol absoluto para eliminar el agua restante. Posteriormente, los filtros se montaron en portas mediante el medio de montaje Vectashield®, que a su vez actúa como un agente anti-apagado de la fluorescencia.

Los recuentos de la abundancia total de bacterias (DAPI), y de cada uno de los grupos bacterianos (sondas con CY3), se realizaron mediante un microscopio de fluorescencia ZEISS III RS, con diferentes filtros preparados para poder diferenciar entre la sonda fluorescente CY3 y el DAPI.

2.12.2. Extracción de ADN

La extracción de ADN se llevó a cabo a partir de los filtros conservados previamente en tampón de lisis. Las muestras se incubaron con lisozima, proteinasa K y SDS en tampón de lisis (Dumestre et al. 2002). La extracción de ADN se llevó a cabo mediante fenol:cloroformo:alcohol-isoamílico (25:24:1, vol/vol/vol) y con cloroformo:alcohol-isoamílico (24:1, vol/vol) seguido de precipitación con isopropanol (Agogué et al. 2005).

2.12.3. Estudio de la diversidad molecular bacteriana mediante técnicas de clonación

La amplificación de fragmentos de ADNr 16S y su incorporación en vectores de clonación bacterianos es una herramienta de gran utilidad para el estudio de la diversidad bacteriana. Esta técnica nos va a permitir aislar de manera precisa fragmentos de ADNr 16S para su posterior secuenciación. De esta se obtuvieron de manera rápida y precisa, librerías clónicas de secuencias de ADN que podrán emplearse para caracterizar la diversidad bacteriana presente en un ecosistema.

Procedimiento

a) Amplificación de fragmentos de ADNr 16S

La amplificación de los fragmentos génicos del ADNr 16S se realizó directamente sobre el ADN extraído de las muestras del Lago Limnopolar, empleándose para ello los "primers" universales para clonación de bacterias 27f (AGAGTTTGATCMTGGCTCAG) y 1492r (GGTTACCTTGTTACGACTT). Las condiciones de la PCR se establecieron en 1 paso inicial de desnaturalización de 94ºC durante 5 minutos, seguidos de 35 ciclos de (1 minuto 94ºC, 1minuto 55ºC, 2 minutos 72ºC), tras estos ciclos se añadió un paso de elongación final de 1 minuto a 72ºC. Una vez finalizada la PCR, las muestras se mantuvieron a 15ºC. En todo momento se trabajó sobre una campana de flujo laminar para evitar la contaminación aérea. Todos los productos de PCR fueron analizados mediante una electroforesis de agarosa 1% (p/v) empleando como marcador 2.5 µl de "Gel Red" para 50 ml de agarosa.

b) Purificación de los productos de PCR

Se empleó un kit comercial (Invitrogen®) para la purificación de los productos de PCR. Fueron transferidos 50 µl de cada producto de PCR a un tubo eppendorf

de 2 ml añadiéndose 250 µl de solución tampón agitándose posteriormente el tubo para obtener una buena mezcla. Todo el volumen se pasó a una columna cromatográfica incorporada en el kit. Se centrifugó durante 1 minuto a 13000 rpm. Se descartó el volumen sobrante añadiéndose de nuevo 750 µ de tampón PE. Se centrifugó de nuevo a 13000 rpm durante 1 minuto descartándose el volumen sobrante. Este paso se repitió 1 vez. Se cambió el tubo colector de la columna por un eppendorf de 1.5ml añadiéndose posteriormente 30 µl de tampón EB sobre la columna. Se dejó durante 1 minuto a temperatura ambiente para que se produjera la elución y se centrifugó durante 1 minuto a 13000 rpm. El producto de PCR purificado fue recogido en el eppendorf de 1.5ml.

c) Adicción de la cola Poli-A a los productos de PCR

Se empleó un kit de poliadenilación (Invitrogen®). Para ello añadimos 4.5µl de tampón de reacción (10X Buffer, MgCl2, dATP, TaqPol) y 30µl de producto de PCR purificado en un tubo eppendorf de 1.5 ml. El tubo se mantuvo durante 10 minutos a 72ºC guardándose posteriormente a 4ºC hasta llevarse a cabo el protocolo de clonación.

d) Clonación

Para la clonación empleamos un kit comercial (TOPO TA Cloning® Kit for Sequencing. Invitrogen). Añadimos 4µl de producto de PCR sobre un eppendorf de 200µl, al cual se añadió 1 µl de la sal incluida en el kit y 1 µl del vector TOPOTA (10ng/µl). Se incubó durante 20-30 minutos a temperatura ambiente. Durante este proceso fueron descongeladas las células competentes almacenadas a -80ºC. 2 µl de la mezcla anterior se introdujeron en cada uno de los tubos de células competentes. Se incubó 30 minutos en hielo seguido de un choque térmico de 30 segundos a 42ºC. Tras este paso se añadieron 250µl de medio SOC al eppendorf y se incubó durante 1-2 minutos en hielo y seguidamente durante 1 hora a 37ºC en agitación constante. Posteriormente, 100µl de la solución anterior fueron sembrados en placas petri con medio de cultivo LB-Amp dejándose toda

la noche a 37ºC. El medio LB-Amp se elaboró con 5g de LB, 3g de Agar para 200ml de H2O, una vez autoclavado y no demasiado caliente se añadió 200 µl de Ampicilina 10µg/ml.

e) Amplificación de las secuencias clonadas

Mediante palillos estériles se transfirieron las colonias crecidas sobre placas multipocillo grandes a las cuales se les había añadido previamente 200µl de medio LB-Amp. Una vez inoculadas las placas con el total de colonias, se dejaron incubar toda la noche a 37ºC Una vez crecido se llevó a cabo otra PCR para amplificar los fragmentos de ADN clonados previamente con el vector TOPO TA ®. Para ello se emplearon los "primers" T3-T4 incorporados en el kit de clonación, específicos de dicho vector. Las condiciones de la PCR fueron un paso previo de desnaturalización de 5 minutos a 95ºC y posteriormente 30 ciclos de (30seg 95ºC, 1 minuto 60ºC, 2 minutos a 72ºC). La PCR se finalizó con un paso de elongación final de 10 minutos a 72ºC. Mediante una electroforesis en gel de agarosa 1% (p/v) se determinaron los pocillos con presencia de producto de PCR. Los productos de PCR con resultado positivo se transfirieron a una nueva placa multipocillo, sellándose con tapas de plástico. Posteriormente esta placa fue enviada a un servicio externo de secuenciación (MacroGen, Korea).

2.12.4. Estudio de la diversidad molecular bacteriana mediante técnicas de DGGE

Al igual que con las técnicas de clonación, las técnicas separativas de DGGE nos permiten estudiar la diversidad molecular bacteriana. A diferencia de los resultados que se obtienen mediante clonación, esta técnica permite obtener de forma rápida un perfil de la diversidad existente en la comunidad al cual llamaremos "screening", este puede ser empleado de forma comparativa entre diferentes ecosistemas como un indicador de la diversidad presente en forma de bandas de ADNr 16S o UTO´s (entendiéndose cada banda como una unidad taxonómica diferente).

Procedimiento

A partir de extractos de ADN, se llevó a cabo una amplificación mediante PCR, empleando los "primers" universales de bacterias para DGGE (341fGC-907r) como se describe en (Casamayor et al. 2000, 2002). A partir de los productos de PCR obtenidos se realizó una electroforesis DGGE con gradiente desnaturalizante 35% a 65% (100% Urea 7M y 4% formamida desionizada).

En cada uno de los pocillos de electroforesis se cargaron aproximadamente 500 ng de producto de PCR. Se mantuvo durante 15h a 120V y a temperatura constante de 60ºC, mediante el sistema de DGGE de Bio-Rad de manera que tuvieran tiempo suficiente para obtener una buena separación de las bandas de ADN. Tras este paso, los geles fueron teñidos durante 30 minutos con el agente cromóforo SybrGold, visualizándose posteriormente en un captador de imagen con UV. Las imágenes digitales fueron obtenidas con un sistema de documentación de geles UVldoc (UVltec Limited, Cambridge, UK) y guardadas en archivos digitales para su posterior procesado y análisis de imagen.

2.12.5. Tratamiento estadístico de datos

Cuando se comparan las bandas de DGGE de diferentes lagos, se puede llevar a cabo un análisis mediante la construcción de una matriz binaria de presencia ausencia de bandas. Mediante estos datos se realizó una matriz de disimilaridad basada en el coeficiente de Jaccard (Sj). A partir del método de UPGMA (unweighted pair group average linkage method) se elaboró un dendograma mostrando las relaciones entre los distintos sistemas acuáticos estudiados.

Empleando la matriz de presencia/ausencia, se elaboró un análisis de correspondencia para agrupar los lagos estudiados por su similitud en el patrón de bandas de la DGGE, agrupando los lagos con comunidades bacterianas más similares. Adicionalmente, empleando datos de variables físicas, químicas y biológicas, se

elaboró un análisis de componentes principales. Los resultados de ambos análisis fueron comparados, teniendo en cuenta la diversidad molecular bacteriana y las características físicas, químicas y biológicas, para encontrar la relación existente entre ambos. Estos análisis fueron llevados a cabo empleando el programa estadístico MVSP (Fourcans et al. 2004).

3. A close link between bacterial community composition and environmental heterogeneity in Maritime Antarctic lakes

Villaescusa JA, Casamayor EO, Rochera C, Velázquez D, Chicote A, Quesada A, Camacho A (2010) A close link between bacterial community composition and environmental heterogeneity in maritime Antarctic Lakes. International Microbiology 13:67-77

Received 14 February 2010 · Accepted 30 May 2010

3.1. Abstract

Seven maritime Antarctic lakes located on Byers Peninsula (Livingston Island, South Shetland Islands) were surveyed to determine the relationship between planktonic bacterial community composition and environmental features. Specifically, the extent to which factors other than low temperature determine the composition of bacterioplankton assemblages of maritime Antarctic lakes was evaluated. Both deep and shallow lakes in the central plateau of the Peninsula, as well as a coastal lake, were studied in order to fully account for the environmental heterogeneity of the Peninsula's lakes. The results showed that shallow coastal lakes display eutrophic conditions, mainly due to the influence of marine animals, whereas plateau lakes are generally deeper and most are oligotrophic, with very limited inputs of nutrients and organic matter. Meso-eutrophic shallow lakes are also present on the Peninsula; they contain microbial mats and a higher trophic status because of the biologically mediated active nutrient release from the sediments. Diversity studies of the lakes' planktonic bacterial communities using molecular techniques showed that bacterial diversity is lower in eutrophic than in oligotrophic lakes. The former also differed in community composition with respect to dominant taxa. Multivariate statistical analyses of environmental data yielded the same clustering of lakes as obtained based on the DGGE band pattern after DNA extraction and amplification of 16S rRNA gene fragments. Thus, even in extremely cold lakes, the bacterial community composition parallels other environmental factors, such as those related to trophic status. This correspondence is not only mediated by the influence of marine fauna but also by processes including sediment and ice melting dynamics. The bacterial community can therefore be considered to be equally representative as environmental abiotic variables in demonstrating the environmental heterogeneity among maritime Antarctic lakes.

Keywords: maritime Antarctic lakes, bacterioplankton, aquatic trophic status

3.2. Introduction

The maritime Antarctic region covers the western part of the Antarctic Peninsula and nearby islands. Its climate is less extreme than that of continental Antarctica, with an annual mean precipitation of 700–1000 mm per year (Bañón 2001) and an average temperature around 0ºC during the austral summer. These characteristics allow the appearance of many water bodies that are free of ice. Byers Peninsula, extending from the western end of Livingston Island (South Shetland Islands), is among the largest ice-free areas in the maritime Antarctic, and includes a large number of lakes (Toro et al. 2007). It has been declared an Antarctic Special Protected Area (ASPA No. 126: Byers Peninsula, Livingston Island, South Shetland Islands) due to the importance of its biological communities as well as its archaeological and geological features. Previous studies in this area (Camacho 2006a, Fernández-Valiente et al. 2007, Toro et al. 2007) as well as in other areas of the maritime Antarctica (Schiaffino et al. 2009) have revealed a correspondence between certain aspects of the biological communities and the limnological characteristics of the water bodies in which they live.

In Antarctic lakes, the harsh environmental conditions explain the dominance of microorganisms (Ellis-Evans et al. 1998). However, the microbial communities inhabiting the lakes' planktonic and benthic environments consist of a low number of taxa, including viruses, bacteria, archaea, heterotrophic protists, algae, and metazooplankton (Hansson et al. 1996, Izaguirre et al. 2003, Laybourn-Parry et al. 1995, 2001). In many polar lakes, the benthic environment of the lakes and their catchments also contain cyanobacterial mats, a type of microbial community with widespread occurrence on Earth (Aguilera et al. 2001, Berlanga et al. 2008). Similar mats may have an important trophic function in the maritime Antarctic lakes of Byers Peninsula due to their high productivity and nutrient input into the water column (Fernández-Valiente et al. 2001, Fritsen & Priscu 1998, Quesada & Vincent 1997, Toro et al. 2007, Vincent et al. 1993). Recent studies have used culture-independent molecular techniques to evaluate the main components of the microbial communities

of maritime Antarctic lakes (Pearce et al. 2003, 2007). The results of these analyses suggested that changes in bacterial diversity are related to variations in nutrient concentrations (Pearce 2005, Schiaffino et al. 2009) and supported the use of rRNA-gene based molecular techniques to describe the environmental heterogeneity of microbial communities present in these lakes.

Here, we report the most relevant environmental variables of several contrasting water bodies of Byers Peninsula, together with the results of genetic fingerprinting of the associated planktonic bacterial communities. This survey included selected lakes within the central plateau of the Peninsula. Additionally, a coastal lake influenced by nutrient input from marine fauna (elephant seals, marine birds), and thus with a higher trophic status, was included. Byers Peninsula has been selected as an Antarctic reference site for the study of coastal, terrestrial, and limnetic ecosystems (Quesada et al. 2009), as the maritime Antarctic is among the areas on Earth currently undergoing intense warming (Quayle et al. 2002). Increased knowledge of the Byers Peninsula's ecological features and biological communities is essential to model the possible effects of rising temperature occurring in the region (Steig et al. 2009) and may help to establish Byers Peninsula as a global climate change observatory.

3.3. Material and methods

3.3.1. Study area

The studied area was located on Byers Peninsula (Livingston Island, South Shetlands Islands, Antarctica), between 62º34´35´´–62º40´35´´ S and 60º54´14´´–61º13´07´´ W. Compulsory permits for working in this ASPA were obtained from the Spanish Polar Committee (CPE) in coordination with the Scientific Committee on Antarctic Research. Byers Peninsula is characterized by the presence of a large number of water bodies that undergo melting during the austral summer. The thaw is accompanied by the intense development of microbial communities, both planktonic and benthic (Ellis-Evans et al. 1998). In this study, seven such lakes were selected in order to carry

out a comparative study of their physical-chemical features and bacterioplankton assemblages. The selection was designed to cover the environmental heterogeneity of lakes within this area.

Lakes Limnopolar, Midge, Chester, Chica and Turbio are located on the central plateau of the Peninsula, with maximal depths ranging from 4 to 8.2 m. Lake Somero is also in the plateau but is very shallow (0.5 m maximum depth) and has an important coverage of microbial mats, mainly in shore areas. This lake shows a high abundance of fairy shrimps (*Branchinecta gaini*), which together with the wind-induced resuspension of sediments facilitates nutrient recycling and release to the water column. All of these lakes are only weakly influenced by the marine fauna. Finally, Lake Refugio is also shallow but is located at South Beaches, next to the sea, and, accordingly, receives strong inputs of nutrients by marine fauna. The lake's high mineral content is also due to its proximity to the sea. Additional details of the environmental features of the lakes and the studied area are provided in Toro et al. (2007).

3.3.2. Sampling techniques and sample handling

Measurements of selected variables (electrical conductivity, pH, and dissolved oxygen) were conducted in situ using an YSI 556 MPS multiprobe. Subsurface water samples (i.e., 0.3 m depth for the shallow lakes Somero and Refugio, and 1 m for the others), were collected in acid-washed plastic containers of 5 and 15 liters. Since all lakes were free of ice and were not stratified at the time of sampling, the samples can be considered as representative and comparable for the whole set of lakes. Samples were obtained from the central zone of the lakes in January February 2007. A fraction of the sample was quickly filtered at the lake shore through fiber-glass filters (Whatman GF/F). Both the filtered water in plastic bottles, used for nutrient analyses, and the filters for further analysis of photosynthetic pigments were stored at –20ºC. The remaining volume was brought to the laboratory at the camp site and processed within not more than 5 h as follows: For the bacterioplankton and virioplankton

counts, sub-samples were fixed with 2% glutaraldehyde and stored at –20ºC and 4ºC, respectively. Another fraction was separated in acid-washed plastic containers and stored at –20ºC for quantification of total nitrogen and total phosphorus. For the analyses of dissolved organic carbon (DOC), water was filtered through 0.2-µm cellulose nitrate filters and kept in acid-washed glass bottles after fixation with 0.2 ml of 1 N HCl. For DNA analyses, water was pre-filtered through a 30-µm Nytal mesh and a 3-µm polycarbonate filter (Whatman) and then filtered by means of a 0.2-µm polycarbonate filter (Whatman) and stored frozen in cryovials with 1.5 ml of lysis buffer, as described by Casamayor et al. 2000, 2001. All samples were shipped under the selected storage conditions to our laboratories in Spain, where the analyses were performed as described below.

3.3.3. Physical, chemical and biochemical analyses

Inorganic dissolved nutrients were quantified following standard methods APHA, AWWA and WEF 1992. Photosynthetic pigment analyses were carried out by HPLC as described by Fernández-Valiente et al. 2007 and Pickney et al. 1996. Chlorophyll concentration was used as a marker for the trophic status of the lakes (Jeffrey et al. 1998). Phaeophytin is a chlorophyll degradation product that is commonly found in the sediment. Fucoxanthin was used as a marker of diatom (Bacillariophyceae) abundance and can likewise be used as a marker for chrysophytes (Chrysophyceae), which are significant members of the phytoplankton in Antarctic lakes. Lutein and other carotenoids, such as violaxanthin and antheraxanthin, were used as markers of chlorophytes (Chlorophyceae) abundance, and zeaxanthin as a pigment marker for the relevant presence of cyanobacteria (Jeffrey et al. 1998).

Samples stored for DOC quantification were also used to quantify chromophoric dissolved organic matter (CDOM) by means of the excitation emission matrix (EEM) method (Stedmond 2005) using a F-7000 Hitachi fluorescence spectrophotometer. Two main types of CDOM were detected. One was of protein type, tyrosine-like and tryptophan-like, with EE maxima at 280–344 and 275–344 nm, respectively. The

other was of the humic type and could be further subdivided in two subtypes: humic acids of terrestrial origin (THA, EE maximum at 240–448 nm) and humic acids resulting from animal fertilization (FHA, EE maximum at 325–428 nm). Standards for protein-like CDOM were prepared with bovine serum albumin (BSA). The standard for humic-acid-type CDOM was prepared with quinine sulfate (QS).

3.3.4. Microbial enumeration

The virioplankton (virus-like particles, VLP) fraction was extracted by filtering 0.5 ml of sample water through 0.02-µm Anodisc filters (Whatman). The particles retained on the filters were then dyed with SYBR Green-I and quantified on a Zeiss III epifluorescence microscope as described by Noble & Furhrman 1998. Bacterioplankton counts were carried out by flow cytometry. One-ml aliquots of water samples previously fixed in 2% glutaraldehyde were incubated for 20 min at room temperature and then stained with SYBR Green-I. The abundance of autotrophic picoplankton (APP) was also measured by flow cytometry by exploiting the natural fluorescence of chlorophyll. Samples were analyzed on a Beckman Coulter flow cytometer (Cytomics FC 500 MPL) with five fluorescent channels, following the procedures described by Gasol & del Giorgio 2000. Side-angle light scatter (SSC) served as a proxy for bacterial cell size (Trousellier et al. 1999). Fluorescents beads of 0.5 and 1 µm were used as size markers. Clusters of bacteria were counted by logical gating from FL2 vs. SSC histograms. Total bacterioplankton abundance and the relative abundance of bacterioplankton groups I (low DNA) and II (high DNA) were obtained (Li et al. 1995). The active cell index (ACI) was calculated by dividing the number of HDNA bacterioplankton cells in each sample by the total abundance, and expressed as a percent (Jellet et al. 1996). The VLP per bacteria ratio (VBR) was calculated by dividing the number of virus-like particles by the number of bacteria in the sample. Molecular techniques to describe bacterioplankton diversity. DNAwas extracted by adding lysozyme, proteinase K, and SDS to filters containing the bacterial biomass that had been stored frozen with lysis buffer (40 mM EDTA; 50 mm Tris pH 8.3; 0.75 M sucrose), as described by Dumestre et al. (2002). DNA separation

and purification were carried out using phenol:chloroform:isoamyl-alcohol (25:24:1, v/v) and chloroform:isoamylalcohol (24:1, v/v) followed by isopropanol precipitation according to Agogué et al. 2005. PCR amplification of 16S rRNA gene fragments was carried out with the universal bacterial primers 341f GC with GC clamp (5´- CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC C CCT ACG GGA GGC AGC AG-3´) and 907r (5´-CCG TCAATT CCT TTG AGT TT-3´), following Casamayor et al, (2000,2002). The quality of the PCR products was analyzed by electrophoresis on a 2% (w/v) agarose gel. Denaturing gradient gel electrophoresis (DGGE) was run on a denaturing gradient of 40–70% (100% = urea 7 M and 40% deionized urea) at 120 V and 60ºC for 15 h in a DGGE D-Code system (Bio-Rad Laboratories, USA). Approximately 500 ng of PCR product were carried into the electrophoresis gel for each lane. The gels were dyed for 30 min with SybrGold. Digital images were obtained with the UVldoc gel documentation system (UVltec Limited, Cambridge, UK) and kept in digital archives. Prominent bands in the DGGE gel were excised and resuspended in 25 µl of Milli-Q (Millipore) ultrapure water and stored at 4ºC overnight, as described by Casamayor et al. 2001. A 2- to 5-µl aliquot of supernatant together with the original primer set was used for PCR re-amplification. The PCR product was purified and then sequenced at external sequencing facilities (www.macrogen.com), and the sequences submitted to a BLAST search (Altschul et al. 1997) for a preliminary identification of the closest relative in the database. The ARB program package (www.arb-home.de) was used for sequence alignment. Partial sequences were inserted into the optimized and validated tree available in ARB (derived from complete sequence data) by using the maximum-parsimony criterion and a special ARB parsimony tool that did not affect the initial tree topology. Chimeric sequences were checked by CHECK_CHIMERA from the Ribosomal Database Project [http:// rdp.cme.msu.edu] and by visual inspection of sequence alignments. Nucleotide sequences from the 18 main sequences were submitted to the EMBL database, where they were assigned the accession numbers FN398055 to FN398072.

3.3.5. Data analyses

DGGE bands identified in the fingerprinting of the seven lakes were classified in 19 different band types (from A to S). A binary data matrix was created, considering the presence or absence of the individual bands. A dissimilarity matrix based on the Jaccard coefficient (Sj) was then calculated, and a dendrogram built using the UPGMA method (unweighted pair group average linkage method). A correspondence analysis (CA) was carried out using the binary matrix to also group the lakes by bacterioplankton fingerprint similarity. Finally, principal components analysis (PCA) was carried out using data from selected physical, chemical, and biological variables other than bacterioplankton diversity. All of the statistical analyses were done using the MVSP program (Fourcans et al. 2004).

3.4. Results

3.4.1. Physical and chemical features

The selected lakes differed markedly in their chemistries, with respect to both salt and nutrient content (Table 3.1). The low mineralization of the plateau was evident by their low conductivity (40-70 μS/cm) whereas the marine influence on coastal Lake Refugio resulted in a higher conductivity (131 μS/cm). The pH values reflected the catchment geology as well as the differences in productivity among the lakes, with the highest values in the most productive lakes. The concentrations of nutrients, mainly nitrogen and phosphorus compounds, identified three levels of nutrient abundance: (i) low concentrations, measured in all deep lakes from the central plateau; (ii) higher N and P concentrations, but mostly in the particulate form, in the shallow Lake Somero, also located in the central plateau; (iii) very high N and P concentrations, both in the particulate and dissolved forms, in coastal Lake Refugio, reflecting the direct influence of marine fauna.

Table 3.2. Values of the lakes' environmental variables in samples corresponding to those from which bacterioplankton DNA samples were obtained. Data on catchment areas are as given by Toro et al. 2007

Variables	Limnopolar	Somero	Midge	Chester	Chica	Turbio	Refugio
Catchment size (km^2)	0.58	0.06	0.27	0.09	0.01	0.58	0.12
Maximum depth (m)	5.5	0.5	8.2	5	4	7.8	0.5
Conductivity ($\mu S\ cm^{-1}$ 25°C)	66	70	68	52	40	58	131
pH	7.2	7.2	6.9	6.9	7.0	6.6	7.7
NH4 (µM)	0.42	0.62	0.68	0.43	<0.1	<0.1	0.5
NOx (µM)	0.15	0.14	0.64	0.21	1.144	1.04	8.36
SRP (µM)	0.06	0.15	0.04	0.03	0.066	0.06	5.27
SRSi (µM)	71.84	64.87	10.23	46.52	21.95	59.55	62.88
Total Nitrogen (µM)	2.56	4.5	0.97	1.50	2.03	1.32	37.15
Total Phosphorus (µM)	0.73	0.98	0.23	0.22	0.52	2.86	23.10
Chl *a* (µg/l)	0.04	0.47	0.10	0.07	0.21	0.15	18.92
Fucoxanthin (µg/l)	0.001	0.101	0.013	0.002	0.001	0.007	ND
Lutein (µg/l)	0.001	0.033	0.009	0.002	0.011	0.008	2.122
Violaxanthin (µg/l)	0.001	ND	0.002	0.001	0.003	0.002	0.456
Antheraxanthin (µg/l)	ND*	0.025	ND	ND	0.003	ND	0.242
Zeaxanthin (µg/l)	ND	0.065	ND	ND	ND	ND	ND
Phaeophytin (µg/l)	ND	0.196	ND	ND	ND	ND	ND

*ND: Not detected

Fluorometric analyses of CDOM were carried out by means of EEM. The results of Figure 1A showed that in most of the plateau lakes protein-type CDOM values did not exceed 0.02 mg/l BSA, while even lower values were measured for the humic acid type. Nevertheless, in Lake Somero, the concentration of protein-like CDOM was almost triple those of the other studied lakes of the plateau, and that of humic acid THA-CDOM almost four times higher. Remarkably, Lake Refugio presented a more equilibrated abundance of the four CDOM subtypes and, among the lakes studied, was the sole lake in which FHA was detected, again due to the influence of marine animals along the sea shore.

3.4.2. Photosyntetic pigments characterization

Chlorophyll a concentrations (Table 3.1) indicated that the trophic rank of most of the lakes ranged from ultraoligotrophic to oligotrophic. The exception was eutrophic Lake Refugio. Relatively high levels of the chlorophyll-a degradation product phaeophytin-a (Jeffrey et al. 1997), which accumulates in sediments following the death of phototrophic organisms, were detected in Lake Somero, possibly due to sediment resuspension processes.

3.4.3. Bacterioplankton and virioplankton abundance

Total bacterioplankton abundances in the subsurface of the lakes (Figure 3.1B) was in the range of 1–12 × 106 cells/ml. Lake Refugio had by far the highest bacterial abundance, in agreement with its high trophic status and abundance of organic matter. The opposite was observed for APP, which was essentially absent from the lakes. Flow cytometry analysis divided bacterioplankton into two groups (Figure 3.1B). The first corresponded to the LDNA fraction, which had a low fluorescence (FL2) signal and was located in the lower part of the flow cytometer plot, and the second to the HDNA fraction, corresponding to a high fluorescence (FL2) signal and located in the upper part of the plot. The ratio HDNA vs. total bacterioplankton, expressed as the

ACI, was lower in the most oligotrophic lakes (Limnopolar, Midge, Chester, Chica, and Turbio) and higher for lakes with a higher trophic status (Somero and Refugio).

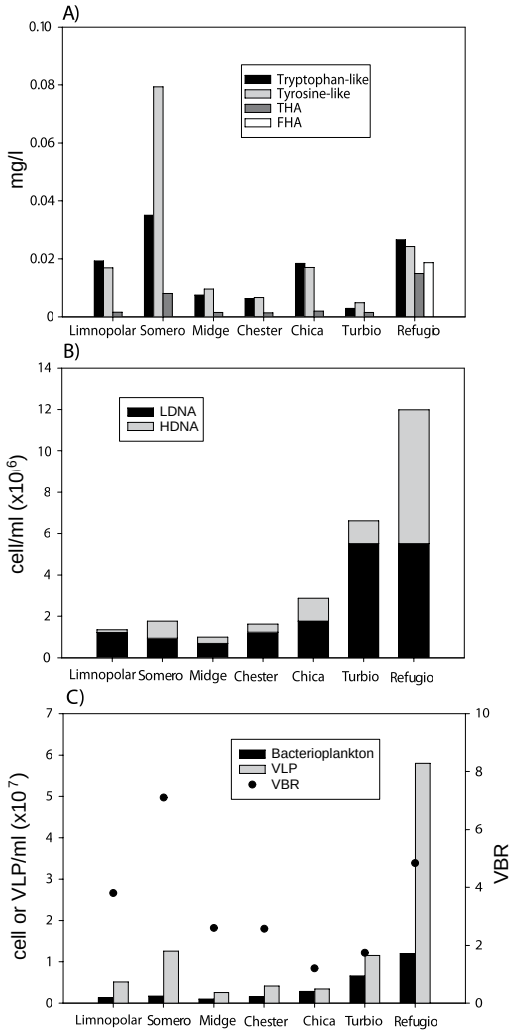


Figure 3.1. (A) Fluorescent dissolved organic matter concentration (CDOM) for protein-like CDOM (tryptophan-like and tyrosine-like), reported in mg/l bovine serum albumin, and humic-like CDOM (terrestrial origin "THA" and fertilization origin "FHA") in mg/l quinine sulfate. (B) Total bacterioplankton abundance (cell/ml × 106) was analyzed by flow cytometry, which resolved two groups based on low and high DNA content (LDNA and HDNA, respectively). (C) Bacterioplankton densities (cell/ml) and virioplankton (VLP) densities (VLP/ml), with VBR as the ratio between virioplankton and bacterioplankton.

The VLP counts (Figure 3.1C) ranged between 0.2 and 5.8 × 107 VLP/ml. Again, higher abundances were found in Lake Somero and, mainly, in Lake Refugio, with concentrations in the latter being one order of magnitude higher than those of the remaining lakes. These two lakes also had higher VLP/bacteria ratios (VBR), with the highest ratio in Lake Somero.

3.4.4. Molecular analysis of bacterioplankton community

Overall, up to 80 bands were observed in the DGGE fingerprints from 19 different positions (A–S, Figure 3.2). Sequencing of the prominent bands yielded 18 different sequences that were subsequently assigned to different operative taxonomic units (OTUs). The sequences belonged to the following band types: type A (18, 26, 34), type B (1, 15), type C (2, 19, 22, 27), type D (16), type J (28), type K (29), type L (23), type M (17, 30), type N (21, 36), and type Q (33), with the phylogenetic affiliations given in Figure 3. The sequences were ordered in two main differentiated groups, clustering within the α-subclass of Proteobacteria and within the phylum Bacteroidetes (Figure 3.3). Among those belonging to α-Proteobacteria, sequences clustering with members of the genus Sphingomonas dominated in the fingerprints of Lake Limnopolar and Lake Somero, which are physically connected by a short stream. Within the Bacteroidetes group, sequences closer to the genus Flavobacterium were present in most of the lakes, and those closer to Flectobacillus only in Lake Refugio.

Cluster analysis based on the Jaccard coefficient (Sj) (Figure 3.4) grouped together the five oligotrophic lakes from the central plateau (Limnopolar, Midge, Chester, Chica, and Turbio). Lake Somero, although also located in the plateau and connected to Lake Limnopolar by a short stream, did not cluster tightly with the plateau lakes. As expected based on its location, Lake Refugio was clearly separated from these lakes.

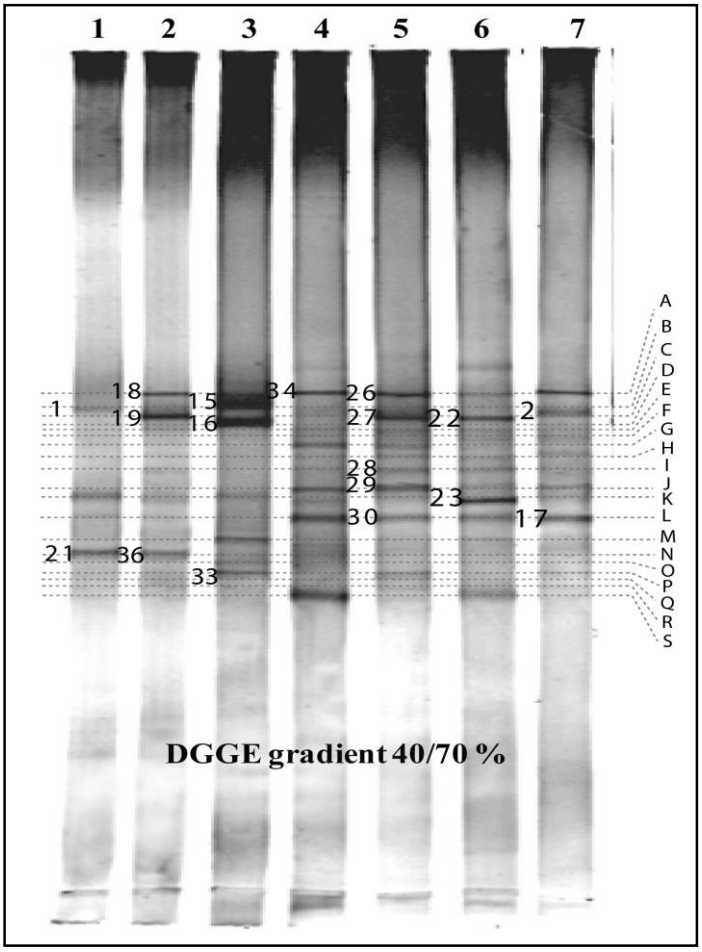


Figure 3.2. Fingerprinting from 16S rRNA gene fragments for samples of the seven lakes identified 19 different types (A-S) of DGGE bands, 18 of which were successfully sequenced. The lakes (number codes in the upper part of the figure) are: 1-Somero, 2- Limnopolar, 3-Refugio, 4-Turbio, 5-Midge, 6-Chica, and 7-Chester.

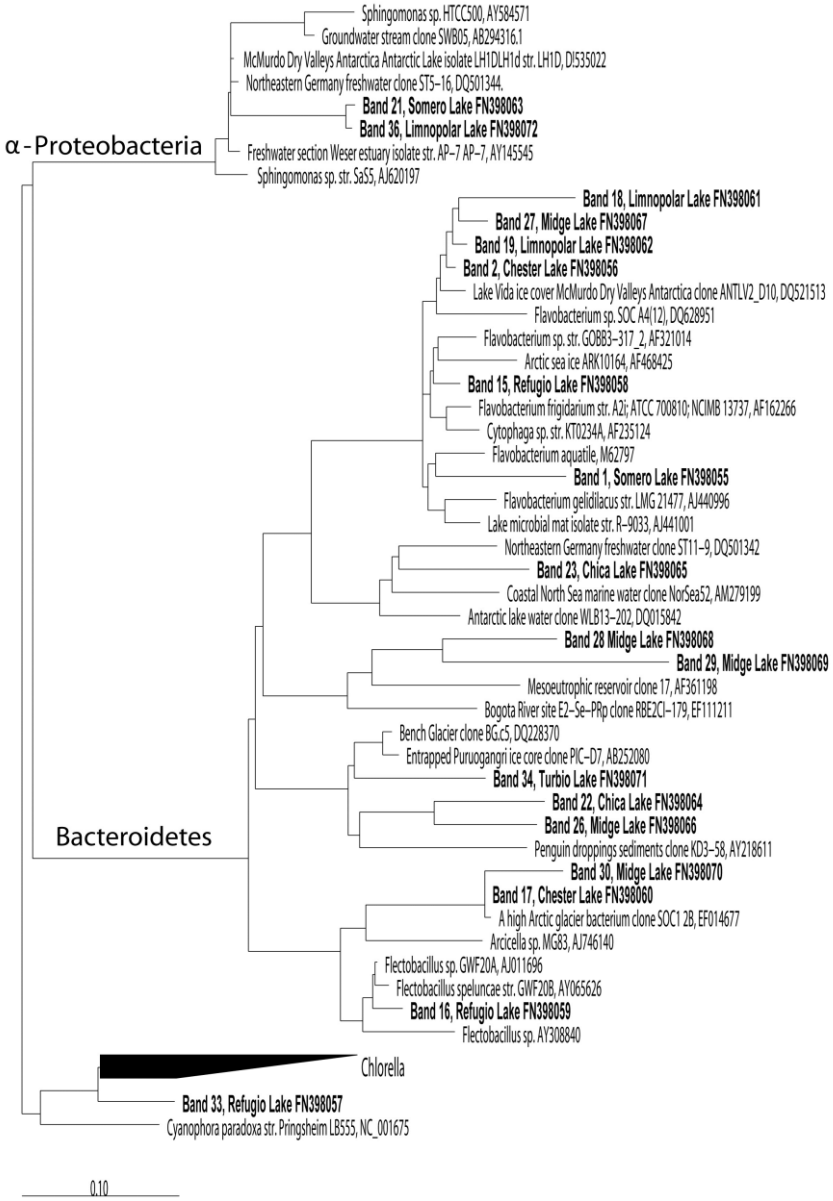


Figure 3.3. Phylogenetic tree with the affiliation of the dominant bacterioplankton 16S rRNA gene fragments sequences (in bold) found in the seven lakes. Band 33 corresponds to plastid DNA.

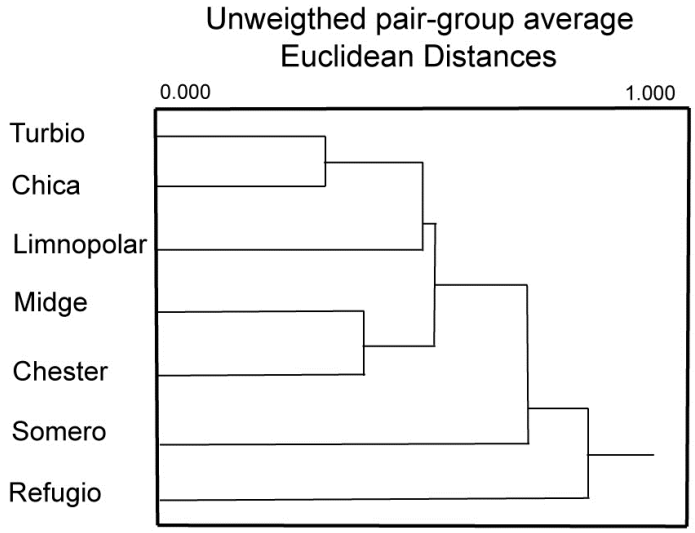


Figure 3.4. UPGMA dendrogram resulting from cluster analysis of the data reporting the presence/absence of DGGE bands in the seven studied lakes.

3.4.5. Comparative ordination analysis: principal component analysis vs. correspondence analysis

Physical, chemical, and biological data were used to conduct a PCA, the results of which (Figure 3.5) showed a separation of the lakes into three types, with the two main components explaining close to 90% of the variance. Deep lakes of the plateau (Limnopolar, Midge, Chester, Chica, and Turbio) grouped together and were distinct from Lake Somero because of its shallowness as well as the dominance of diatoms (high fucoxanthin to lutein ratio), high ACI, and high nutrient and organic matter content. Lake Refugio was strongly separated from the others, with the highest factor score for the principal component I, which explained 71.5% of the variance. This axis can be interpreted as representing a combination of the variables related to higher trophic status, i.e., soluble reactive phosphorus (SRP), DIN, and Chl-a concentration,

with a contribution, in this case, also by the conductivity because high trophic status is also related to the proximity of the sea, with its influence of marine animals.

The presence/absence binary matrix of the genetic fingerprinting results was used in a CA (Figure 3.6), which grouped lakes with the same band patterns. The two first axes were found to explain 75% of the variance, forming a homogeneous group that included the deep lakes from the plateau (Limnopolar, Midge, Chester, Chica, and Turbio), whereas Lake Somero appeared in the upper part of the graph and Lake Refugio highly separated from the others.

A similar distribution pattern of the lakes' features was obtained when the two ordination analyses were compared. Lakes Somero and Refugio were highly separated from each other whereas the remaining lakes (Limnopolar, Midge, Chester, Chica, and Turbio) clustered together. These results matched those obtained with the cluster analyses performed using the binary matrix data, thus suggesting a close relationship between the environmental features of the lakes and the composition of the bacterioplankton assemblages.

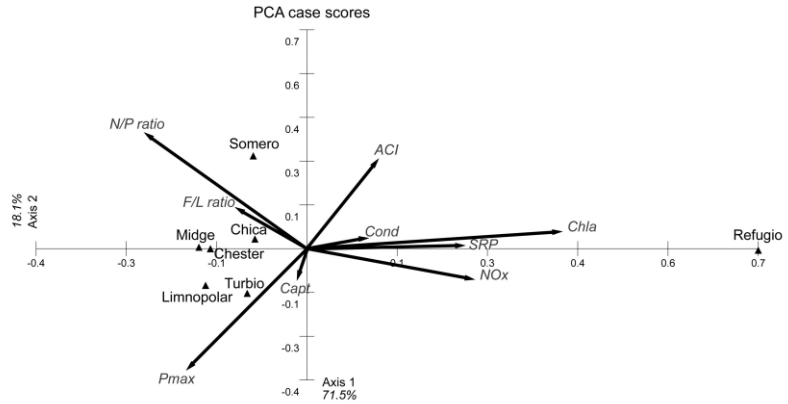


Figure 3.5. Biplot corresponding to the PCA for the seven lakes (black triangles) and the most significant environmental variables: Capt (catchment size), Pmax (maximum depth), Cond (electrical conductivity), Chla (chlorophyll concentration), SRP (soluble reactive phosphorus), NOx (oxidized inorganic nitrogen forms), N/P ratio (total nitrogen/total phosphorus ratio), ACI (active cell index), and F/L ratio (fucoxanthin/lutein ratio).

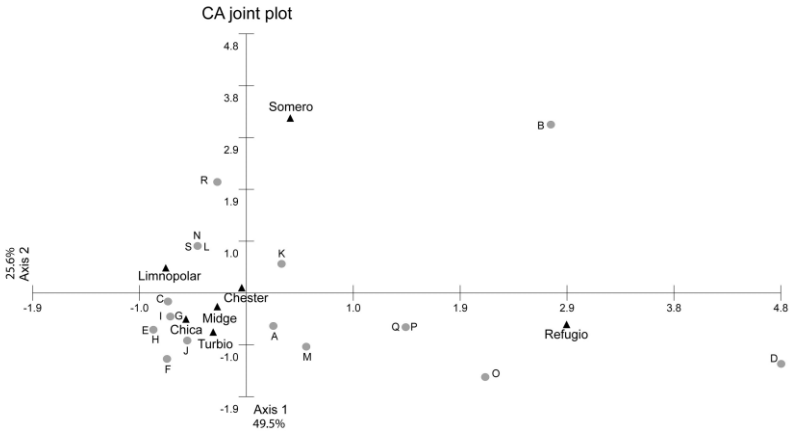


Figure 3.6. Biplot resulting from the CA using the presence/absence matrix of the 19 different types (A–S) of DGGE bands (gray circles) of the seven studied lakes (black triangles).

3.5. Discussion

Antarctic lakes are often perceived, like many other terrestrial Antarctic ecosystems, as very unproductive, in which physical limitations such as low temperature and low light (energy) availability restrict biological productivity (Camacho 2006a). Lakes from maritime Antarctica, however, are located in the less harsh area of the continent. Especially during the austral summer, conditions are less extreme, allowing a limited variety of life forms to flourish (Camacho 2006a). Under these circumstances, in which light availability is relatively high and temperatures are not too low, other factors, such as nutrient availability, play a major role in controlling the productivity of maritime Antarctic lakes.

The chemical and biological features of the lakes included in our study were indicative of a contrasting trophic status very much dependent on the influence of external inputs but also modulated by nutrient recycling within the lake basins, which, in turn, is somewhat linked to the lakes' morphology and ice dynamics. Sediment

removal, as occurs in shallow lakes such as Lake Somero or in the deeper Lake Turbio (in this case because of ice dynamics), can promote greater development of bacterioplankton since both organic and inorganic nutrients released from the sediments become partly available for heterotrophs, thus supporting a greater bacterioplankton abundance (Figure 3.1). The trophic pattern reflected by the nutrient concentrations was confirmed by other chemical and biological variables. For instance, concentrations of chlorophyll and carotenoids, which serve as markers of phytoplankton abundance (and, in shallow lakes, also of tychoplankton abundance), were very low in lakes from the plateau. This was not the case for Lake Somero, where pigment concentrations were higher, including that of the chlorophyll degradation product phaeophytin, which accumulates in the sediment. In the oligotrophic lakes from the plateau, the amounts of both protein-like and humic-type CDOM were also relatively low, which indicated that inputs of organic matter were less than in richer lakes (Stedmond 2005). In Lake Somero, however, because of its shallowness, both the removal of nutrients from the sediments and their recycling are favored by physical (e.g., wind resuspension) and biological (sediment turn over by fairy shrimps) processes, in contrast to the deep lakes of the plateau. This was also confirmed by the much higher abundance of autochthonous protein-like CDOM in the plateau lakes than in the other inland lakes.

At the other trophic extreme, the coastal shallow Lake Refugio was characterized by higher nutrients and chlorophyll a concentrations, but in contrast to Lake Somero the main reason for its high trophic status was its proximity to the coast, where seals and penguins are an important source of nutrients. This lake also had high concentrations of humic type CDOM and was the only one with significant amounts of FHA CDOM, clearly indicating animal inputs (Stedmond 2005). In this lake, phytoplankton composition switched to a dominance of chlorophytes, as indicated by the strong increase in the abundance of the specific carotenoids lutein and violaxanthin, which also evidenced the influence of nutrient status on protist community structure. Additionally, lake productivity was also reflected by other physical and chemical variables; for example, the pH of the plateau lakes varied

between 6.64, at Lake Turbio, to 7.21, at Lake Somero, and was 7.65 at coastal Lake Refugio. In low buffered waters of relatively similar mineralization, this indicates an increase in primary production with increasing pH. Variability in the trophic status of the lakes close to the sea was previously described for lakes located in maritime Antarctica. Commonly, lakes that are far from the sea have much lower nutrient concentrations than coastal lakes (Izaguirre et al. 2003), as the latter, unlike the more inland lakes, usually have high nutrient inputs from marine fauna, especially seals and penguins (Hansson 1992, Hansson et al. 1996, Izaguirrre et al. 2001, 2003, Mataloni et al. 1998). This is also the case for Byers Peninsula, although, as our results highlight, other processes (e.g., sediment suspension and nutrient recycling) govern the differences among lakes with low external nutrient inputs.

Trophic status was also observed to influence bacterioplankton abundances, with a much higher abundance found for the coastal Lake Refugio than for other eutrophic Antarctic lakes (Schiaffino et al. 2009). However, as discussed, the modulation by trophic status of bacterioplankton abundance may be influenced not only by external inputs, but also by sediment release, benthic production, and ice-cap dynamics. In addition to trophic status, a role for predation could also be claimed to explain the control of bacterioplankton abundance in plateau lakes, since trophic cascades involving metazooplankton and bacteriophagous protists, releasing grazing pressure on bacterioplankton, have been suggested to occur in oligotrophic polar lakes (Camacho 2006a). In addition to these top-down effects, viral impacts could influence bacterioplankton abundance and growth. Thus, among all the studied lakes, Lake Somero presented the highest VBR (Figure 3.1C). Virioplankton proliferation can result in a high mortality in bacterioplankton but, when nutrients are available, as typical of Lake Somero (compared to the other plateau lakes), the bacterioplankton community can enhance growth, thus compensating for the viral effect (Personnic et al. 2009). Although we did not directly measure bacterial growth or bacterial production, the ACI, defined as the percentage of HDNA bacterioplankton as a function of the total bacterioplankton population, can be used to some extent and together with other variables, e.g., CDOM-type concentrations, as an indicator of

bacterial activity and growth (Gasol & del Giorgio 2000, Jellett et al 1996). Among the plateau lakes, both the ACI and the amount of protein-like CDOM were highest in Lake Somero, indicating more active bacterial growth dynamics. Both parameters were also much higher in Lake Refugio, consistent with the common pattern of a high correlation of HDNA cell abundance with chlorophyll-a concentration and productivity, as reported by Li and co-workers (Li et al 1995).

The limitations of the DGGE method to describe microbial diversity are well known (Casamayor et al. 2000, Pedrós-Alió 2006). However, since this technique selectively amplifies the dominant taxa of bacterioplankton, band number serves as a good indication of the minimum number of dominant phylotypes present (Pearce 2005, Schiaffino et al. 2009). Although not necessarily conclusive, our DGGE results (Figure 3.2) showed a higher number of dominant bands, and consequently a high OTU richness in the deep oligotrophic lakes, with 11–16 different bands in the DGGE gel, compared to the lower richness in the shallow lakes Somero and Refugio (8 and 9 OTUs, respectively). As a general ecological pattern, eutrophic conditions can produce a strong competitive exclusion, reducing diversity. Pearce (2005) found a similar pattern of reduced bacterioplankton diversity with increasing trophic status in lakes from the nearby Signy Island, although other studies performed in lakes of the maritime Antarctic failed to find a clear pattern (Schiaffino et al. 2009).

Sequencing of the dominant bands in the DGGE fingerprints showed that most of them corresponded to uncultivated bacteria (Figure 3.3). The majority of the dominant OTUs detected in this study were affiliated with α-Proteobacteria and Bacteroidetes, both of which are dominant in other maritime Antarctica lakes, together with β-Proteobacteria and Actinobacteria (Pearce 2005, Pearce et al. 2007), although in a recent revision Pearce and Galand (Pearce y Galand 2008) stated that β-Proteobacteria might be the most abundant group in polar freshwater ecosystems. However, recent studies performed in other lakes of the maritime Antarctic, demonstrated the dominance of bands whose sequences clustered within the Bacteroidetes (Schiaffino et al. 2009), which is consistent with our results.

Most of the detected bands clustered with sequences coming from high-latitude or glacier habitats, typical of samples of freshwater Antarctic bacterioplankton (Schiaffino et al. 2009), with many of the taxa showing a bipolar distribution (Pearce et al. 2007). Cluster analysis defined three main groups of organisms. Within those grouped with α-Proteobacteria, sequences clustering with members of the genus Sphingomonas (bands 21 and 36) corresponded to some of the dominant bands in lakes Limnopolar and Somero. Despite their marked differences, these lakes are connected by a short stream, which would explain their shared dominant taxa. Sphingomonas has been found in diverse environments, including aquatic (both freshand seawater) and terrestrial habitats from Antarctica (Van Trappen et al. 2002) and temperate zones. The second group clustered within the Bacteroidetes, specifically, with sequences present in cold habitats, including sequences from uncultivated bacteria of the genus Flavobacterium. Species of this genus (e.g. *Flavobacterium antarticum*, *F. hibernum*), some of which are psychrophilic, are usually found in cold aquatic habitats with low salinity, typically in polar lakes (Van Trappen et al. 2002), although also in rivers, springs, or soils. The third group, also clustering within the Bacteroidetes, included a sequence with high similarity to three Flectobacillus sequences (band 16 from Lake Refugio). This genus is commonly found in Arctic and Antarctic aquatic cold environments (McGuire et al. 1987) but also in the lakes and springs of temperate zones. Within the third group, but of low similarity to the above-mentioned sequences of Flectobacillus, were bands 17 and 30, from lakes Chester and Midge, which are located quite close to one another but are not connected. These sequences clustered with a sequence retrieved from an Arctic glacier.

Cluster analysis (Figure 3.4) gave a preliminary indication of the similarity among the bacterial assemblages dominating the different types of lakes. The most homogeneous cluster was that associated with oligotrophic deep lakes from the plateau. These lakes shared many of the dominant bands and many environmental features. Among them, the tight clustering of sequences from lakes Midge and Chester, which are located quite close to one another, reflected the high similarity of

the dominant bacterioplankton taxa in these lakes. The same was true of lakes Chica and Turbio, which also share specific environmental features, such as the presence of ice dams that suddenly break down, which also occurs in Lake Limnopolar.

The existence of three differentiated types of lakes was shown by PCA of the environmental variables (Figure 3.5), confirming the results of the cluster analysis. The first group included the deep oligotrophic lakes from the plateau (Limnopolar, Chester, Midge, Chica, and Turbio). Within the first axis, which explained a high percentage of the variance (71.5%) and can be interpreted as related to trophic status, lakes with more active suspension of the sediments because of ice dynamics, such as Chica and Turbio, were located closer to another plateau lake, Somero, whose sediments dynamics are more active. However, Lake Somero was segregated by axis 2, in which several variables, such as lake depth, strongly scored, thus separating this lake from the other plateau lakes. Finally, Lake Refugio was extremely separated from all other lakes, with its appearance at the higher end of axis 1 indicating its much higher trophic status. The same pattern of similarity among lakes was obtained in a comparison of environmental and fingerprinting analyses, regardless of whether they were performed with data from environmental variables (PCA, Figure 3.5) or from determination of dominant bacterioplankton diversity, and either after classification (cluster techniques, Figure 3.4) or ordination (CA, Figure 3.6) methods were applied. Thus, the composition of the bacterioplankton assemblages matched very well with the environmental diversity among the studied lakes.

We have shown that the trophic status of the lakes of Byers Peninsula mediates both the abundance and the composition of bacterioplankton assemblages. Trophic status, in turn, is regulated not only by external inputs from marine fauna, as often reported (Izaguirre et al. 2003, Schiaffino et al. 2009), but also, as demonstrated here for the first time for maritime Antarctic lakes, by lake morphometry and sediment dynamics, with the latter influenced by physical and biological processes.

4. Bacterioplankton summer dynamics in a Maritime Antarctic lake

Villaescusa JA, Rochera C, Velázquez D, Rico E, Quesada A, Camacho A (2013) Bacterioplankton summer dynamics in a maritime Antarctic lake. Limnética 32(2):253-268

"Premio al mejor artículo publicado en Limnética por un joven investigador en el bienio 2012-2013, galardonado por la Asociación Ibérica de Limnología"

Received 14 February 2010 · Accepted 30 May 2010

4.1. Abstract

Maritime Antarctica shows less severe weather conditions than continental Antarctica, thereby allowing an important thaw process during the austral summer that increases both light availability for primary production and the inputs of nutrients and organic matter through run-off. Thus, a rapid development of certain phytoplankton groups is favored at the beginning of the austral summer as a direct effect of the higher availability of light and nutrients after the ice thaw. Inputs of allochthonous organic matter from microbial mats and moss carpets distributed across the catchment also enhance the abundance of heterotrophic bacterioplankton. Indeed, the bacterioplankton abundance and the availability of organic matter are correlated for each studied depth, revealing a possible differential use of the organic matter. Additionally, the availability and dynamics of different types of dissolved organic matter (autochthonous and allochthonous) may influence the diversity and abundance of the different bacterioplankton groups along the water column and throughout the summer, with a relative abundance of each group that differs between the surface and bottom waters. The study of DGGE patterns reveals the existence of changes in the abundance of different bacterial taxa during the summer and the different relative importance of each bacterial group at each depth. This result can also be related to the organic matter dynamics during the austral summer, as evidenced by the correlation between the concentration of chromophoric dissolved organic matter (CDOM) and abundance of bacterioplankton at each depth. Therefore, the regional temperature regime, which allows ice thaw of the lakes and their catchments during summer, plays a key role in the onset of biological processes and the length of the productive season throughout the austral summer.

Key words: Maritime Antarctic Lakes, Summer Dynamics, Bacterioplankton, CDOM, Temperature, Bacterial Diversity.

4.2. Introduction

Livingston Island (South Shetland Islands), which is located in the maritime Antarctic region, displays a less extreme climate than continental Antarctica (Vincent 2000, Bañón et al. 2001, Convey 2011), allowing many water bodies (lakes, ponds, pools and streams) to be free of ice during the austral summer (Rochera et al. 2010). Byers Peninsula, located on Livingston Island, is one of the largest ice-free areas of this maritime region of the continent (SCAR 2003) exhibiting a high number of aquatic ecosystems (Toro et al. 2007, Villaescusa et al. 2010). Previous studies in Byers (Fernández-Valiente et al. 2007, Toro et al. 2007) and in other areas of maritime Antarctica (Ellis-Evans 1996, Schiaffino et al. 2009) have revealed the existence of a strong relationship between these lakes and their landscape via run-off from the catchment. Additionally, these lakes are particularly sensitive to temperature changes, which are reflected in the timing of the ice-free period, thus affecting the length of the productive season (Rochera et al. 2010).

Pelagic production in permanently ice-covered lakes is mainly based on the internal carbon supply, as observed in the continental region of Antarctica (Azam et al. 1991). In these lakes, phytoplankton dynamics respond mainly to over-wintering strategies, such as mixotrophy or resting-stage forms (McKnight et al. 2000). However, maritime Antarctic lakes receive important allochthonous inputs of inorganic nutrients and organic matter that can support lake productivity (Camacho 2006a, Schiaffino et al. 2011), favouring the uncoupling between the internal primary production and secondary production (Thingstad 2000), particularly in catchments with microbial mats.

Additionally the existence of benthic mosses covering the lake bottom (Toro et al. 2007, Villaescusa et al. 2013b) can provide an alternative source of organic matter to the pelagic community. Both allochthonous and benthic inputs of organic matter may affect the heterotrophic bacterioplankton community structure in Antarctic lakes, demonstrating the relevance of the trophic status over the microbial community

structure in these lakes (Villaescusa et al. 2010). These allochthonous inputs can also represent an important parameter for explaining differences in the taxonomic bacterial composition throughout the water column. Changes in the bacterioplankton community structure during the holomixis period are commonly displayed by Antarctic lakes and are associated with an increase in the levels of nutrient input and in the timing and length of the lake ice cover (Pearce 2005). The present study aims to explore the relationship between different limnological factors of Antarctic lakes and the bacterioplankton dynamics during the austral summer in Lake Limnopolar as a model lake, highlighting the relevance of the different compartments that subsidize the organic supply to the water column.

4.3. Material and methods

4.3.1. Sampling site

Byers Peninsula is one of the largest ice-free areas of maritime Antarctica and is situated at the western end of Livingston Island (62°34´35´´- 62°40´35´´ S and 60°54´4´´-61°13´07´´ W), in the South Shetland Islands. The climate on Byers Peninsula is less extreme than in continental Antarctica, with mean summer temperatures of approximately 1-3 °C (Bañón 2001, Rochera et al. 2010). These summer temperatures above the melting point of water contribute to the presence of diverse open-water bodies with profusely settled planktonic and benthic microbial communities (Toro et al. 2007, Villaescusa et al. 2010).

Lake Limnopolar is a small (2.2 ha), relatively shallow (4.5 m maximum depth during the summer ice-free period) water body located in the central plateau of Byers Peninsula. The lake has a scarcely vegetated drainage watershed of 0.582 km^2 that is covered mainly by microbial mats and moss carpets. Most of the catchment run-off flows into the lake through a small stream. An important feature is the occurrence of benthic mosses (*Drepanocladus longifolius*) covering the lake bottom. The lake food

web presents a low complexity, dominated mainly by components of the microbial loop (Camacho et al. 2006a, Toro et al. 2007, Rochera et al. 2010).

4.3.2. In situ physical and chemical parameters

Meteorological data from 2006/07 and 2008/09 Antarctic campaigns were recorded by an automatic weather station equipped with a Campbell CR10X logging unit. Fieldwork was conducted at Lake Limnopolar during two sampling periods in the austral summer of 2006/07, from November to February, and 2008/09, from December to February. The sampling site was located over the point of maximum depth. The vertical profiles of temperature, conductivity, pH and dissolved oxygen were obtained using a 6920 YSI multi-parametric probe. The photosynthetically active radiation (PAR) profiles were measured as µmol photons $m^{-2}s^{-1}$ at 0.5 m depth intervals using a 2π sensor (Li-192SA), with uniform response in the range of 400-700 nm (PAR), attached to a Li-COR datalogger (Li-1000).

4.3.3. Nutrient and biological measurements

Water samples from three different depths (0.5, 2 and 4 m) at every sampling event of 2006/07 and 2008/09 were collected using a hydrographic bottle (Uwitech) and 5-litre acid-washed plastic carboys, in parallel to the in situ physical-chemical survey at the deepest point of the lake. A fraction of each sample was rapidly filtered through glass-fiber filters (Whatman GF/F). Both the filtered water collected in plastic bottles, which was used for nutrient analyses, and the filters used for the further analysis of photo-synthetic pigments, were stored at -20 ◦C. A portion of the remaining volume was processed for bacterioplankton counts, fixed with 2 % glutaraldehyde and stored at -20 ◦C. Another fraction of unfiltered water was separated in acid-washed plastic containers and also stored at -20 ◦C for the quantification of the total nitrogen and total phosphorus. The remaining water was immediately filtered through 0.2 µm cellulose nitrate filters, placed in acid-washed glass bottles and fixed with 0.2 ml of 1N HCl to remove the dissolved inorganic carbon; the samples were then stored for the analyses

of the chromophoric dissolved organic matter (CDOM, stored at 4 ˚C) and TOC (stored frozen). Both the dissolved and particulate forms of nitrogen and phosphorus were quantified following Standard Methods (APHA 1992). CDOM was determined using the excitation-emission matrix (EEM) method (Coble 1996, Stedmond & Markager 2005) with an F-7000 Hitachi fluorimeter. Two forms of CDOM were described: autochthonous organic matter as protein-like CDOM (excitation-emission of 280-344 nm), which was quantified using bovine serum albumin (BSA) standards, and allochthonous organic matter as terrestrial humic acid ("THA") CDOM (excitation-emission of 250-412 nm and 250-448 nm), which was quantified using quinine-sulphate (SQ) standards (Villaescusa et al. 2010). The THA concentration primarily reflects the allochthonous input of organic matter from the catchment (i.e., microbial mats and mosses) and from the briophytic in-lake community.

Photosynthetic pigment analyses were performed by HPLC, as described by Pickney et al. (1996) and Fernández-Valiente et al. (2007). The concentrations of these photosynthetic pigments were used to define the structure of the phytoplankton community in the lake. The chlorophyll-a concentration was used as a marker for the trophic status of the lake (Jeffrey et al. 1997). Fucoxanthin was used as a marker of the diatoms (Bacillariophyceae) abundance and can similarly be used as a marker for chrysophytes (Chrysophyceae), which are significant members of the phytoplankton in Antarctic lakes. Lutein was used as a marker of the prasinophytes (Prasinophyceae) abundance (Jeffrey et al. 1997).

Bacterioplankton counts were performed using flow cytometry. Aliquots of 1ml water samples, previously fixed in 2 % glutaraldehyde, were incubated for 20 min. at room temperature and then stained with SYBR Green-I. The samples were analysed using a Beckman Coulter flow cytometer (Cytomics FC 500 MPL) with five fluorescent channels, following the procedures described by Gasol & del Giorgio (2000).

4.3.4. Fluorescence in situ hybridisation (FISH) and DGGE analyses

Changes in the diversity of the main bacterial groups present in the studied Antarctic lake were monitored during the summer of 2008/09 at every sampled depth (0.5, 2 and 4 m) using FISH analysis. Samples fixed with 2 % formaldehyde were previously filtered through 0.2 µm white polycarbonate filters (Whatman); the FISH hybridisation process followed Cottrel & Kirchman (2003). Although this method has directly been used in the characterization of Antarctic lakes (Pearce 2003), different limitations are implied and should be considered, such as those mentioned in the Discussion section. DAPI was used to count the total bacteria for comparison with the FISH analysis. The probes used for the monitoring were focused on the different groups previously described as dominant in Antarctic lakes (Pearce 2003) and particularly in Lake Limnopolar (Villaescusa et al. 2010). The probes used were as follows: a general probe for Bacteria (Eub338, 51-GCTGCCTCCCGTAGGAGT-31), alpha-Proteobacteria (Alf1b, 51-CGTTCGYTCTGAGCCAG-31), beta-Proteobacteria (Bet 42a, 51-GCCTTCCCACTTCGTTT-31), gamma-Proteobacteria (Gam42a, 51-GCCTTCCCACATCGTTT-31) and Bacteroidetes (CF319a, 51-TGGTCCGTGTCTCAGTAC-31).

Table 4.3 Meteorological conditions in Byers Peninsula during the summers of 2006/07 and 2008/09. Max and Min refer to the average of the maximum and minimum daily values. The 2001/04 data show the average values for the summer period of three years between 2001-2004 (Rochera et al. 2010).

Summer	Air temp. (ºC)	Soil temp. (ºC)	Water temp. (ºC)	Radiation (Kj m^{-2})	Wind speed (km h^{-1})	Max wind speed (km h^{-1})	Ice free period (days)
2006/2007							95
Mean	1.2	2.1	4.2	14350	24	51	
Max	3.2	6.0	9.7	29985	46	95	
Min	-0.3	-0.3	-0.2	3735	10	25	
2008/2009							115
Mean	1.4	2.1	4.3	14520	25	34	
Max	5.3	8.5	12.9	27750	56	86	
Min	-2.0	-2.1	1.2	4615	1	4	
2001/2004							69
Mean	0.9	1.3	3.1	12474	23	51	
Max	5.2	8.4	12.4	28565	48	111	
Min	-5.0	-4.4	-0.9	1491	8	19	

To describe the changes in the bacterial structure during the summer and the differences within the vertical profile, a DGGE (Denaturing Gradient Gel Electrophoresis) profile was performed for the three last sampling dates of the summer of 2008/09. The methods used for DNA extraction and amplification and the DGGE fingerprinting of microbial community are described in Villaescusa et al. (2010). A matrix with the per cent contribution of each band to the total of that lane was built for the different DGGE lanes; the percent contribution of each band was calculated using an image analysis program based on the light intensity profile for each lane (Díez et al. 2001). A dendrogram based on this matrix was developed using an average linkage method (UPGMA, unweighted pair group method with arithmetic mean).To describe the changes in the bacterial structure during the summer and the differences within the vertical profile, a DGGE (Denaturing Gradient Gel Electrophoresis) profile was performed for the three last sampling dates of the summer of 2008/09. The methods used for DNA extraction and amplification and the DGGE fingerprinting of microbial community are described in Villaescusa et al. (2010). A matrix with the per cent contribution of each band to the total of that lane was built for the different DGGE lanes; the percent contribution of each band was calculated using an image analysis program based on the light intensity profile for each lane (Díez et al. 2001). A dendrogram based on this matrix was developed using an average linkage method (UPGMA, unweighted pair group method with arithmetic mean).

4.3.5. Statistical analyses

The results for the physical-chemical and biological variables for the two years sampled (06/07 and 08/09) were compared using a Principal Component Analysis (PCA) including the data for the three depths sampled (0.5, 2 and 4 m) and for all the sampling dates. Another PCA analysis using the per cent of each group measured in the FISH analysis was also performed to study the distribution of the different groups along the water column.

4.4. Results

The meteorological data from Byers Peninsula during the studied period are shown in Table 4.1. The summer of 2008/09 showed a slightly higher average air temperature, approximately 0.2 ˚C warmer than 2006/07, and the average maximum and minimum temperatures in 2008/09 showed higher and lower values, respectively. These characteristics reflect a more extreme 2008/09 summer in comparison to 2006/07. The temperature during these two years was warmer compared to the summer periods of 2001 through 2004 (Table 4.1), and this increase in temperature was also observed in the higher duration of the ice-free period compared to the 2001-2004 summer periods. During 2006/07, the ice melted at the beginning of December, and the lake remained free of ice for a period of 95 days; during 2008/09, the ice cap melted completely during November, and the lake remained ice free for a period of 115 days. At the end of December (already the ice-free period) in both years, approximately 5-20 % of the superficial incident light reached the lake bottom, and the total irradiance reaching the lake bottom significantly increased to 30 % by the end of January in both years. The water temperature and conductivity profiles in both summers were characterized by relatively uniform values along the water column (data not shown).

The dissolved inorganic nitrogen (DIN) concentrations ($NOx + NH_4$) were near the detection limits throughout the sampling periods of both years, usually below 2 µM (Figure 4.1A and 1B). During the ice-free period of 2006/07, the highest concentrations of DIN were measured at the beginning of February, mainly dominated by oxidized forms (NO^-), and in the 2006/07 summer season soluble reactive phosphorus (SRP) concentrations varied from undetectable levels (< 0.03 µM) to a maximum of 0.149 µM by mid-January. Conversely, during the summer 2008/09 SRP concentrations were lower, with maximum values approximately 0.08 µM at the end of January. Concerning the possible existence of a vertical gradient of nutrient concentrations, an increase in the SRP concentration was detected in the 2 m samples of mid-January in both years. However, no significant differences in depth throughout the summer were detected for the remaining analyzed samples. The TN/TP molar

ratios were lower (between 6 and 48) during 2006/07 compared to the summer of 2008/09, a time when the values were above 60 in mid-January.

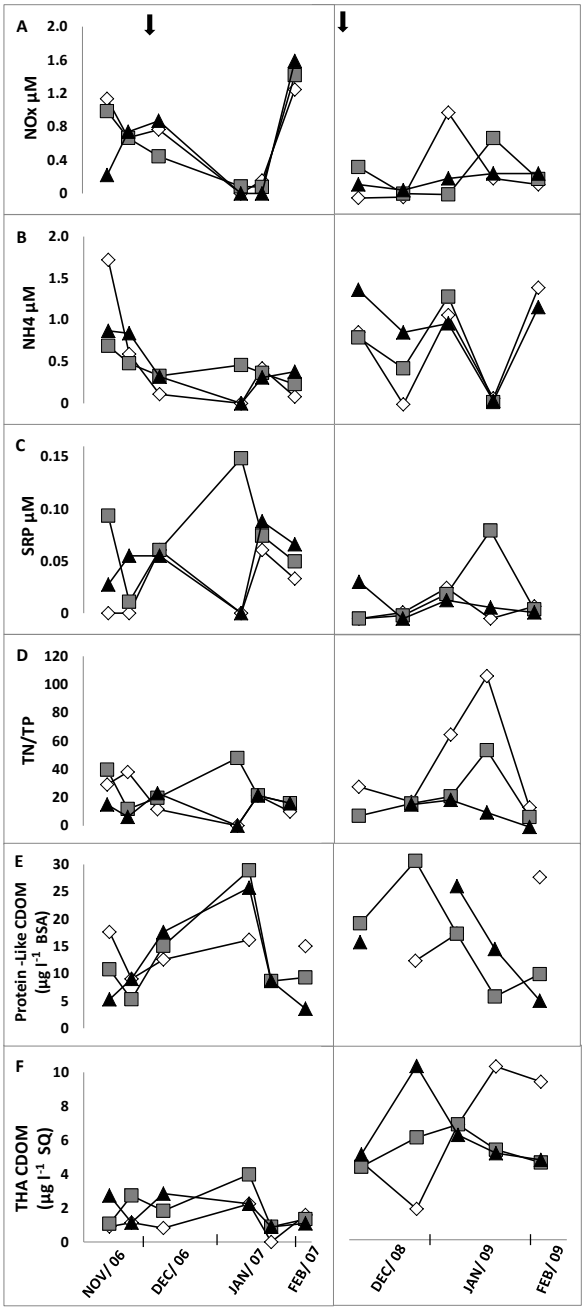


Figure 4.1. Dynamics of the main chemical parameters at three different depths (diamond = 0.5 m; square = 2 m; triangle = 4 m) in Lake Limnopolar during the summer periods of 2006/07 and 2008/09. A (NOx), B (NH_4), C (SRP), D (Total N/Total P), E (protein-like CDOM) and F (THA CDOM). The arrows indicate the date of ice melt.

The fluorometric CDOM analyses showed that the protein-type concentrations did not exceed 30 µg l^{-1} BSA for both years (Figure 4.1E). The highest concentrations appeared after the ice melt, specifically in the deeper samples (2 and 4 m), most likely due to an increase in the lake primary production, as shown by an increase in phytoplankton abundance. The protein-type CDOM concentration was homogeneous throughout the water column during the remainder of the sampling period. The terrestrial humic CDOM "THA" (Figure 4.1F) concentrations were different in both years. The THA concentration was stable along the summer of 2006/07 and did not exceed values of 4 µg l^{-1} SQ. However, the THA concentration pattern during 2008/09 showed higher values, with a maximum of 10 µg l^{-1} SQ. For this year, the THA measurements during December were higher at the bottom of the lake, whereas the THA peak was measured in the upper layers at the end of January.

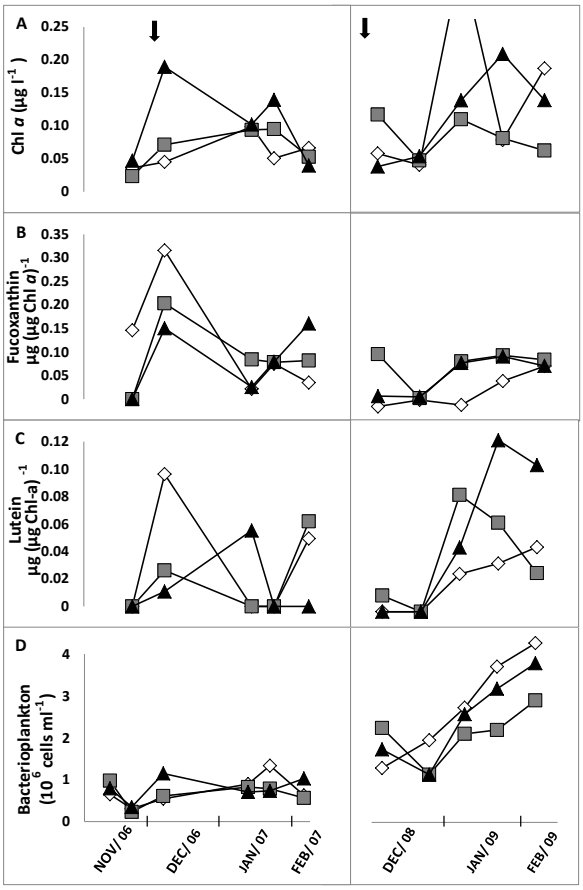


Figure 4.2. Dynamics of the photosynthetic pigments concentrations and abundance of heterotrophic bacterioplankton at three different depths (diamond = 0.5 m; square = 2 m; triangle = 4 m) in Lake Limnopolar during the summer periods of 2006/07 and 2008/09. A (Chl-a), B (fucoxanthin/Chl-a ratio), C (lutein/Chl-a ratio), D (bacterioplankton abundance). The arrows indicate the date of ice melt.

The phytoplankton biomass (Figure 4.2A), measured as the concentration of chlorophyll-a (Chl-a), was very low in both of the studied seasons, with an average summer concentration of 0.07 µg l^{-1} in 2006/07 and 0.1 µg l^{-1} in 2008/09. During 2006/07, the maximum values of Chl-a concentration were observed in the bottom layers after the ice cap thaw at the beginning of January.

However, this early summer increase in Chl-a concentration was not detected in 2008/09, most likely due to an anticipated ice cap thaw during this year. Conversely, an increase in the Chl-a concentration occurred in the surface layers during mid-January (2008/09 summer) and at the lake bottom at the end of January. The concentrations of the main taxa-specific carotenoids are shown in Figure 4.2B and 4.2C. The fucoxanthin concentration, which was used as a marker for diatoms and chrysophytes, showed different patterns in both summers. During 2006/07, the ratio between fucoxanthin/Chl-a showed an increase immediately following the ice melt; however, it suffered a slight decrease during the rest of the summer, reaching concentrations of 0.03 µg fucoxanthin (µg Chl-a)$^{-1}$. In contrast, this increase after the ice cap thaw was not observed during 2008/09, and the fucoxanthin concentration (as a ratio to Chl-a) was stable throughout the summer, with average values of 0.1 µg fucoxanthin (µg Chl-a)$^{-1}$. The lutein concentration as a ratio to Chl-a, which was used as a marker of prasinophytes, was relatively stable during the summer of 2006/07, with average values of lutein/Chl-a of 0.04 µg lutein (µg Chl-a)$^{-1}$. However, after the ice melt, an increase in the surface concentration was registered, with maximum values of 0.10 µg lutein (µg Chl-a)$^{-1}$. During 2008/09 this ratio increased by mid-January mainly in the bottom samples, reaching values of 0.12 µg lutein (µg Chl-a)$^{-1}$.

The bacterioplankton abundance showed significant differences between the two years studied (Figure 4.2D). During 2006/07, the bacterial abundance showed constant average values of approx. $1x10^6$ cell ml^{-1} during the summer. However, during the sampling period of 2008/09, the bacterial abundance showed a progressive increase from $1x10^6$ cell ml^{-1} in December to $4x10^6$ cell ml^{-1} at the end of January and beginning of February. Nonetheless, the bacterial abundance throughout the water

column was comparatively homogeneous within the vertical profile for both years, though the distribution of the dominant groups was not.

The results for the 2008/09 FISH analyses showed hybridisation levels for EUB338 between 64 % at 0.5 and 2 m and 53 % at 4 m when compared to the bacterial counts according to DAPI staining. The identification and quantification of the main groups of bacteria were possible using the available set of probes. The percentage of each group at the different depths is shown in Figure 4.3A. Remarkably, the addition occasionally amounted to even more than 100 % of the DAPI-stained cells due to a slight lack of specificity of the probes that would led to double counting by FISH. The abundance distribution among the different groups showed some differences among the groups studied: beta-Proteobacteria and Bacteroidetes were the most abundant in the lake, whereas gamma- Proteobacteria was least abundant at all depths; alpha-Proteobacteria showed a higher abundance only for the 0.5 m samples. The results of the heterogeneous vertical distribution were also supported by a PCA analysis (Figure 4.3B) that separated the four groups (alpha, beta, gamma and Bacteroidetes) by a "depth" axis (82 % of variance). In this analysis, alpha-Proteobacteria was grouped with the surface samples, beta and gamma-Proteobacteria were grouped with the deeper samples, and Bacteroidetes (C-F) appeared in a middle position due to its higher abundance in the mid-depth lake samples.

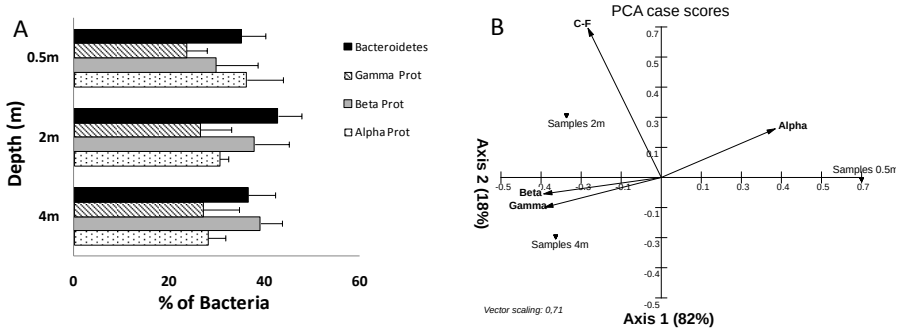


Figure 4.3. A) Relative dominance of the main bacterial groups found at different depths of Lake Limnopolar during the austral summer 2008/09 using FISH counts. B) Biplot corresponding to the Principal Components Analysis of the 2008/09 FISH samples for the different depths sampled. The values shown correspond to the average value of the samples at each depth.

The DGGE profiles for the samples of the 2008/09 Antarctic campaign are shown in Figure 4.4. Although the pattern of band diversity for each depth in Lake Limnopolar was relatively similar during the summer, the DGGE profiles showed differences in band intensity. The band intensity matrix analysed by a Euclidean distance dendrogram, grouped similar band patterns for each depth and date analysed. For the first sampling date (January 10th, 2009) the grouping of the band profiles of the three depths described a homogeneous distribution. The other two sampling dates (January 21st, 2009, and February 2nd, 2009) showed a different group classification. For the second sampling date (January 21st, 2009), the bottom sample (4 m) appeared clearly separated from the other depths (0.5 and 2 m), reflecting a change in the band pattern of the lake bottom sample; for the last date (February 2nd, 2009), the samples of the middle and bottom depths (2 and 4 m) appeared clearly grouped, showing a different pattern compared to that of the surface sample.

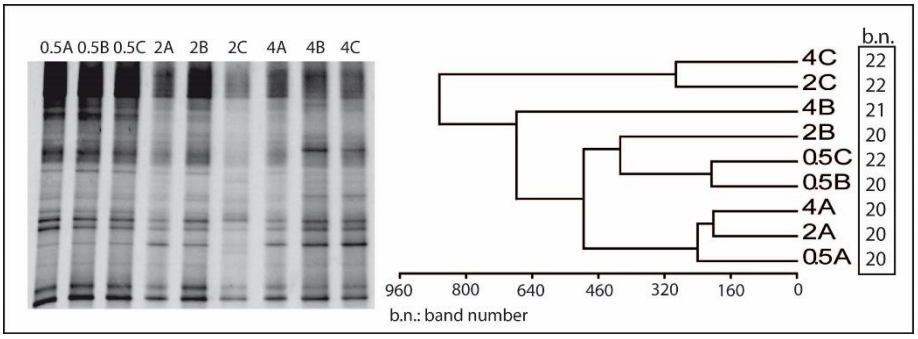


Figure 4.4. Dendrogram of the Euclidean distances between different DGGE profiles obtained at Lake Limnopolar at three different depths (0.5, 2 and 4 m) during the 2008/09 summer. The letters indicate the three different sampling dates: A (January 10th, 2009), B (January 21st, 2009) and C (February 3rd, 2009). b.n. = number of bands.

The principal component analysis (PCA) for the environmental data of the two sampled years (Figure 4.5) showed significant differences among the years. Axis 1 (27 %) separated both years with a clear biological-biochemical component (bacterial abundance and CDOM concentration), though a correlation analysis between the bacterial abundance and CDOM concentration did not display significant differences, with a 0.073 correlation factor ($\alpha = 0.05$, $p = 0.314$). However, correlation analyses comparing the average values for the five sampling events and separately for each

depth showed significant differences among them when the depth was considered for grouping, with a correlation factor of 0.85 ($\alpha = 0.05$, $p = 0.042$). Although the PCA analysis with environmental variables (Figure 4.5) did not show clear differences between the different depth samples, the PCA analysis for the 2008/09 FISH results (Figure 4.3B) showed differences in the bacterial composition with depth: a higher abundance of alpha-Proteobacteria in the surface samples, and Bacteroidetes (C-F in Figure 4.3B) and beta-Proteobacteria most abundant in the middle and deep layers, respectively.

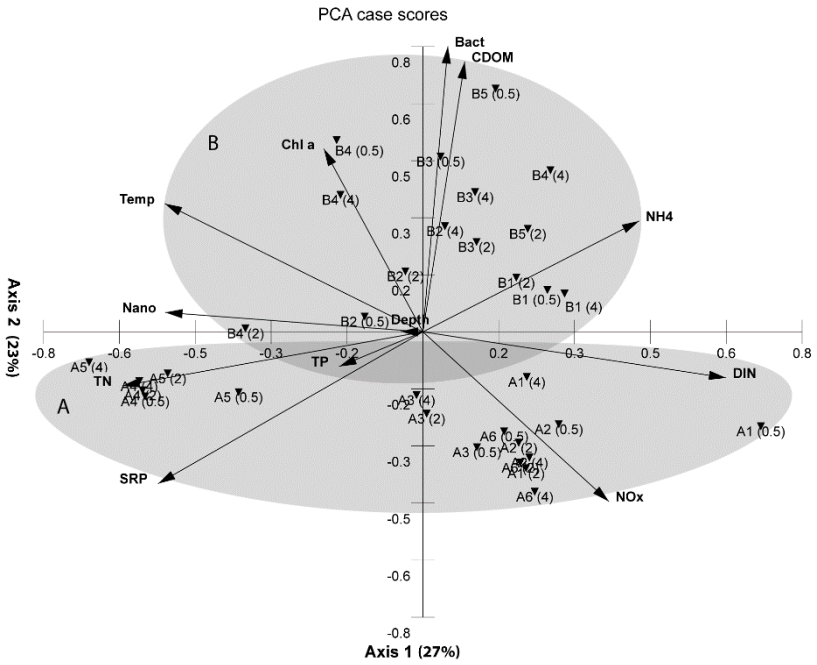


Figure 4.5. Biplot corresponding to the Principal Components Analysis for two years (A = 06/07; B = 08/09). Lake Limnopolar samples enumerated by sampling order by date (1-6) and depth (0.5, 2 and 4 m). The variables shown are as follows: Depth (maximum depth), Temp (temperature), Chl a (chlorophyll concentration), Bact (bacterial abundance), Nano (nanoplankton abundance), CDOM (chromophoric dissolved organic matter), NH_4 (ammonium), NOx (oxidised inorganic nitrogen forms), DIN (dissolved inorganic nitrogen), SRP (soluble reactive phosphorus), TN (total nitrogen), TP (total phosphorus). Note that first component is plotted on the vertical axis.

4.5. Discussion

The results from this work together with data from previous studies (Rochera et al. 2010), are an example of how changes in major physical factors related to climate may drive the functioning of the studied lake. The meteorological data show that the 2006/07 and 2008/09 summers were slightly warmer than the previously monitored years of 2001-2004 (Rochera et al. 2010) and that the mean solar radiation was also higher. These results reflect a high variability in the meteorological conditions in this area for different years. It has been demonstrated that variation in meteorological conditions largely influences the length of a lake ice-free period (Rochera et al. 2010) and may cause important changes in the inputs of inorganic nutrients and organic matter from the catchment, thereby directly affecting the microbial structure of the lake (Pearce 2005). The 2008/09 period (Table 4.1) showed a warmer and extreme summer, with a higher average maximum temperature and lower minimum temperature, reflecting the highest range of variation for this season. Higher temperatures could provoke an advance in the ice melt and favour an increase in nutrient and organic matter inputs into the lake, thus enhancing microbial activity and altering the productive period of the phytoplankton and aquatic mosses within the lake, as well as moss carpets and microbial mats that cover wide areas of the lake catchment (Fernández-Valiente et al. 2007). Because the length of the productive period in maritime Antarctic lakes during the austral summer is controlled by the date of the ice melt in the lake, very low productive sea- sons, such as the 2003/04 summer (Rochera et al. 2010), or long productive periods, such as the 2008/09 summer, can occur.

The low nutrients, Chl-a and carotenoid concentrations reveal that Lake Limnopolar exhibits ultra-oligotrophic characteristics. Such nutrient scarcity has been reported for Byers Peninsula lakes located in the central plateau (Toro et al. 2007, Villaescusa et al. 2010) and for other inland maritime Antarctic lakes (Drago 1989, Izaguirre et al. 2003). The carotenoid ratios showed the existence of a change in the relative importance of the different algal groups during the summer. The increase in

the fucoxanthin/Chl-a ratio immediately following the ice melt during 2006/07 reveals an increase in the abundance of chrysophytes and diatoms in the lake as a response to the increase in light and nutrient availability, in addition to an input of benthic diatoms from the catchment. This increase was not recorded in the 2008/09 summer due to an anticipated melting of the ice cap. Additionally, the strong increase in the lutein/Chl-a ratio in the surface samples after the ice melting 2006/07 reflects the response of the prasinophytes to increased light and nutrient availability. This massive increase in algal abundance after the ice melt has also been reported in studies of the viral diversity in the lake, which showed a high diversity of phytophagous viruses after the ice melt (López-Bueno et al. 2009).

The marked differences in the bacterioplankton abundance during the two years surveyed could be related to the availability of organic carbon and the length of the ice-free period. Despite the inexistence of a statistically significant correlation between the bacterioplankton abundance and CDOM when all the samples were considered together, a statistically significant correlation appeared when the average values for each depth during the summer of 2008/09 were compared by considering the depths separately. This could demonstrate a different response of the bacterial community to the allochthonous and/or moss-produced CDOM (THA) at each depth. This evidence can be supported by the study of the bacterioplankton diversity in Lake Limnopolar using clone libraries (Villaescusa et al. 2013a), demonstrating the existence of a biological gradient in the bacterial community diversity, which may be responding differentially at each depth. Additionally, the evident differences between the 2006/07 and 2008/09 summers, as shown in the PCA analysis (Figure 4.5), are linked to a higher abundance of organic matter and bacterioplankton. In addition to these factors, the selective effect of temperature and zooplankton predation could also exert a parallel control over the bacterioplankton community (Camacho 2006a). The allochthonous THA CDOM concentration during the 2006/07 summer was stable, as was the bacterioplankton abundance (Figures. 4.1 and 4.2), reflecting a comparatively stable season. However, the abundance of allochthonous THA CDOM along the water column was not homogeneous during the 2008/09 summer, and the higher CDOM concentration found in the lake bottom after the ice melt might be the result of a higher

photosynthetic activity by benthic mosses (Toro et al. 2007, Li et al. 2009) promoted by the increase in light availability. However, the evident increase in THA CDOM in the surface samples during the last part of the summer could be related to the activity of the catchment vegetation (Coble 1996, Stedmond & Markager 2005, Velázquez et al. 2013), which, in our case, was composed mainly by microbial mats and moss carpets (Fernández-Valiente et al. 2007). Thus, bacterioplankton production in Lake Limnopolar could be enhanced by both benthic mosses and the terrestrial communities of primary producers within the catchment, albeit only partly because THA CDOM is relatively refractory to bacterial degradation. The evident differences in the abundance of autochthonous and allochthonous organic matter along the water column could influence the existence of a bacterial diversity gradient in the lake (Villaescusa et al. 2013a) and, perhaps, the differential use of organic matter.

The FISH analyses of the 2008/09 summer samples showed a community largely dominated by Bacteroidetes and beta-Proteobacteria at all depths but mainly in the middle and deeper parts of the lake, respectively, and a dominance of alpha-Proteobacteria in the upper part of the lake. Despite the known limitations of the FISH method related to low specificity and signal, the existence of differences in our samples is statistically demonstrated by the results of the PCA analysis (Figure 4.3B) that correctly describes the bacterial group distribution. Our results agree with other studies using FISH probes, indicating that the beta subdivision of Proteobacteria appears to be globally distributed in both non-polar (Wagner et al. 1993) and polar freshwater environments, regions where these taxa are particularly abundant (Pearce 2003, Pearce et al. 2007).

The Euclidean distance dendrogram performed using the DGGE band intensity profile for the 2008/09 samples showed that the main bacterial taxa distribution of approx. 20-22 bands remained stable throughout the summer; however, the relative abundance of the different taxa varied along both time and depth (Figure 4.4). These results demonstrate the existence of a change in the relative dominance of the different bacterial groups in the deep waters during the last part of the summer. These dynamics in bacterioplankton group abundance could be related to the observed changes in the

allochthonous THA CDOM concentrations (Figure 4.1F) along the water column. Therefore, the differential availability of organic matter could be influencing alterations in the abundance of the main bacterial groups (alpha, beta, gamma Proteobacteria and Bacteroidetes).

The changes in the bacterial group abundance along summer and depth, together with the known bacterial diversity differences between the upper and lower parts of the lake (Villaescusa et al. 2013a), represent relevant differences in biological composition. Vertical differences in the dominance of the bacterial groups occur even though a physical stratification did not occur, as shown by the PCA analysis (Figure 4.5), thus suggesting a major role of the benthic compartment (i.e., benthic mosses) and allowing the existence of a certain "biological stratification" in the system. Therefore, the organic matter produced by lacustrine benthic mosses and microbial mats within the catchment is one of the most significant factors determining the structure of the bacterioplankton community in Lake Limnopolar, as also suggested for other Antarctic lakes (Pearce 2005).

The variation in the duration of the productive season due to an early or late ice thaw affects the inputs of nutrients and organic matter into the lake by run-off processes. Consequently, these dynamics produce important changes in the composition and abundance of the bacterial community. Additionally, the allochthonous input of organic matter favours the appearance of an uncoupling in the biological process in the lake relative to the available organic carbon supplied by planktonic primary production, thereby increasing the heterotrophic bacterioplankton abundance and promoting changes in its composition during summer and along the water column. The significant warming of this area (Quayle et al. 2002) provided our study with a great relevance to understand the changes that are occurring in maritime Antarctica lacustrine ecosystems.

5. Uncoupling of planktonic primary production and bacterial production in an oligotrophic Antarctic lake

Villaescusa JA, Rochera C, Velázquez D, Rico E, Quesada A, Camacho A. Uncoupling of planktonic primary production and bacterial production in an oligotrophic Antarctic Lake. (In preparation to be submitted to Limnology and Oceanography)

5.1. Abstract

Lakes located in the maritime Antarctic region are subjected to less severe climatic conditions compared with the ice covered lakes situated in Continental Antarctica. During the summer, maritime Antarctic lakes lose their ice surface layer due to important melting processes caused by the increasing temperature and solar radiation. This ice melt causes the appearance of run-off processes from the catchment that provide the lakes with nutrients and organic matter. Due to this, primary production rates suffer a clear increase, reaching high values more closely related to those observed for higher latitude lakes like Patagonian lakes. This primary production should be sufficient to support the bacterioplankton community, however, the organic matter contributions derived from benthic mosses activity and from allochthonous catchment microbial mats exert an additional carbon source for the bacterioplankton community. In this situation where the external contributions exceed the internal primary production, an uncoupling between planktonic primary production and bacterioplankton production appears.

Additionally, the importance of temperature increase over the planktonic communities was evaluated using a thermostatic bath experiment assaying five different temperature scenarios. In this way, the strong response of maritime Antarctic lakes to temperature changes has been evaluated, making them as an exceptional tool to study and monitor the climatic change in the Antarctic Peninsula and surrounding islands.

Keywords: Phytoplankton, Bacterioplankton, Primary and Bacterial production, Antarctic lakes

5.2. Introduction

Phytoplankton primary production in lakes is mainly controlled by environmental features like incident irradiance and temperature. However, some other stochastic factors such as nutrient availability, mixing depth and non-algal light attenuation could exert an important role in some lakes. The importance of these variables has been studied in lakes around the world, taking into consideration that the incident irradiance and temperature represent the most important factors affecting the primary production (Lewiss 2011). Nonetheless, the latitude represents an important feature that affects the previously mentioned variables. In this way, lakes situated in polar areas suffer from a permanent or non-permanent ice cover that represent an important issue to take into account due to its impact to the primary production. In particular, lakes situated in the Maritime Antarctic region display a non-permanent ice cover that thaw during the austral summer, this feature along with the major availability of incident irradiance and the increase in temperature allow a great development of the phytoplankton. The run-off processes associated with the ice melting in this area produce important contributions of nutrients and organic matter for the lakes. Thus, these allochthonous contributions represent another important factor controlling the planktonic production rates, especially for the bacterioplankton community.

Heterotrophic bacterioplankton is an important consumer of dissolved organic matter (DOM) in aquatic systems (Cammack et al. 2004) and can also be an important source of different kinds of DOM (Guillemete & del Giorgio 2012). Hence, in most of the epicontinental water systems, dissolved organic matter (DOM) from the primary production becomes an important source to support heterotrophic bacterioplankton production (Kritzberg et al. 2004). According to this, some works have described the coupling between phytoplankton and bacterioplankton production in temperate lakes e.g. Fouilland & Mostajir (2010). However, in high-latitude lakes, and especially in those situated in polar areas, this coupling between the primary and secondary production is not so obvious. In this study, the evidence of the uncoupling mentioned above has been observed in the Antarctic Lake Limnopolar, situated in the

maritime Antarctica. Lakes situated in this area receive important contributions of dissolved organic carbon, mainly produced by microbial mats and mosses situated in their catchment, during the beginning of the summer due to the run-off caused by the ice melting (Fernández-Valiente et al. 2007, Toro et al. 2007, Villaescusa et al. 2010). In this situation, where allochthonous inputs of organic matter from microbial mats are higher, the dependence of heterotrophic bacterioplankton on the phytoplankton autochthonous organic matter will be lower allowing the appearance of an uncoupling in the system. Moreover, the existence of a benthic mosses community in these systems will involve an additional source of nutrients and organic matter to support the bacterioplankton production (Moorhead et al. 2005, Fernández-Valiente et al. 2007, Fouilland & Mostajir 2010). Therefore, allochthonous inputs of organic matter from the catchment and the benthic mosses community contributions could be driven the planktonic bacterial community dynamics in Lake Limnopolar during the austral summer. In this way, the system displays an uncoupling between phytoplankton and bacterioplankton production (Thingstad 2000).

As previously mentioned, temperature represents an important variable for the planktonic production in lakes. Thus, the strong local warming reported in the area of maritime Antarctica (Meredith & King 2005) represents an important issue that directly affects the planktonic and benthic communities in this area. Due to this warming, the study of the community response against different temperature scenarios represents an important tool to understand how these communities respond against temperature. Therefore, our study includes a temperature manipulation experiment to evaluate the response of the different biological compartments in the lake and how they are sensitive to temperature changes.

5.3. Material and methods

5.3.1. Study area

The studied lake is located on Byers Peninsula (Livingston Island, South Shetlands, Antarctica), between 62º34´35´´- 62º40´35´´ S and 60º54´14´´- 61º13´07´´W. Byers Peninsula is characterized to display many heterogeneous water bodies that lose their ice cover during summer by melting processes, favoring an intense development of planktonic and benthic microbial communities (Toro et al. 2007). The study showed here was conducted in Lake Limonpolar, a relatively deep lake located in the central plateau of the peninsula, from mid-December of 2008 to February of 2009 covering the warmest season in which the lake is ice-free. Some of the limnological and biological characteristics of the lake, are detailed in Tables 5.1 and 5.2.

5.3.2. Sampling and in-situ production assays

Sampling was conducted in five days spread over December, January and February during the austral summer of 2008/09. Physical parameters such as temperature, pH and conductivity were measured in-situ using a 6920 YSI multi-parametric probe.

Photosynthetically active radiation (PAR) was measured at 0.5 m depth during the incubations (between 12:00-16:00) using a 2π sensor (Li-192SA) attached to a Li-COR datalogger (Li-1000). Every sampling date, a volume of water from three different depths (0.5, 2 and 3.5 m) was taken using a hydrographic bottle (Uwitech) and stored in 5 liter acid-washed plastic carboys for the quantification of the different physical and biological variables, see Tables 5.1 and 5.2. The results for the different physical and biological variables were integrated in a global value using the data obtained from the 3 sampled depths. The water samples obtained were filtered through glass-fiber filters (Whatman GF/F) and stored both (filtered water samples and glass-fiber filters) at -20ºC for the subsequent analysis of dissolved nutrients and photosynthetic pigments quantification. Some of the non-filtered water was fixed with

2% glutaraldehyde and stored at -20ºC for the bacterioplankton counts. Another fraction of the non-filtered water was filtered through 0.2 µm cellulose nitrate filters and fixed with 0.2 ml of 1N HCl; these samples were stored in acid-washed glass bottles and stored at 4ºC for the analyses of chromophoric dissolved organic matter (CDOM) and dissolved organic carbon (DOC). The filters were stored at -20ºC for the quantification of particulate organic matter (POC).

Dissolved and total fractions of nitrogen and phosphorus were quantified following Standard methods (APHA 1992). CDOM composition and concentration was determined by means of the excitation-emission matrix (EEM) method (Stedmond 2005) using an F-7000 Hitachi fluorimeter. Two forms of CDOM were described, the first was characterized as autochthonous organic matter derived from lake internal processes named as protein-like CDOM (excitation-emission 280-344nm) and the second was allochthonous organic matter from the lake catchment named as humic acid CDOM (excitation-emission 250-412nm and 250-448nm) (Villaescusa et al. 2010). Dissolved organic carbon (DOC) was analyzed using a TOC (TOC-V CSN Shimadzu) analyzer. Particulate organic carbon (POC) was analyzed using an Elemental analyzer (LECO CHNS-932).

Bacterioplankton counts were carried out by flow cytometry using a Beckman Coulter flow cytometer (Cytomics FC 500 MPL) with five fluorescent channels, following the procedures described by Gasol & del Giorgio (2000).

Photosynthetic pigments were analyzed using an HPLC as described by Picazo et al. (2013). Chlorophyll-a concentration was used as a marker for the trophic status of the lake and the different measured carotenoids were used as marker of the different algae groups on the lake. Fucoxanthin was used as a marker of diatoms (*Bacillariophyceae*) and Chrysophytes (*Chrysophyceae*) abundance. Lutein and violaxanthin were used as marker of Clorophytes (*Clorophyceae*) and Prasinophytes (*Prasinophyceae*) abundance. Antheraxanthin was also used as marker of

Prasinophytes (*Prasinophyceae*) abundance. Zeaxanthin and Myxoxantophyll concentration were used as marker of cyanobacteria abundance.

Table 5.1. Biological parameters measured at Lake Limnopolar during austral summer 08-09. During this year the ice cover thaw occurred during the first week of December.

Date	PAR* ($\mu E\ m^{-2}\ s^{-1}$)	T[a] (ºC)	pH	Cond ($\mu S\ cm^{-2}$)	SRP (μM)	DIN (μM)	DOC ($mg\ l^{-1}$)	POC ($mg\ l^{-1}$)	Humic CDOM ($\mu g\ l^{-1}$ SQ)	Protein CDOM ($\mu g\ l^{-1}$ BSA)	Chl a ($\mu g\ l^{-1}$)	BACT (10^6 cell ml^{-1})
Lake Limnopolar												
13-12-08	477	1.22	6.8	53	<0.03	1.15 ± 0.31	1.23	0.16	4.37 ± 0.34	17.06 ± 2.28	0.07	1.63
29-12-08	260	4.56	6.7	52	<0.03	0.50 ± 0.62	1.15	0.05	5.68 ± 3.97	20.85 ± 12.19	0.05	1.43
10-01-09	241	5.76	7.3	59	<0.03	1.45 ± 0.40	0.96	0.26	6.21 ± 0.34	19.62 ± 4.75	0.19	2.29
21-01-09	277	5.52	7.5	67	<0.03	0.45 ± 0.21	1.63	0.38	6.48 ± 2.72	10.15 ± 5.81	0.12	2.81
03-02-09	319	5.13	7.7	81	<0.03	1.44 ± 0.08	0.83	0.20	5.83 ± 2.55	13.97 ± 11.22	0.13	3.39

*Average surface radiation during incubation (12:00-16:00)

Table 5.2. Production rates of the different microbial groups at Lake Limnopolar and their catchment during austral summer 08-09. (PPr: plankton primary production, PPm: benthic mosses primary production, BP: bacterial production, PPmats: microbial mats primary production). During this year the ice cover thaw occurred during the first week of December.

Date	PPr ($gC.m^{-2}day^{-1}$)	PPmosses ($gC.m^{-2}day^{-1}$)	BP ($gC.m^{-2}day^{-1}$)	PPmats ($gC.m^{-2}day^{-1}$)
Lake Limnopolar				
13-12-08	0.026	0.067	N.A.D.	0.576
29-12-08	0.035	0.125	N.A.D.	0.720
10-01-09	0.090	0.209	0.050	0.770
21-01-09	0.017	0.022	0.115	0.860
03-02-09	0.007	0.043	0.089	0.880

N.A.D.: no available data (samples lost)

5.3.3. Primary and secondary production

When we refer to methods for quantifying the primary and secondary production in Antarctic and sub-Antarctic lakes, ^{14}C-labeled molecules incorporation methods have been traditionally used (Priscu et al. 1998, Laybourn-Parry et al. 2004, 2006). However, these methods have some drawbacks in their use and management in protected areas, especially in those where the human activity is restricted. Byers Peninsula, where our work takes place, is an Antarctic specially protected area (ASPA) where the use of radioactive material may be harmful to the researchers and the environment due to logistical difficulties related to working in these areas. Due to these drawbacks, non-radioactive methods based on ^{13}C stable isotopes can be employed as a reliable alternative to the commonly used radioactive methods (Pimenov et al. 2008). Despite of displaying a lower sensibility, these non-radioactive methods are recommended as an alternative due to their absolute environmental safety (Pimenov et al. 2008).

During the previously described sampling events, some primary and bacterial production assays were done for the same three different depths. Collected water samples for each depth were placed in 500 ml incubation bottles Nunclon® in duplicate and were incubated for 2-3 hours at its corresponding depth. A similar treatment was done for the benthic mosses and for the microbial mats. In the first case, weighted pieces of mosses were introduced inside incubation bottles for a period of 2-3 hours at the lake bottom. In the case of the microbial mats, small fractions with known weight and surface were introduced in transparent incubation bags Whirl-pack® and incubated for a period of 2-3 hours on the surface. For each of the production assays previously mentioned, non-radioactive isotopes of ^{13}C (Pimenov et al. 2008) were employed. For the quantification of planktonic, benthic and mosses production, 1.5ml of a 1g l^{-1} $NaH^{13}CO_3$ solution was used. For the bacterial production 250 µl of 13.3 g l^{-1} ^{13}C-Leucine solution was used. After the incubation, the samples were immediately filtered in glass fiber filters GF/F and stored at -20ºC. Also, a

portion of the assayed benthic mosses and microbial mats were stored at -20ºC for the subsequent analysis.

The samples were analyzed in a mass spectrometer (ICP-MS NexION 300XX Perkin-Elmer) and the results were expressed as percentage of each isotope. Primary production rates for the plankton, benthic mosses and microbial mats in the catchment were calculated using the equations described in Fernández-Valiente et al. (2007) and represented per unit area and as daily total values. Daily total production values were calculated by means of an interpolation equation using the three depth production values assayed. The bathymetric profile of Lake Limnopolar was taken into account for the calculation of the total daily production values. Additionally, specific planktonic primary production rate was calculated and referred per chlorophyll-a concentration, mol of photons (Einstein) and time to display the effect of the incident light over the planktonic community. Bacterial production was quantified by a variation of the method mentioned in Kirchman et al. (1985). In this case the radioactive molecule of ^{14}C was replaced by the stable molecule of ^{13}C-Leucine. In the calculation, it was assumed that the natural concentration of leucine in the lake water was negligible. The following equation was used:

$$V_c = \frac{\%^{13}C_m - \%^{13}C_b}{\left(\left(100 \cdot \frac{^{13}C_a}{^{13}C_a + LEU}\right) - \%^{13}C_b\right) \cdot T}$$

Vc: Leucine specific incorporation rate (h^{-1})

% ^{13}Cm: ^{13}C percent in the sample after treatment

% ^{13}Cb: ^{13}C percent in the sample before treatment

^{13}Ca: ^{13}C-Leu concentration added

LEU: ^{13}C-Lue concentration in the lake

T: incubation time (h)

Once the specific leucine incorporation rate was calculated, the carbon incorporation rate for the bacterioplankton was estimated. For this purpose, we used the factor of 1.5 kg of carbon incorporated x mol of leucine incorporated (Simon & Azam 1989). Bacterial production rates were represented per unit area and as daily total values. Also, the specific bacterioplankton production rates were represented per cell.

5.3.4. Manipulation experiments

An experimental primary and bacterial production "ex-situ" assay was done using thermostatic baths. The objective of this experiment was the study of the planktonic and benthic community response, reflected in the production rates, to different temperature scenarios.

To simulate these different temperature scenarios, that could reflect the warming affecting the area of maritime Antarctica, five thermostatic baths with different temperatures (0, 4, 8, 12 and 16ºC) were employed. In each of the thermostatic baths different samples of previously collected water from the lake surface (0.5m sample) and samples of benthic mosses were introduced in small "whirl-pack" bags. The different samples were incubated in the period of maximum irradiance for 3-4 hours. The methods used for the assay and storage of the primary and bacterial production were the same described in the in-situ production measurements.

5.4. Results

During the austral summer of 2008-2009, Lake Limnopolar showed a strong melting process occurring in the beginning of December. Thus, during the end of that month the major part of the ice cover had disappeared and then about 5-20% of the incident light had reached the lake bottom. The percent of PAR reaching each studied depth (0.5, 2 and 3.5m) remained stable along the summer and was established around 62%,

37% and 26% for each depth respectively. Surface photosynthetically active radiation (PAR) showed average values 241 to 477 µE $m^{-2}s^{-1}$ (Table 5.1).

Table 5.3. Lake Limnopolar daily values of ^{13}C uptake (PPr: plankton primary production, PPm: benthic mosses primary production, BP: bacterial production, PPmats: microbial mats primary production). Values for daily water Lake inflow and Lake outflow. Daily values of DOC (dissolved organic carbon) and POC (particulate organic carbon) inputs and outputs

Date	PPr (kg C.day^{-1})	PPm (kg C.day^{-1})	BP (kg C.day^{-1})	PPmats (kg C.day^{-1})	Lake inflow (m^3.day^{-1})	Lake outflow (m^3.day^{-1})	DOC input (kg C.day^{-1})	DOC output (kg C.day^{-1})	POC input (kg C.day^{-1})	POC output (kg C.day^{-1})
Lake Limnopolar										
13-12-08	0.38	1.01	N.A.D.	3.31	N.A.D.	N.A.D.	N.A.D.	N.A.D.	N.A.D.	N.A.D.
29-12-08	0.49	1.91	N.A.D.	4.13	1816	2122	1.46	1.58	0.06	0.06
10-01-09	1.29	3.18	0.73	4.42	8320	924	4.84	1.25	1.11	0.14
21-01-09	0.21	0.33	1.56	4.94	1022	1723	N.A.D.	1.23	0.15	0.22
03-02-09	0.10	0.66	1.31	5.05	1467	3009	3.55	8.22	0.20	0.63

N.A.D.: no available data

Accordingly, average water column temperature values increased through the summer from initial values of 1.22 ºC in the beginning of December, to maximum values of 5.8ºC at the first half of January (Table 5.1). Such an increase in temperature favored the ice melt process, allowing the change to a completely ice free lake and favoring the mix of the water column. This mixing process is reflected in homogeneous values for the physical and chemical variables along the water column. Hence, features like dissolved oxygen saturation was above 85% throughout the water column. Conductivity showed increasing values along the summer, from 53 µS cm^{-1} in December to values of 81 µS cm^{-1} at the end of February. The pH values increased from 6.7 at the beginning of the summer to 7.7 during February.

Concentrations of the main inorganic nutrients (SRP and DIN), dissolved and particulate organic carbon (DOC and POC) and colored dissolved organic matter (CDOM) are shown in table 5.1. Soluble reactive phosphorus (SRP) values were lower than the detection limit of 0.03 µM. The concentration of dissolved inorganic nitrogen (DIN) fluctuated through the summer with values from 0.5 to 1.5 µM, with maximum observed values at the beginning of January when the planktonic communities were more productive. Cromophoric dissolved organic matter (CDOM) is shown in the table 5.1. Dissolved organic carbon (DOC) showed an increase at the last part of January with maximum values of 1.63 mg l^{-1}. Particulate organic carbon showed a similar dynamic to DOC with a maximum increase at the last part of January with maximum values of 0.38 mg l^{-1}. Humic CDOM increased along the summer from 4.37 ng l^{-1} SQ at the first part of December to values of 6.48 ng l^{-1} SQ at the end of January. Protein-like CDOM decreased along the summer from 20.85 ng l^{-1} BSA at the last part of December to values of 10.15 ng l^{-1} BSA at the end of January.

Chlorophyll-a concentrations are shown in Table 5.1. They ranged 0.02-0.2 µg l^{-1} for the summer period, with maximum concentration found at the first half of January. The relative abundance of the carotenoid groups is shown in Figure 5.1. Carotenoid pattern along the three studied depths showed clearly marked differences. During December, the surface sample was dominated by lutein compounds,

describing an explosive growth of Prasinophytes coinciding with the ice cover melt. During this period, the non-surface samples showed a high relative abundance of fucoxanthin, describing dominance of diatoms and chrysophytes. During the first part of January, the abundance of lutein drastically decreased in the surface samples, however the deeper samples showed an increase in this compound. In this period the diversity of carotenoid groups increased, appearing other compounds like violaxathin describing the appearance of clorophytes and other groups of prasinophytes. During January, other carotenoids like myxoxantophyll appeared, revealing the presence of different groups of cyanobacteria. The increase in cyanobacterial carotenoids was higher in the 3.5m sample and more marked with the appearance of zeaxanthin during the last part of January and the first part of February. Thus, the abundance of cyanobacteria was lower in the surface and mid samples, showing a more balanced phytoplankton composition with a higher abundance of prasinophytes and clorophytes. The abundance of diatoms and chrisophytes in the deeper samples decreased through the summer but it was always present in the algae composition.

Bacterioplankton abundances during the studied period are shown in Table 5.1. Bacterioplankton population increased from values of 1.2x106 cell ml^{-1} to maximum values of $3.96x10^{6}$ cell ml^{-1} at the beginning of February.

Production rates of the different microbial groups studied are shown in Table 5.2. The values of planktonic primary production (PPr) displayed an increase in the first part of the summer reaching maximum values of 0.09 gC m^{-2} day^{-1} in mid-January. The values of primary production for the benthic mosses showed a similar pattern, with maximum values of 0.209 gC m^{-2} day^{-1} in mid-January. However, the increase in primary production was less marked compared with the increase for the plankton. Bacterial production rates displayed an increase in the last part of January, reaching maximum values of 0.115 gC m^{-2} day^{-1}. Production rates showed an increase during the first part of the summer, reaching stable values around 0.8 gC m^{-2} day^{-1} for the rest of the summer.

The seasonal effect of temperature over the primary production was evident, however, the effect of light over planktonic primary production was also studied and represented in the specific phytoplankton ^{13}C uptake (Figure 5.2). This figure showed lower values of primary production for the surface samples (0.5m) during December compared to the 2 and 3.5m samples, this represent a photoinhibition effect negatively affecting planktonic primary production due to the high initial incident light reaching values of 477 $\mu E\ m^{-2}\ s^{-1}$. By mid January the production rates of the surface sample increased, reaching values slightly higher to the observed values of the mid and deeper samples. At the end of January, production rates in the lake decreased to values similar to observations made during the first part of December.

The specific bacterial production rates showed higher values during the last part of February, mainly for the 2 and 3.5m samples. This increase in bacterial production coincided with a decrease in the specific primary production rates during the same period. These results displayed a clear time uncoupling between primary planktonic production and bacterial planktonic production. In this situation where the external inputs of organic matter display a great importance in the system, the bacterial planktonic community gets subsidized by this allochthonous source and the planktonic community suffers an uncoupling between the primary and bacterial productions.

Daily production values for the studied group of organisms (Table 5.3) showed that microbial mats located in the lake catchment contributed to the carbon balance of the lake with the highest production rates reaching values around 5 $kgC\ day^{-1}$. These values contrast with the observed production values for the planktonic primary producers with maximum values around 1.3 $kgC\ day^{-1}$ for the whole lake in mid January. The production rates for the benthic mosses population displayed maximum values around 3.2 $kgC\ day^{-1}$. These values showed the importance of microbial mat communities over the aquatic systems. However, not all the organic matter produced by the microbial mats have a direct effect on the lake system, Table 5.3 shows the input and output values for dissolved organic matter in Lake Limnopolar. These

values represented a positive balance with more inputs than outputs during the last mid and last part of January, becoming an important source of organic matter for the bacterioplankton community that display their maximum production values (around 1.56 kgC day^{-1}) during this period. The values of particulate organic carbon (POC) also represented an important input of organic matter with a positive balance between input/output of 0.97 kgC day^{-1} during the mid-part of January.

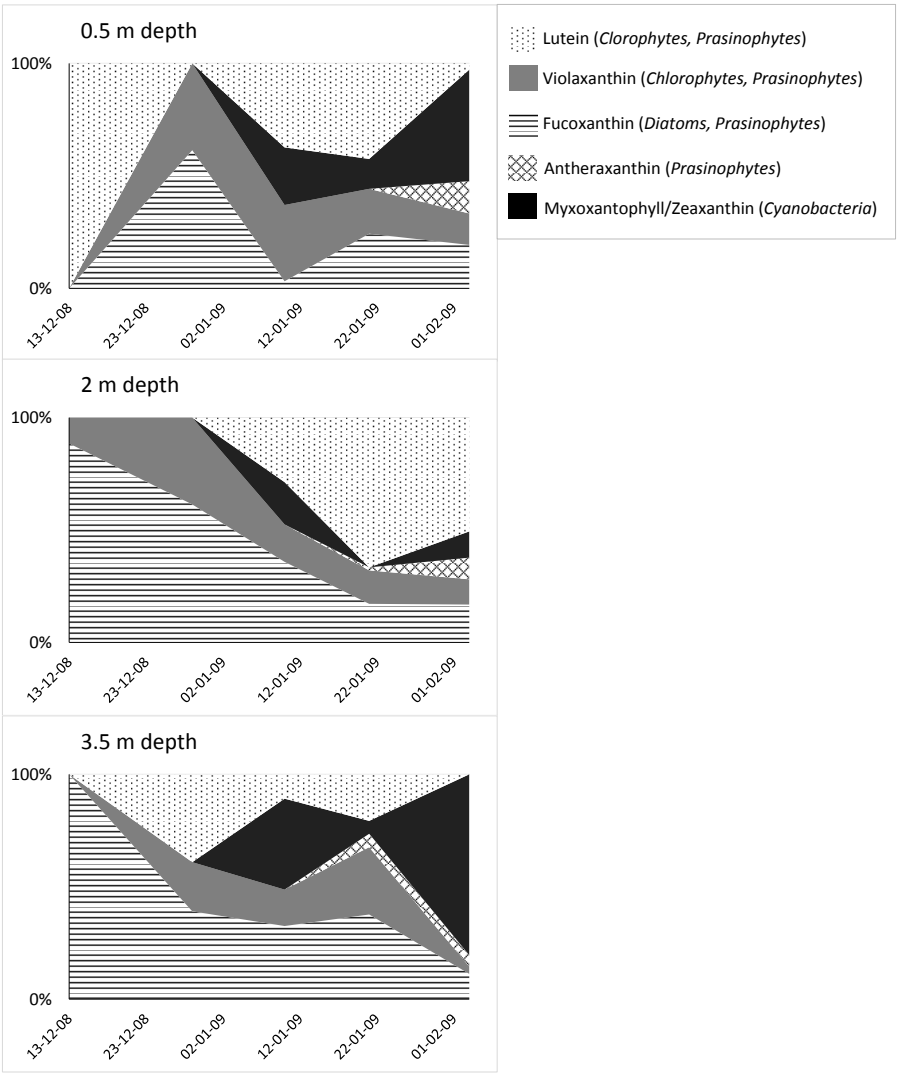


Figure 5.1. Lake Limnopolar carotenoids relative abundance during the austral summer of 08-09. The different algae groups appeared described by their main carotenoid types.

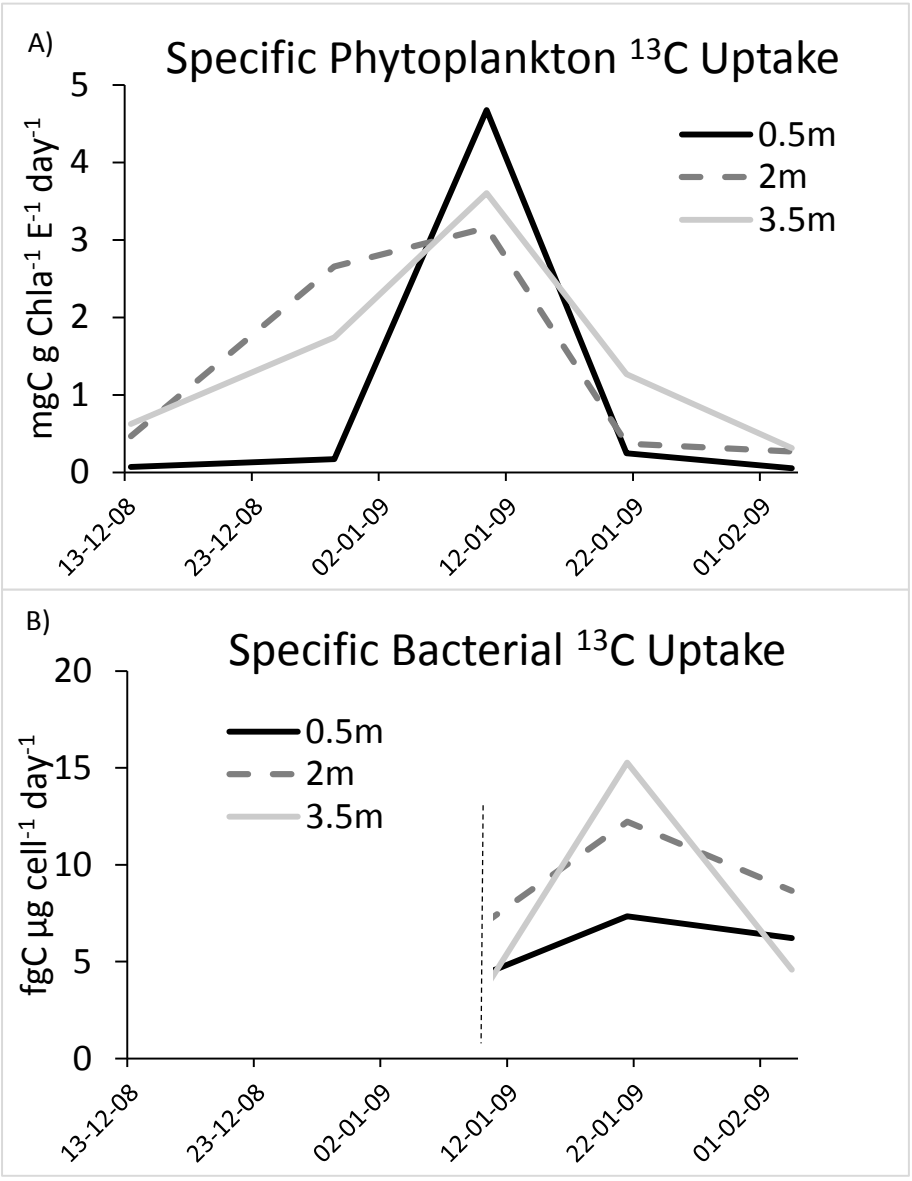


Figure 5.2. Values of specific 13C uptake: A) phytoplankton 13C uptake, B) bacterial 13C uptake. Data of bacterial 13C uptake before 10-1-09 were not available (these samples were lost during transportation).

The experiments carried out using thermostatic baths showed the response of the primary and bacterial production in the lake to the temperature (Figure 5.3). Phytoplankton C-fixation strongly responded to the increases in temperature with a temperature coefficient (Q10) of 2.9. Is important to note that the response between temperature and phytoplankton production in the manipulation experiment was significantly and highly correlated (p=0.002, r=0.96). In the case of the benthic mosses, the C-fixation rates increased slightly compared with the planktonic, showing a higher response for the 16ºC treatment. However the correlation between temperature and benthic mosses was not significant (p=0.138, r=0.5). The Q10 value for the benthic mosses was 3.9. The results for the bacterial production showed a slight increase in the production rates with temperature. The temperature coefficient (Q10) was 1.86 between 0 and 12ºC. The effect of temperature over bacterial production between 0ºC and 12ºC was significant (p=0.006, α=0.05) and correlated (r=0.98). However, an increase in temperature above 12ºC produced a reduction in the bacterial production, reflecting a negative effect over bacterioplankton probably by the appearance of psychrophilic activities over the bacterioplankton.

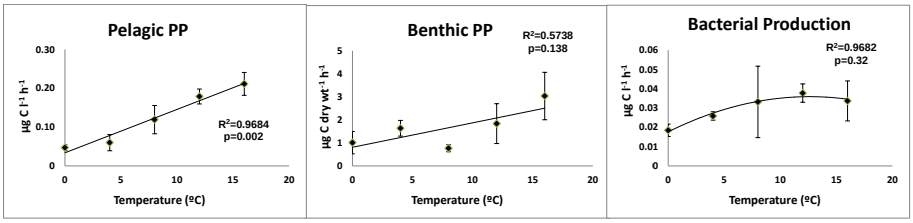


Figure 5.3. Results of pelagic ^{13}C uptake for phytoplankton (Pelagic PP), benthic ^{13}C uptake for mosses (Benthic PP) and bacterial production in the bath experiments testing five different temperatures.

5.5. Discussion

The biological features described for Lake Limnopolar (Table 5.1) showed a lake with a marked oligotrophic status. In this system, the increase in temperature, light availability (PAR) and the contributions of nutrients during the austral summer represents an important effect over the planktonic microbial community and the benthic mosses community. These processes are triggered by the melt of the lake ice cover during the last part of December, increasing at first the light availability and allowing major contributions of inorganic nutrients and organic matter from the catchment run-off processes. The different humic CDOM types of the organic matter (Figure 5.1) support the idea of these allochthonous contributions of organic matter. These dynamics are characterized to activate the productive processes in the lake and have already been described for some maritime Antarctic systems (Montecino et al. 1991, Rochera et al. 2010, Izaguirre et al. 2012, Villaescusa et al. 2013b).

Therefore, the increase in the plankton primary production rates during the first half of January (Table 5.2) is stimulated by the high temperature and light availability. These processes contribute to important inputs of inorganic nutrients and organic matter into the lake by runoff processes after the ice melts, mainly produced by the mosses and microbial mats in the catchment area. Due to these features, Lake Limnopolar displays higher primary production rates compared to those lakes situated in the continental area of Antarctica that are mainly closed systems where the ice cover doesn't disappear during the summer (Priscu 1998, Dore & Priscu 2001). Thus, Lake Limnopolar display biological features closer to Patagonian lakes, like Lake Morenito (Navarro et al. 2009) subjected to less severe climate conditions. Also, the measured values of primary production in Lake Limnopolar can be compared with ultra-oligotrophic mountain lakes such as Lake La Caldera (Morales-Baquero et al. 1992), which in spite of being situated in the southern part of Spain, displays typical characteristics of a sub-polar lake due to their extreme conditions.

The higher planktonic primary production rates are also followed by an increase in the heterotrophic bacterioplankton production rates during the summer period (Table 5.2), showing their maximum values during the second half of January. The observed values of bacterial production were higher compared to the values of continental Antarctic ultra-oligotrophic lakes like Lake Beaver (Laybourn-Parry et al. 2006), or Lake Crooked (Laybourn-Parry et al. 2004). In fact, Lake Limnopolar bacterial production values are closest to those observed in patagonian lakes (Navarro et al. 2009).

In this way, the increase in planktonic production rates during the austral summer directly translates into high abundances of bacterioplankton and phytoplankton (measured as Chl-a concentration) (Table 5.1). However, if we focus our attention on the phytoplankton abundance among the different algal groups, we found a non homogenous composition through the summer. The carotenoid pigments abundance (Figure 5.1) reflects a change in the composition of algae groups through the summer. It is important to note the abundant presence of prasinophytes and clorophytes on the surface of the lake during early austral summer just after the ice cover thaw process. This significant bloom of *Clorophyta*, in particular prasinophytes, has been previously argued in Lake Limnopolar by virus abundance studies, showing a great presence of prasinophytes phytophagous viruses after the ice cover melts (López-Bueno et al. 2009). This algal bloom can be explained as an opportunistic response to the increase in light and available nutrients immediately below the ice due to melting. In contrast, the rest of the water column appears dominated by diatoms and chrysophytes, being the main algae representatives in these ecosystems (Izaguirre et al. 2012). Finally, during mid-January some cyanobacteria carotenoid pigments increased their relative abundance describing an important change in the phytoplankton structure coinciding with the decrease in the specific phytoplankton uptake (Figure 5.2).

Although the planktonic primary production is clearly increasing during the first half of January, it seems that these processes are not homogeneous along the water column. The specific planktonic production rates (Figure 5.2) showed low values in

the surface sample after the ice melts, probably due to a photoinhibition process caused by the high irradiance values observed during the first part of December. The high relative abundance of prasinophytes in the upper layer of the lake during December could be related to a residual presence of this group of algae that usually inhabit under the ice cover during the winter (Lovejoy et al. 2007). In this way, studies of virus diversity in Antarctic lakes have reported a high abundance of phytophage virus that predate over the prasinophytes population during December, just after the ice cover thaw, supporting the high presence of this algal group in the upper layers (López-Bueno et al. 2009). This study has also demonstrated that the transition from an ice-covered lake during the austral summer to an open system produces important changes in the virus composition, possibly reflecting a seasonal change in algal host organisms.

After this initial period, the specific primary production increased up to their maximum values coinciding with the change in the composition of the algae groups during mid-January. After reaching its maximum value, planktonic primary production decreased during the last part of the summer, coinciding with a change in the composition of the algae groups. However, if we compare these results with the specific bacterial production rates (Figure 5.2) an evident difference can be observed. The maximum values of bacterial production appeared with a marked delay in comparison with the maximum values of phytoplankton. This uncoupling could be related to the external inputs of organic matter from the catchment area that subsidize the bacterial production during this period.

If we want to explain the observed uncoupling between phytoplankton and bacterial production, we must rely in other biological compartments that can be autochthonous, like benthic mosses, or allochthonous, like microbial mats growing in the lake catchment. In the first place, if we attend to the benthic mosses communities, mainly composed by *Depranocladus longifolius*, a similar carbon uptake pattern was observed compared with the observed in the phytoplankton (Table 5.2), with maximum values during mid-January. The presence of benthic mosses communities

has been described in other maritime Antarctic systems like King George lakes (Montecino et al. 1991) and other Antarctic lakes (Imura et al. 2003, Moorhead et al. 2005, Ask et al. 2009). Benthic mosses total carbon production per unit area (Table 5.3) represent a value three times higher compared to the observed for the phytoplankton. Thus, the benthic mosses community and its activity act as an additional source of organic matter for the bacterial production. However, we must not forget that the net primary production of these benthic mosses is clearly dependent on the availability of radiation that is controlled by the date of the ice cover melting. Studies on Artic lakes (Riss et al. 2014) have predicted that the change in the length of the productive period in polar lakes due to the climate change could provoke dramatic effects on the annual net production of mosses and thus whole lake primary production. This also suggests possible changes in the dominance patterns of benthic mosses species in these systems.

However, carbon contributions by benthic mosses seems not to be the only source of carbon to subsidize the planktonic production. Microbial mats located in the catchment of Antarctic lakes and other minor external sources like mosses may represent an important source of carbon production for these systems (Fernández-Valiente et al. 2007, Villaescusa et al. 2010). Results of total carbon production (Table 5.3) reveal values up to four times higher compared to those of phytoplankton production. These differences are also existent within benthic mosses production and microbial mats production, showing the higher relative importance of microbial mats over benthic mosses. Nevertheless, microbial mats are not an autochthonous element in Antarctic lakes, therefore, the contributions of organic carbon from these mats communities in the lakes depends on the existence of run-off processes. The analysis of the water inflow and outflow (Table 5.3) along the austral summer revealed important allochthonous inputs of dissolved organic carbon (DOC) during the first part of January supporting a strong run-off process related to the snow and ice melt on the lake catchment. This dynamic was also observed for the particulate organic carbon (POC). During February the allochthonous inputs of organic matter decreased due to less water inflow into the lake and the absence of snow in the catchment. This

decrease in the organic matter inputs and the high observed water lake outflow, derived in a reduction in the concentration of DOC and POC in the lake (Table 5.1) negatively affecting the bacterial production during this period.

Thus, the allochthonous contributions of organic carbon from the microbial mats and other minor external sources like mosses, along with the autochthonous contributions of organic carbon from the benthic mosses, subsidize the bacterial production in the lake and support the observed uncoupling between primary and bacterial production. This uncoupling between bacterioplankton and phytoplankton production could seem strange in Antarctic systems mainly based on the internal carbon supply (Dore & Priscu 2001) but as said before, our system receives external inputs of allochthonous organic matter from the catchment (mainly produced by microbial mats) that can subsidize the lake bacterial production as has been described in other systems (Priscu et al.1998, Kritzberg et al. 2004). These dynamics produce an uncoupling between the primary production and bacterial production, which become subsidized by the external inputs of organic matter (Thingstad 2000).

The effect of temperature over the systems and their microbial communities was one of the principal reasons explaining the observed dynamic during the austral summer. The temperature manipulation experiments showed the existence of a high correlation between temperature and planktonic primary production (Figure 5.3). These results support that planktonic communities on Lake Limnopolar actively respond to the increase in temperature during the austral summer. The existence of this direct relationship between temperature and planktonic primary production have also been reported in other Antarctic systems (Montecino et al. 1991, Laybourn-Parry et al. 2006). Also, the measured values of Q10 for the plankton was 2.9 being closely similar to the values of Q10 measured for the lakes of Signy Island (Hawes 1993) with similar characteristics. However, Q10 values for temperate lakes are higher than 3 and higher than 4 for marine systems.

Unlike planktonic assays, results for the benthic mosses showed a weaker response against temperature. However, benthic mosses display a clear response to

temperature for the high temperature treatments >8ºC. This weaker response against temperature could be caused by the existence of a damping effect by the water column, reducing the response of benthic communities to the increase in temperature. This effect has been described in some Antarctic lakes (Montecino et al. 1991, Imura et al. 2003) as in other non-Antarctic systems (Vadeboncoeur et al. 2008). In another way, Riss et al. (2014) reported the clearly effect of radiation availability and the length of the productive period on these benthic moss communities that could affect the dominance patterns species in these systems.

On the other hand, the results of the bacterioplankton production assays (Figure 5.3) showed a high correlation between production and temperature for the interval of 0 to 12ºC. The marked decrease in bacterial production over 12ºC could be explained by the psychrophilic behavior of some bacterioplankton species, which has been previously described in other Antarctic lakes (Spring et al. 2003). The existence of psychrophilic communities in the lakes of maritime Antarctica reflects the vulnerability of these communities to the climate warming that is affecting this area (Meredith & King 2005, Steig et al. 2009). In this way, a possible future scenario of high temperature in this area could change the composition of the bacterioplankton community with non-psychrophilic species better adapted to higher temperatures.

According to the observed sensitive response of the biological community against temperature (Figure 5.3), the intense warming occurring in this area (Meredith & King 2005) should be considered as an important issue. This sensitive response has been demonstrated in Lake Limnopolar by the development of an ecological model (Camacho et al. 2014). The results of this model conclude that the increase in temperature regimen in maritime Antarctic lakes could exert important changes in the system. As a result, the earlier summer ice cover thaw and the changes in the stream dynamics could derive in a higher contribution of inorganic nutrients and organic carbon, increasing the length of the productive period. Also, the change in the dynamic of these summer events, force the appearance of changes in the planktonic microbial community group composition (Izaguirre et al. 2012, Villaescusa et al. 2013a). Thus,

the ecological lake models act as an excellent tool to describe the dynamics of these systems and predict the possible irreversible changes in their biological composition and dynamics (Reid & Croud 2008, Herbei et al. 2010).

In conclusion, we can affirm that the planktonic community of Lake Limnopolar is clearly regulated by the external contributions of organic matter from the microbial mats covering their catchment. The allochthonous organic matter subsidizes the planktonic production in these systems, allowing the appearance of an uncoupling between the primary and bacterial production. However, these contribution events are ultimately controlled by the change in the temperature regimen in this area. As a response to the increase in temperature, the lake systems display a major availability of light (PAR), that exerts an effect over the algae group compositions, and major contributions of inorganic nutrients and organic matter, which ultimately control the planktonic community abundance and composition.

6. Heterogeneous vertical structure of the bacterioplankton community in a non-stratified Antarctic lake

Villaescusa JA, Casamayor EO, Rochera C, Quesada A, Michaud L, Camacho A (2013) Heterogeneous vertical structure of the bacterioplankton community in a non-stratified Antarctic Lake. Antarctic Science 25(2):229-238

Received 97 August 2012 · Accepted 13 August 2012

6.1. Abstract

Bacterial community composition during summer was analysed in surface and bottom waters of the oligotrophic shallow (4.5 m) Lake Limnopolar (Livingston Island, South Shetland Islands, Antarctica), using 16S rRNA gene clone libraries and sequencing. Up to 61% of the 16S rDNA sequences found were closely related to sequences retrieved from lakes, glaciers or polar systems. The distribution of these sequences was not homogeneous, with vertical differences found in both bacterial taxa composition and isolation source of the closest match from GenBank. In the surface sample 86% of the sequences were related to bacteria found in soils, seawater or gut microbiota, probably explained by waterborne transport from the catchment, by wind through sea sprays, or local bird activity. Conversely, in the deep samples, 95% of the sequences were closer to bacteria typically described for lakes, glaciers or polar systems. The presence of benthic mosses covering the bottom of the lake favours a more stable deep layer leading to the existence of this biological heterogeneity through the water column, although the lake does not show physical-chemical stratification in summer. This study illustrates a strong influence of external factors on the microbial ecology of this model Antarctic lake.

Keywords: bacteria, biological stratification, maritime Antarctic lakes, vertical heterogeneity, 16S rRNA gene

6.2. Introduction

Antarctic lakes located in the ice-free regions represent only the 2% of the total continental ice-free surface, and include both freshwater and saline systems. Most of these water bodies have experienced little or no anthropogenic impact and, therefore, still harbour pristine biotopes. In Antarctic lakes the combination of extreme environmental stressors (i.e. low temperature, low photosynthetically active radiation, nutrient limitation, extended periods of ice-covering, limited availability of liquid water, and short growing season) leads to simple truncated food webs with no fish and a limited presence of grazing and burrowing organisms (Michaud et al. 2012). Such systems can be considered as sensitive indicators of environmental changes because snow and ice cover variations markedly affect all ecological variables (Quayle et al. 2002, García-Jurado et al. 2011). These environments may also be considered analogous to those present prior to the evolution of metazoans, and they are generally dominated by organisms of the microbial loop, including bacteria, protozoa and phytoplankton (Laybourn-Parry 2002, 2009).

Studies on the microbial ecology of Antarctic ecosystems have focused on the structure and dynamics of microbial communities, both bacteria and protists, using traditional black-box approaches (Laybourn-Parry et al. 1991, 1995, Bell & Laybourn-Parry 1999, McKnight et al. 2000, Duarte et al. 2005). However, during the last decade studies have focused on the application of molecular techniques based on direct amplification of 16S rRNA ribosomal gene sequences by using PCR and subsequent molecular sequencing or hybridization techniques such as FISH (Pearce & Butler 2002, Pearce et al. 2003, Pearce 2003, 2005, Unrein et al. 2005, Laybourn-Parry & Pearce 2007). These studies have been a major step forward in the characterization of the microbial communities in Antarctic systems.

Byers Peninsula is one of the largest ice-free areas of Maritime Antarctica. The area is characterized by the presence of a large number of water bodies, which support the development of important planktonic microbial communities during the summer

(Toro et al. 2007, Villaescusa et al. 2010). Lake Limnopolar is one of the lakes located on Byers Peninsula (Livingston Island, South Shetland Islands, Antarctica), that has been selected as a model lake for many studies (Camacho 2006a, Toro et al. 2007), as it is characteristic of most features of inland lakes in this region of Antarctica. This lake has been the focus of study over the last decade by the Spanish research team Limnopolar (www.uam.es/limnopolar). Part of this work has focused on investigating the ecological relationships implicit in the microbial food web, where the copepod *Boeckella poppei* Mrázek appears to be the main predator (Camacho 2006a, Toro et al. 2007), as in other Maritime Antarctic lakes (Butler et al. 2005). Metazooplankton may play an important role in Lake Limnopolar determining a top-down effect on the food web that extends to bacterioplankton (Camacho 2006a). A comparative study by genetic fingerprinting of the diversity of surface bacterioplankton in different lakes of Byers Peninsula was previously carried out (Villaescusa et al. 2010) showing a close relationship between physical and chemical features and the influence of lake's catchment processes on bacterial diversity. However, vertical heterogeneity within the bacterial assemblages was not previously investigated, as most of these lakes are shallow or cold monomictic, which results in thermal homogeneity and a non-stratified water column during summer. In such shallow oligotrophic systems, the physical and chemical variables remain relatively similar throughout the water column during the summer, causing a high degree of homogeneity in the system. However, the bottom of these lakes is often covered by large populations of the benthic moss *Drepanocladus longifolius* (Mitt.) Broth. ex Paris (Toro et al. 2007). These occurrences may generate stability in the bottom waters (Montecino et al. 1991, Imura et al. 2003), and thus may enhance vertical biological stratification. A previous study (Villaescusa et al. 2010) showed the influence of this benthic community on bacterioplankton diversity. The importance of stratification effects in lacustrine systems are well known, favouring the development of distinct microbial populations in the deep part of the lakes (Camacho 2006b). Even with the absence of a conspicuous physical-chemical stratification, the differences between surface and bottom waters due to the influences of catchment processes and the bottom coverage by mosses (also described in other Antarctic lakes, e.g. Sombre Lake in Signy Island, Pearce & Butler

2002), can be an important source of bacterial diversity in these aquatic systems. In the present study we have explored the possible vertical heterogeneity of the planktonic microbial community of Lake Limnopolar by 16S rRNA gene cloning and sequencing, analysing vertical changes in the bacterial community composition that could be explained by the influence of the moss carpet on the bottom waters and the influence of the catchment effects on surface waters.

6.3. Material and methods

6.3.1. Study area and sampling

Lake Limnopolar is on Byers Peninsula located between 62834'35"- 62840'35"S and 60854'14"- 61813'07"W. Lake Limnopolar is small (2.2 ha) and relatively shallow (4.5m maximum depth). It is a cold monomictic (showing only winter stratification) lake, which is ice-covered for most of the year. The ice-free period usually starts at the beginning of December, lasting approximately three months, but the ice-free time is closely related to winter dynamics and can be different each year (Rochera et al. 2010). The lake is oligotrophic due to low inorganic nutrients and organic matter inputs, which are primarily derived from the microbial mats within the lake basin (Fernández-Valiente et al. 2007).

The lake food web has been described as having low complexity and is mainly dominated by microorganisms (Camacho 2006a). The lake is c. 3 km from the ocean and the input from marine animals is limited to visiting birds, skua pairs and Antarctic terns, as demonstrated by repeated field observations. On 25 January 2007 sampling was done at Lake Limnopolar over the point of maximum depth. Vertical profiles of temperature, conductivity, pH and dissolved oxygen were obtained with a 6920 YSI multiparametric probe. Water samples from two depths (0.5m (surface waters, but not including neuston) and 4m (bottom layer)) were collected with a hydrographic bottle (Uwitech), using 5 l acid-washed plastic carboys. Physical, chemical and biological characteristics (electrical conductivity, temperature, pH, dissolved oxygen,

chlorophyll a (chl-a) concentrations, inorganic nitrogen and phosphorus, nanoflagellate abundance), were measured as described in Villaescusa et al. (2010). Bacterioplankton abundance was measured using flow cytometry after dying the samples with SYBR Green-I.

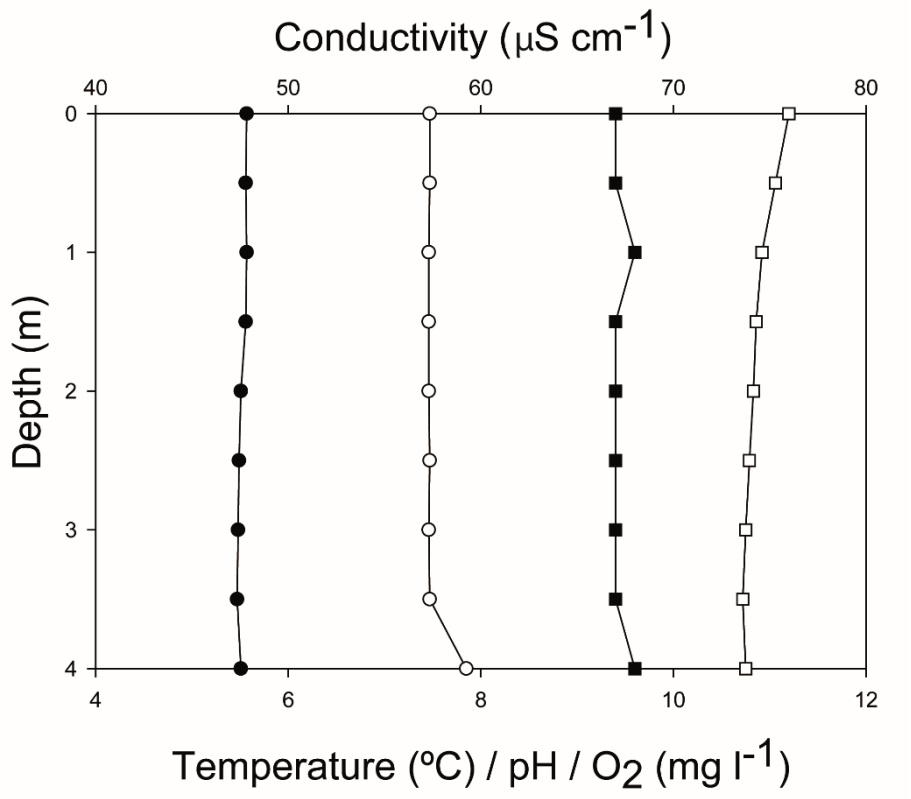


Figure 6.1. Environmental variables measured in Lake Limnopolar at the sampling date, showing the homogeneity of the vertical structure of the lake. Variables shown are temperature (ºC) = black circles, pH = white circles, conductivity (mS cm^{-1}) = black squares, and O_2 (mg l^{-1}) = white squares

Table 6.1. Environmental variables measured at the selected depths of Lake Limnopolar at the sampling date

	Lake Limnopolar	
	0.5m	4m
NH_4^- (mg l^{-1})	0.008	0.006
NO_3^- (mg l^{-1})	0.01	BDL
PO_4^- (mg l^{-1})	0.006	0.008
Chl a (µg l^{-1})	0.051	0.139
Bacteria (10^6 cell ml^{-1})	0.63	0.56
Nanoflagellates (ind l^{-1})	BDL	801

BDL: below detection limits

6.3.2. DNA extraction, 16S rRNA gene amplification, cloning and sequence analysis

For the study of bacterial diversity in each sample a clone library was developed. Approximately an hour after the samples were collected, a volume of 300 ml of water from the 5 l plastic carboys from each depth was filtered through 0.2 mm polycarbonate filters and preserved in DNA lysis buffer (40mM EDTA, 50mM Tris pH 8.3, 0.75 sucrose) at -20ºC. DNA was phenol extracted after enzymatic digestion (Dumestre et al. 2002).

Amplification of 16S rRNA gene fragments was performed by using bacterial universal primers 27f (AGAGTTTGATCMTGGCTCAG) and 1492r (GGTTACCTTGTTACGACTT), and PCR conditions were established at 558C annealing as previously described by Hervàs & Casamayor (2009). Amplified DNA fragments were purified using a purification kit (Invitrogen®), cloned (TOPO TA cloning® kit for sequencing, Invitrogen®), and sent for one-reaction, onestrand sequencing (MacroGen, Korea). To minimize PCR limitations we were cautious in the number of PCR cycles run to avoid the 'plateau' phase and in using the same amount of template in each reaction. The samples that we compared were run in the

same PCR run and analysed in exactly the same way. Any PCR bias should have been the same in all the samples and therefore, semi-quantitative comparison between these samples would be still valid.

Table 6.2 Comparison of bacterial diversities in the clone library for the two depths (alpha diversity) and the whole lake (Total, beta diversity) by means of the Chao1 richness estimator and the reciprocal Simpson's dominance index. OUT = operational taxonomic unit.

	Lim 0.5m	Lim 4m	Total
Number of clones	76	101	177
Number of OTU´s	24	21	42
Chao1 estimator	38	22	55
Reciprocal Simpson Index	14	14	27
Number of unique OTU´s	11	5	13

Next relatives of clones/isolates were determined by comparison to 16S rRNA gene sequences in the NCBI GenBank and the EMBL databases using BLAST, and the ''Seqmatch'' and ''Classifier'' programs of the Ribosomal Database Project II (http://rdp.cme.msu.edu/, accessed July 2012).

All sequences with similarity ≥ 97% were considered to represent one phylogenetic group or phylotype (operational taxonomic unit (OTU)). Sequences were further aligned using the program Clustal W (Thompson et al. 1994) to the most similar orthologous sequences retrieved from database. Each alignment was checked manually, corrected and then analysed using the neighbour-joining method (Saitou & Nei 1987) according to the model of Jukes-Cantor distances. A phylogenetic tree was constructed using the MEGA 5 (Molecular Evolutionary Genetics Analysis) software (Tamura et al. 2011). The robustness of the inferred trees was evaluated by 500 bootstrap re-samplings.

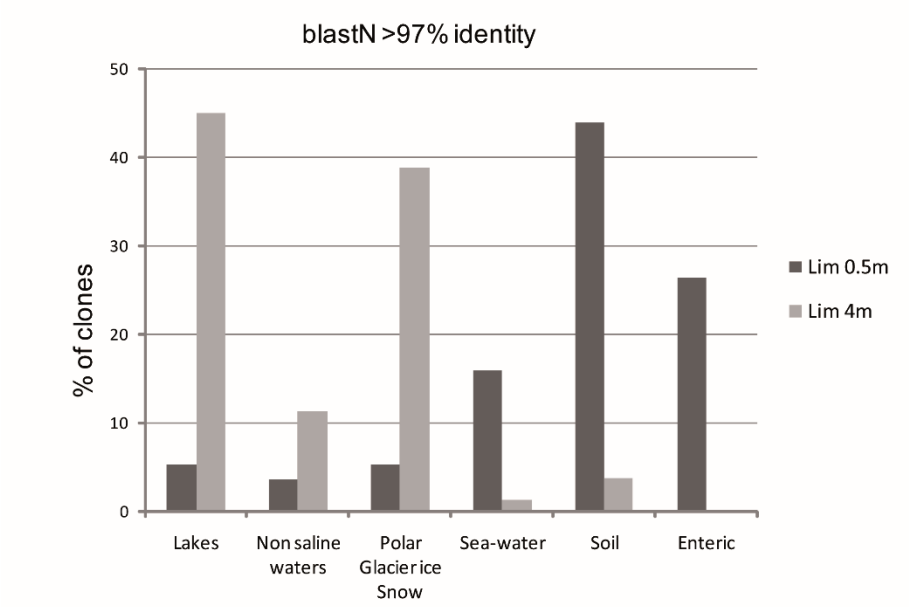


Figure 6.2. Isolation sources of the closest BLAST match found in GenBank (search done September 2011) for each of the analysed samples. Only BLAST matches with more than 97% identity were considered (species level).

Coverage values were calculated in order to determine how efficiently the clone libraries described the complexity of the original bacterial community. The coverage (Good 1953) value is given as C51 - (n1/N) where n1 is the number of clones which occurred only once in the library (singletons). The significance of differences among clone libraries was tested with Libshuff and the microbial composition significantly differed between samples (Libshuff test, $P < 0.001$) (Singleton et al. 2001).

All 16S rRNA gene sequences were submitted to GenBank under the accession numbers FR 848645-FR 848824. Sequences grouped at >97% identity were classified as different OTUs. In addition, we calculated the Chao1 index as a statistical estimator of richness, and the reciprocal Simpson's dominance index as an estimator of diversity for each single depth and for the whole lake (Xing et al. 2009).

6.4. Results

According to previous studies our sampling period is considered as the climax of the midsummer biological activity in the lake, occurring about one month after ice melt and water temperatures reaching the maximum annual values. Vertical profiles from Lake Limnopolar showed fairly homogeneous physical and chemical variables throughout the water column (Figure 6.1); at this time temperature and specific conductivity were stable around 5.5ºC and 68-69 mS cm^{-1}, respectively. This resulted in almost no vertical density gradient and thus a complete mixing of the water column was expected. This is especially the case given the strong winds typical of this region. Among physical-chemical variables, the most remarkable difference is shown by the increase of c. 0.4 units of the pH at the depths where mosses extensively cover the lake bottom. Chemical and biological data (Table 6.1) also showed an increase, such as that in chl a concentration at the bottom of the lake. This increase in chl a concentration was accompanied by a slight decrease in inorganic nitrogen concentrations (both ammonium and nitrate) and bacterioplankton abundance, similar to what was found in previous summers. Nanoflagellates were only detected in the deep part of the lake.

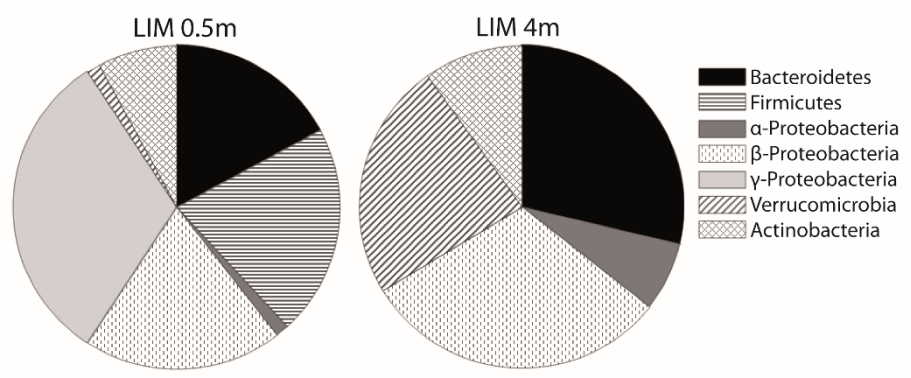


Figure 6.3. Differences in the phylum (or Class) assignation among bacterial composition between lake surface (0.5 m) and lake bottom (4 m) in Lake Limnopolar.

For the study of the bacterial community composition a total of 177 clones were processed and analysed (76 belonging to 0.5m depth, 85% of coverage, and 101 to 4m depth, 92.3% of coverage). The number of identified OTUs (Table 6.2), 21-24 per depth sampled, was similar to that obtained in other limnetic systems when the same sampling effort was undertaken. The Chao1 index and the Reciprocal Simpson's dominance index are shown in Table 6.2.

Overall, the results show (Figure 6.2) that c. 61% of identified clones (with at least .97% identity) were related to sequences from temperate aquatic non-saline systems, polar systems or ice glaciers, while c. 20% of clones were related to soil bacteria. The rest of clones belonged to seawater bacteria and enterobacteria (gut microbiota). When comparing the surface with bottom water samples, a clear vertical segregation was observed. In surface waters up to 76% of the retrieved sequences were affiliated to bacteria found in soils, microbial mats, seawater, or even enteric bacteria, whereas in the deep samples almost all the sequences (95%) were related with epicontinental water bodies even from non-polar regions and glaciers.

The comparison between sequences isolated from the surface and bottom waters showed differences in the relative abundance of each phylum (Figure 6.3). The major differences appeared in the phylum *Firmicutes*, representing 21% of the recovered sequences in the surface sample but these were not found in the bottom sample. Examining the branch of the phylogenetic tree (Figure 6.4) for the phylum *Firmicutes*, these isolated sequences belong primarily to the order *Clostridiales* with a single genus, Clostridium. Our environmental sequences were closely related to cultured species such as *C. psychrophilum*, *C. frigoris* or *C. lacusfryxellense*. These sequences are closely related (>99% identity) to Antarctic psychrophilic bacteria isolated from microbial mats.

Representative sequences of *Verrucomicrobia* and *Bacteroidetes* phyla were relatively more abundant at the bottom of the lake, and this is especially true for the former, although both groups also were observed in the surface waters.

Verrucomicrobia sequences were split into two different orders, *Opitutales* and *Verrucomicrobiales*, with some representatives closely related (>97% identity) to sequences from ultra-oligotrophic lakes such as Crater Lake, USA.

Bacteroidetes were grouped in three orders, *Sphingobacteriales*, *Cytophagales* and *Flavobacteriales*. The order *Sphingobacteriales* appeared in both, surface and bottom samples. Inside this order were a large amount of sequences of >98% identified with *Sediminibacterium* sp. In contrast, in the bottom of the lake, this phylum was mainly dominated by *Cytophagales* and *Flavobacteria*, represented by *Flavobacterium* sp. and *Flectobacillus* sp. These sequences were related (>97% identity) with bacteria isolated from ultra-oligotrophic lakes, high mountain glaciers and polar areas. Some of them were also found in Antarctic lakes and others matched with sequences of uncultured bacteria from Arctic glaciers.

Sequences within the *Actinobacteria* phylum were found throughout the water column of the lake. These sequences were closely related (>99% identity) to sequences of uncultured *Micrococcus* sp. commonly retrieved from oligotrophic lakes and Arctic glaciers. The phylum *Proteobacteria* was the main group represented in the whole set of retrieved sequences. The relative contributions of *Proteobacteria* sequences as a whole in the surface and bottom lake samples were very similar. However, the classes *Alpha-*, *Beta-* and *Gammaproteobacteria* showed important qualitative and quantitative differences in the vertical profile (Figure 6.3). Representatives of *Alpha-* and *Betaproteobacteria* were much more abundant in the bottom sample and the Gamma group was only found in the surface sample. Within this class the retrieved sequences were related to five orders: *Enterobacteriales*, *Alteromonadales*, *Oceanospirillales*, *Methylococcales* and *Pseudomonadales*. The *Enterobacteriales* sequences were closely related (>99% identity) to *Kluyvera* sp. and *Hafnia alvei*, these two taxa are widely distributed in nature and are also found in the gut of some animals. Both microorganisms also have psychrotrophic characteristics. For the other orders, sequences were closely related (>99% identity) to marine bacteria of the genera *Cobetia* sp., Pseudomonas sp. and *Pseudoalteromonas* sp.

Figure 6.4. Phylogenetic tree of the operational taxonomic units isolated from Lake Limnopolar.

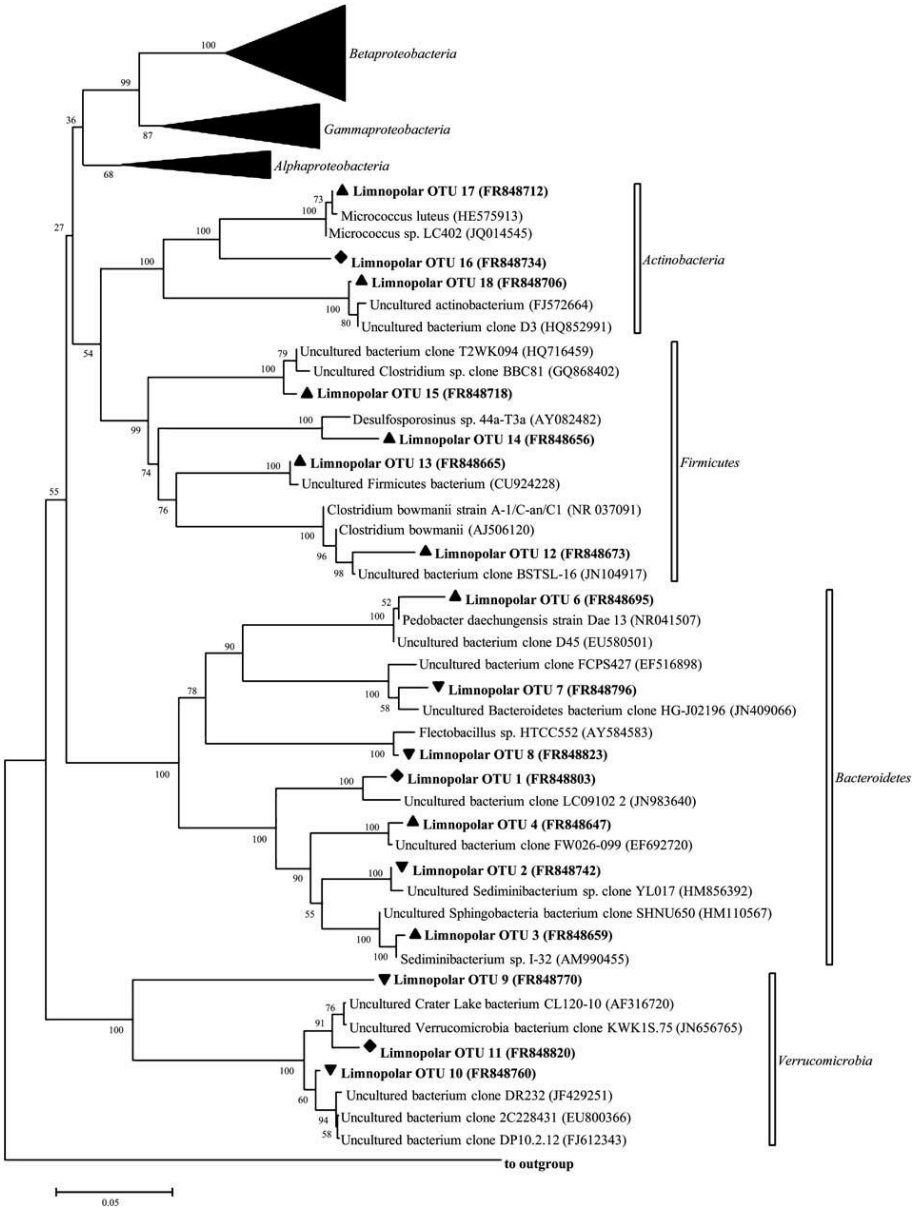


Figure 6.4. Continued.

Alphaproteobacteria also showed differences in species composition between surface and bottom waters. The surface water sample had sequences related (>98% identity) to *Brevundimonas* sp., whereas in the bottom sequences were related (>97% identity) to *Sphingomonas* sp., this latter has been commonly isolated from temperate oligotrophic lakes.

Sequences from *Betaproteobacteria* were the most abundantly recovered after PCR-cloning in Lake Limnopolar. All of them were from a single order, *Burkholderiales*, represented by four families: *Comamonadaceae*, *Burkholderiaceae*, *Methylophilales* and *Oxalobacteraceae*. We noticed differences in the composition of families and species through the water column. In the surface sample, some sequences were related (>98% identity) to bacteria of the genera *Ralstonia* sp., *Comamonas* sp. and *Massilia* sp., whereas in the bottom of the lake, other sequences were related (>99% identity) to *Polaromonas* sp., a genus usually found in glaciers and Antarctic lakes. Sequences closely related (>98% identity) to *Polynucleobacter* sp., a cosmopolitan group commonly found in oligotrophic lakes also appeared.

6.5. Discussion

Lake Limnopolar displays a remarkable oligotrophic status (Toro et al. 2007, Villaescusa et al. 2010, and data herein) and can be used as a model lake for inland lakes of Maritime Antarctica. However, the inputs of organic matter from the microbial mats and surrounding vegetation (mainly composed by mosses and lichens) located within the lake drainage basin (Fernández-Valiente et al. 2007), and the presence of a benthic moss population (Toro et al. 2007, Villaescusa et al. 2010) may be important in bacterioplankton development.

Bacterial beta diversity in the lake (estimated as a whole sample joining the 177 total isolated sequences, Table 6.2) was similar to that observed in temperate lakes (Bosshard et al. 1999, Eiler & Bertilsson 2004, Wu et al. 2007). However, when bacterial diversity was analysed separately for surface and lake bottom waters (alpha

diversity, Table 6.2) it much decreased compared to beta diversity. Separately, these lower alpha diversity values were more similar to those expected in cold-latitude oligotrophic lakes and high mountain lakes (Xing et al. 2009, Llorens-Marès et al. 2012). This difference between alpha and beta diversity is related to the existence of significant differences in terms of taxonomic composition between surface and bottom waters that are commonly attributed to spatial heterogeneity, and commonly is referred to as beta diversity. This point is of interest in further refining the species-area relationship found for microorganisms in lakes (Reche et al. 2005), with a higher increase of beta diversity than expected only from the habitat heterogeneity per unit area as discussed elsewhere (Barberán & Casamayor 2011).

In Lake Limnopolar, it is not unreasonable to think of this vertical segregation of the bacterial assemblages as a biological stratification, even though a clear physical and chemical vertical gradient is not displayed. In this lake, biological interactions play a major role during the summer (Camacho 2006a), and this also seems to be true in determining the composition and distribution of the bacterioplankton community. The analysis of the retrieved sequences with >97% identity (segregation criterion according to Tamames et al. 2010), and the origin of the closest matches found in GenBank, demonstrate the importance of the catchment inflow (soil bacteria), birds (enteric bacteria from birds faeces), and sea spray (airborne bacteria) in explaining the taxonomic composition of the bacterial community in surface waters. Contrastingly, bacterioplankton composition of deep waters is much more similar to that commonly described for other cold freshwater environments. This appears to be the case even though no physical stability of the water column is displayed, meaning that a more stable environment could be provided by the dense carpet of mosses covering the bottom of the lake, which confers a higher resilience to this bacterioplankton community.

The data obtained for Lake Limnopolar are in agreement with the idea of the dominance of *Proteobacteria* in Antarctic lakes (Pearce & Butler 2002, Pearce 2003, Pearce et al. 2003, Mosier et al. 2007) followed in abundance by the *Bacteroidetes*

phylum. If we delve into the *Proteobacteria* group, most clones belong to *Betaproteobacteria*, followed by *Gammaproteobacteria*, whereas *Alphaproteobacteria* were less represented (Figure 6.4). This pattern is commonly found in non-marine aquatic systems (Barberán & Casamayor 2010). Anyway, the interpretation of quantitative data should be carefully considered because of the well-known PCR-cloning limitations, even though we were quite careful with the analytical procedures. With our procedures, any PCR bias should have been the same in all the samples and we therefore consider that semi-quantitative comparison between these samples would be still valid, although we did not use quantitative data in absolute terms.

The presence of psychrotrophic enteric related bacteria, such as *Kluyvera* sp. and *Hafnia alvei* (Park et al. 2006), in the lake surface reflected the proximity of sea birds like skuas (*Catharacta lonnbergi Mathews*) and individual Antarctic terns (*Sterna vittata* Gmelin) in the Byers Peninsula region. The visits of these bird species to the lake has been confirmed and repeatedly reported. Also, the presence of halotolerant and halophilic bacteria (*Cobetia* sp., *Pseudomonas* sp. and *Pseudoalteromonas* sp.) typically isolated from seawater environments also support the influence of bird visits to the lake as well as the effects of sea spray.

The fact of a marked biological heterogeneity in the lake is also evident by the lack of *Gammaproteobacteria* in the deepest part of the lake (Figure 6.3) and the significant changes in specific bacterial composition within *Alpha-* and *Betaproteobacteria*.

The occurrence of many sequences of *Sediminibacterium* sp. (*Bacteroidetes*) in the surface samples which have been previously associated with reservoir and river sediments (Qu & Yuan 2008) is probably related to the important inflows of water entering the lake from the watershed during the summer. This is especially important during the snow melt processes that add runoff of sediments and bacteria from soil and lake basin vegetation, including microbial mats. This is also supported by the

presence of species such as *C. psychrophilum*, *C. frigoris* or *C. lacusfryxellense*, included within the phylum *Firmicutes*, usually associated with microbial mats (Spring et al. 2003).

6.6. Conclusion

Our main conclusion is the vertical heterogeneity in the bacterial community structure in this shallow lake. This heterogeneity is governed by continuous allochthonous inputs into the surface waters making the bacterial community inhabiting this layer quite distinct from the deepest portion of the lake. These inputs are sensitive to catchment variations in physical-chemical and biological processes. On the other hand, the benthic population of mosses appears to be exerting a stability effect in the deep part of the lake allowing the existence of bacterial species more associated with oligotrophic aquatic polar systems. The increase of c. 0.4 units of the pH at the depths where mosses extensively cover the lake bottom (below 3.5m depth) can be attributed to the photosynthetic activity of mosses, which consumes inorganic carbon (i.e. CO_2) and increases pH and the carbonate equilibrium in these soft waters. Our results demonstrate that, although this lake is not thermally stratified, the benthic biological community, namely the moss coverage, also exerts an important role by creating mesohabitats that either hold or stabilize specific bacterial populations, thus allowing a major differentiation of the catchment-influenced surface waters and the benthic community-influenced bottom waters. Therefore, although it is commonly thought that a lake non-stratified from the physical and chemical point of view should provide a homogeneous distribution of the biological community, Lake Limnopolar illustrates a non-physical and chemical stratified lake with an important bacterioplankton stratification, that perhaps could be a common characteristic of Maritime Antarctic lakes where mosses cover the benthos, especially during the summer.

7. Carbon utilization profiles reflect of the high environmental heterogeneity in Maritime Antarctic lakes

Villaescusa JA, Rochera C, Camacho A. Carbon utilization profiles reflect of the high environmental heterogeneity in Maritime Antarctic lakes. (In preparation to be submitted to Aquatic Microbial Ecology)

7.1. Abstract

Studies on the metabolic diversity of the planktonic microbial community in Antarctic lakes can be rapidly assayed and monitored using multiwell plates Biolog Ecoplate™. Accordingly, this study evaluates the degree of utilization over 31 different carbon sources included in the Biolog Ecoplates™, for five lakes located in Byers Peninsula (Livingston Island, Antarctica) with contrasting trophic status and limnological features. The huge differences between the five organic carbon utilization profiles supported the idea of the high environmental heterogeneity in the lakes of the area. The differences over the studied lakes were highlighted by a principal component analysis, revealing a close relationship between trophic status and bacterial physiological diversity in each lake. Lakes with high trophic status showed the highest physiological diversity. However, this idea contrasts with some previous evidence showing a decrease in bacterial taxonomic diversity in lakes with high trophic status. Therefore, our results suggest the existence of dominant bacterial taxa in lakes with higher trophic status, that develop a wider type of metabolic activities as a response of the greater diversity of organic matter types present in their environment. In conclusion, the analysis of carbon utilization profiles with Biolog Ecoplates™ represents an improvement in the analysis of the bacterioplankton physiological diversity in Antarctic lakes and can be used as a rapid tool to compare the physiological complexity of these systems. In addition, the detailed analysis of the different metabolized carbon groups can provide information about the origin and dynamics of the organic carbon in these lakes.

Keywords: maritime Antarctic lakes, bacterioplankton, environmental heterogeneity, carbon utilization profiles, Biolog Ecoplates™

7.2. Introduction

The utilization of carbon sources in soil, sediment and water by microbial communities can be used to study the physiological diversity of the bacterial community (Leflaive et al. 2008, Salomo et al. 2009, Wang et al. 2011). A rapid and standardized tool for conducting such studies is the use of multiwell plates BiologEcoplate™ (Stefanowicz 2006).

The Biolog Ecoplate method for the analysis of microbial communities through inoculation of mixed cultures of microorganisms was originally described by Garland & Mills (1991). This physiological approach has been recognized as a very effective tool to distinguishing spatial and temporal changes in the microbial communities. This system based on plates with commonly used carbon sources was specifically created for the analysis of microbial communities in soils and aquatic systems. However, the fact that many of their substrates are usually present in lakes, especially carbohydrates and amino acids (Chróst et al. 1989, Jørgensen & Jensen 1994), make these plates a useful tool to evaluate the bacterial physiological diversity in lake systems (Choi & Dobbs 1999).

The study of ecological features, and more in particular the interest in diversity, physiology and distribution of planktonic microbial communities in maritime Antarctic systems, have been the object of study for the scientific community during the last years (Pearce et al. 2003, Reche et al. 2005, Schiaffino et al. 2009).

Lakes in maritime Antarctica display a biological community mainly dominated by microorganisms (Ellis-Evans 1996, Laybourn-Parry et al. 2001, Laybourn-Parry 2002, 2009, Michaud et al. 2012). In these systems, bacterioplankton performs an essential role on the microbial planktonic community, however bacterioplankton carbon utilization has not been yet accurately described due to the lack of information about this issue. Our study aims to describe the carbon physiological activity of the

bacterioplankton community in these ecosystems to overcome the lack of information on this concern.

In maritime Antarctic lakes, a great part of the planktonic community functioning is driven by the bacterioplankton dynamics, and organic carbon is essential for the growth of bacterioplankton. As previously stated (chapter 5), planktonic primary production by itself cannot entirely support bacterial production in these lakes. In this scenario, the allochthonous inputs of organic carbon from the microbial mats and mosses growing either in the catchment or in the lake bottom (Fernández-Valiente et al. 2007, Villaescusa et al. 2010, Kenarova et al. 2013), represent an important source to support the lake bacterial production. This organic matter contribution is mainly represented by compounds such as amino acids, carbohydrates and carboxylic acids, which together with organic compounds derived from lake sediment removal processes, such as putrescine or algae polymeric compounds (Decho 1994), supports the bacterioplankton production in the lake.

Previous studies in maritime Antarctic lakes, showed the existence of a close relationship between physical-chemical variables and bacterial taxonomic diversity (Villaescusa et al. 2010, Rochera et al. 2013). These results and the known effect of nutrients on functional and genetic diversity (Leflaive et al. 2008), advised that the relationship between taxonomic and physiological diversity should be studied from a metabolic point of view. Thus, dissolved organic carbon utilization by the bacterioplankton was assayed in the present work using carbon multiwell plates (Biolog Ecoplate^{TM}), allowing a physiological comparative study between lakes with different trophic status. Biolog plates include a diverse set of organic carbon compounds commonly used in the nature, such as amino acids, carboxylic acids, carbohydrates, polymers and amines. Antarctic lake amino acids, in the form of protein organic matter, appear mainly as a product of the phytoplankton and benthic mosses production (Villaescusa et al. 2010), that in the end are metabolized by the bacterioplankton. However, the allochthonous inputs of organic matter include a more complex composition of carbon compounds, mainly composed by carbohydrates and

polymers derived from algal cryoprotectants, algae mucilage and moss exudates (Kenarova et al. 2013). In this way, the Biolog plates contain most of the carbon compound groups that are involved in the Antarctic lake bacterioplankton physiological activities.

7.3. Material and methods

7.3.1. Study area

The studied area is located in Byers Peninsula (Livingston Island, South Shetland Islands, Antarctica), between 62°34'35" - 62°40'35" South and 60º54'14" - 61º13'07" West. Byers Peninsula is one of the largest ice-free areas of maritime Antarctica (SCAR 2003). It is characterized by the presence of a large number of lakes that during the austral summer suffer a thaw process, showing a significant development of planktonic and benthic microbial communities (Ellis-Evans et al. 1998, Toro et al. 2007).

The study was conducted after selecting five different lakes at Byers Peninsula as representatives to reflect the environmental heterogeneity of microbial communities (Villaescusa et al. 2010). Four of the selected lakes have already been included in a previous study on the relationship between physical-chemical variables and bacterial taxonomic diversity in maritime Antarctica (Villaescusa et al. 2010). An additional lake (Lake Maderos) was included in the present study to increase the representation of coastal lakes. The selected lakes can be joined into three groups according to their main ecological characteristics: (i) Lakes Limnopolar and Chica, located in the central plateau of the Peninsula, with an average depth of around 4m and a marked oligotrophic status;(ii) Lake Somero, also located in the central plateau of the peninsula) with a maximum depth 0.5m, showing a strong interaction between the sediment and the water column. In this lake, wind-induced sediment removal increases the availability of nutrients and organic matter, thus showing higher trophic status compared to the previously mentioned lakes). (iii) Lakes Refugio and Maderos,

located next to the coast, with depths not exceeding 0.5 m, which display an eutrophic status due to the activity of marine birds and mammals.

7.3.2. Sampling and in-situ measurements

For each of the studied lakes, some in-situ physical and chemical variables (conductivity, pH, temperature and dissolved oxygen) were measured following the protocols described by Villaescusa et al. (2010). Subsequently, subsurface water samples (0.3m for Lakes Somero, Refugio and Maderos, and 0.5m depth for the rest) were collected and pooled into 5 liter acid washed plastic bottles. For the case of Lake Limnopolar, water of three different depths (0.5m, 2m, 3.5m) was also collected to explore the possible vertical differences linked to the so-called "biological stratification" (Villaescusa et al. 2013a,b). This "biological stratification" is reflected by an increase in the bacterial diversity on the deep layers close to the benthos linked to a higher availability of nutrients by the activity of benthic mosses. Later, some chemical and biological analyses were conducted from the water samples of each lake (Table 7.1), also following the methods described byVillaescusa et al. (2010).

Table 7.1. Environmental variables values for the five studied lakes. Data about catchment area were obtained from Toro et al. (2007).

Chemical Variables	Limnopolar	Chica	Somero	Refugio	Maderos
Catchment Area (km^2)	0.58	0.01	0.06	0.12	2.41
Max Depth (m)	5.5	4	0.5	0.5	0.5
Conductivity ($\mu S\ cm^{-1}$ 25°C)	67	45	70	130	257
pH	7.47	6.82	7.21	8.46	7.4
NH_4 (µM)	0.42	< 0.1	0.62	0.5	0.78
NOx (µM)	0.15	1,144	0.14	8.36	14.96
SRP (µM)	0.06	0.066	0.15	5.27	0.24
Chl-a ($\mu g\ l^{-1}$)	0.04	0.21	0.47	18.92	1.44
Bacterioplankton (10^6 cells ml^{-1})	1.31	2.87	1.77	6.48	3.72

7.3.3. Biolog test

For the development of our comparative study, we used the multiwell BiologEcoplate™ system designed specifically for environmental microbial community studies. These plates hold 31 different types (3 replicates each) of commonly used carbon sources, such as amino acids, amines, carbohydrates, carboxylic acids and polymers.

The wells in the Ecoplates were inoculated with water of the assayed lakes and incubated for 12-13 days at the camp laboratory with a stable temperature of around 5ºC. The detection of metabolic activity was measured by the appearance of a purple color on the plates. This reaction occurs when growing microorganisms are able to degrade each carbon sources, reducing the tetrazolium chromophore molecule bound to the carbon source, resulting in the appearance of a purple color (Figure 7.1). This means that the assayed carbon source can be used as main source of carbon to support bacterial growth.

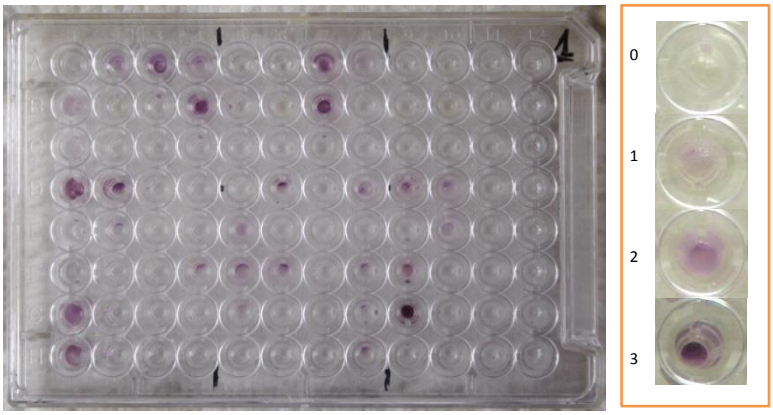


Figure 7.1. Picture showing the tetrazolium molecule reduction on the plates Biolog Ecoplate™. It is also presented the qualitative color scale used in our analysis, the values were ranked from 0 to 3 according to color development.

7.3.4. Data analyses

Due to restricted logistic conditions and the lack of facilities at the camp laboratory, the metabolic activity in each plate was recorded every 24 hours using images taken by a photographic camera Canon EOS 350D, (EF-S 18-55 mm, 1:3.5-5.6 DC). After that, images were analyzed and processed by an image analysis program (ImageJ) to determine the color developed pattern in each well, which can be directly related to the intensity of the metabolic activity on the studied carbon sources.

The intensity of metabolic signal (bacterial growth on the substrate) in each plate was quantified using a qualitative scale from 0 to 3 (Figure 7.1). For each positive carbon treatment, an average value from their three replicates was obtained. Treatments where the color signal only appeared in one of the three replicates were considered as false positives. These average ranked values, with their respective standard deviation, were used as an indicator of metabolic activity for each treatment. Moreover, each carbon compound tested was clustered into one of the different five following groups to facilitate the data analysis: amino acids, carbohydrates, carboxylic acids, polymers and amines (Bradley & Owen 2007).

To compare the functional diversity of the bacterioplankton of the selected lakes, the Average Well Color Development (AWCD) index suggested by Garland and Mills (1991) was used. This index serve as an approach to the physiological diversity in each lake.

$$AWCD = \sum \frac{(n_i - c)}{31}$$

n_i: average qualitative color value of the three wells
c: average qualitative color value of control wells

Additionally, a principal component analyses (PCA) was built using the carbon source average utilization values for each lake. The objective of the PCA analysis was the discrimination between the possible differences in the carbon source utilization

profiles of each lake, also incorporating the three studied depths in Lake Limnopolar as different study cases.

7.4. Results

For each of the studied lakes, the most relevant physical and chemical measured variables are shown in Table 7.1. These results reflected the high environmental heterogeneity between the studied lakes, organizing them into three different groups. If we attend to the values of maximum depth and conductivity, different groups of lakes appear. Shallow lakes (0.5m maximum depth) with relative high conductivity (>100 µS cm^{-1} 25°C) include the coastal lakes Refugio and Maderos. Other group are lakes Limnopolar and Chica, with lower conductivity values (<100 µS cm^{-1} 25 °C) and a wider depth range (4-6 m maximum depth), situated in the central part of the peninsula. The third type, also located in the central, is represented by shallow lakes (0.5m maximum depth) with higher trophic status, like Lake Somero. Comparing the concentrations of major dissolved nutrients (NH_4, NOx and SRP), shallow and coastal lakes displayed the highest concentrations. Biological variables (Chl-a concentration,

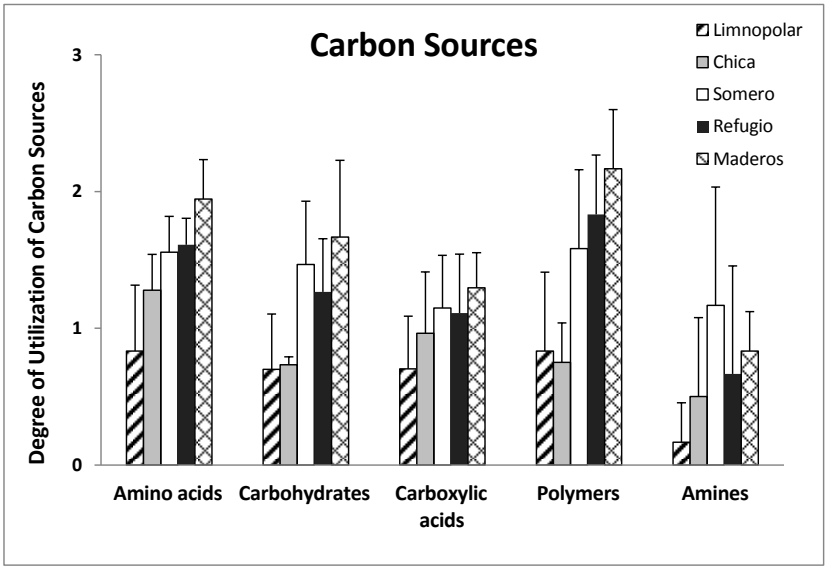


Figure 7.2. Average utilization values for the different carbon source´s groups by the microbial community of the five studied lakes.

featuring the trophic status, and abundance of bacterioplankton) showed the highest values (>1µgl^{-1}Chl-a and> 3.10^{6} cells ml^{-1} of bacterioplankton).

The results of the utilization of commonly used carbon sources (grouped by types) for each lake are shown in figure 7.2. Among the different lakes, the most utilized carbon sources were, on average, amino acids "AA" and polymers "P" and both in Lake Maderos. The AWCD index showed values of 0.71 for Lake Limnopolar, 0.89 for Lake Chica, 1.39 for Lake Somero, 1.32 for Lake Refugio and 1.62 for Lake Maderos, with a pattern of increasing the physiological diversity with increasing trophic status.

Focusing on the utilization profiles for the different groups of organic compounds (Figure 7.3), some patterns can be found. For amino acids "AA", the diversity index AWCD showed the higher values in the Lake Maderos (0.38) followed by the lakes Refugio (0.31) and Somero (0.30). Lake Chica showed a less diverse utilization pattern (0.25) and Lake Limnopolar displayed the lowest utilization values (0.16). It is noticiable that lakes Somero, Refugio and Maderos displayed high utilization values for compounds like L-arginine (AA1) and L-asparagine (AA2) that are very common in nature and are usually metabolized by microorganisms. Less abundant amino acids like L-phenylalanine (AA3) and L-threonine (AA5) were only used in Lake Maderos though with low intensity.

A similar pattern appeared for carbohydrates. Lake Maderos also showed the most diverse utilization values (0.54), followed by Lake Somero (0.47) and Lake Refugio (0.41) whereas the less diverse utilization values appeared for the lakes Chica (0.24) and Limnopolar (0.23). Most carbohydrates were readily used in all lakes, though β-Methyl-Glucoside (C3), which is a monosaccharide derived from glucose, was only slightly metabolized in lakes Refugio and Maderos. Also, phosphorylated compounds like Glucose-1-Phosphate (C8) and Glycerol-Phosphate (C9) showed very low utilization.

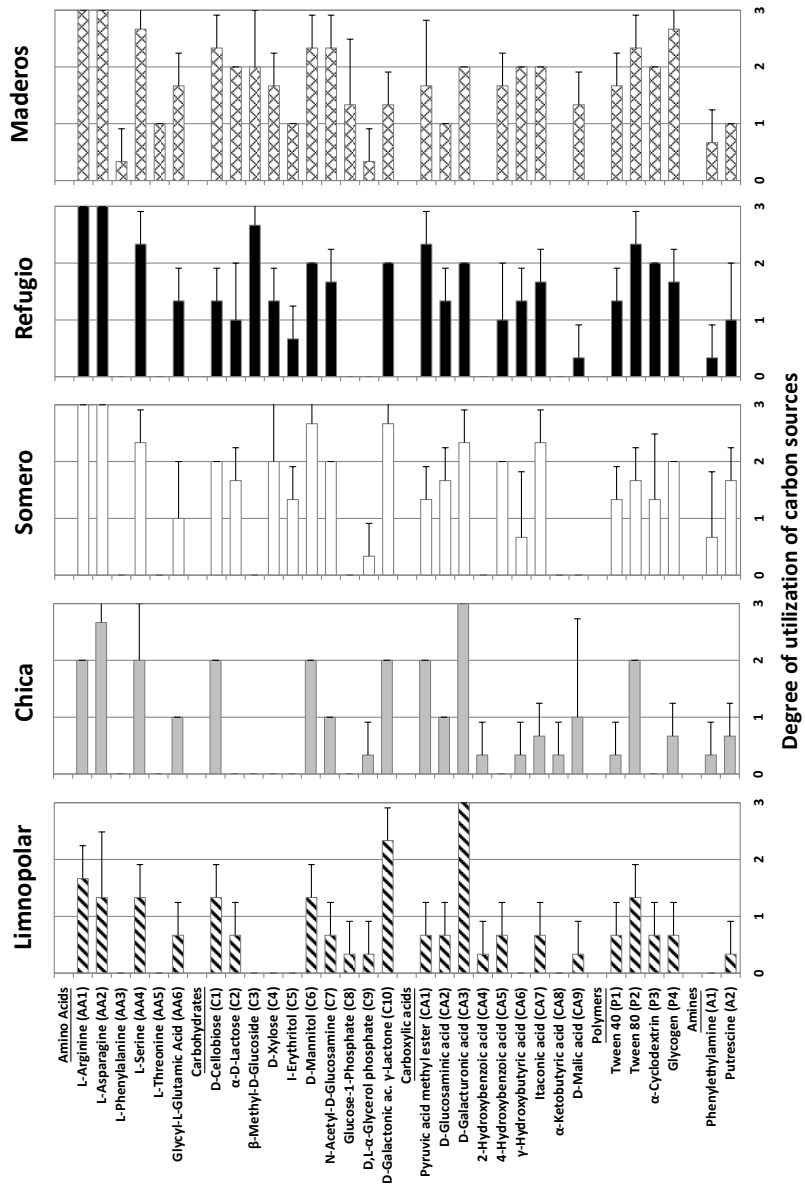


Figure 7.3. Carbon source´s average utilization profiles for each of the studied lakes.

The utilization of carboxylic acids showed a similar pattern than that of amino acids and carbohydrates, however, the differences among the studied lakes were lower than the previously mentioned compounds. Lake Maderos still showed the greater physiological diversity (0.38) over carboxylic acids, followed by Lake Somero (0.33) and Lake Refugio (0.32). The less diverse utilization pattern still appeared for the lakes Chica (0.28) and Limnopolar (0.2). Most carboxylic acids were readily used, although some compounds such as 2-hydroxybenzoic acid (CA4) or α-ketobutyric acid (CA8) hardly were metabolized. It is very noticeable that unlike the rest of compounds, D-galacturonic acid (CA3) was more efficiently metabolized in the oligotrophic lakes Chica and Limnopolar.

Also the physiological activity over polymeric compounds showed a similar pattern than the previously mentioned organic compounds. The more diverse utilization patterns appeared for the Lake Maderos (0.28), followed by the lakes Refugio (0.24) and Somero (0.2). The physiological diversity was also lower for the lakes Chica (0.1) and Limnopolar (0.11). The compound Tween-80 (P2), which is a commercial derivate of oleic acid, showed the highest utilization values for all tested lakes.

Contrasting with the other four types of compounds, the physiological diversity over amines showed a slightly different pattern, since coastal lakes did not showed the highest values. The higher AWCD values appeared for Lake Somero (0.08), followed by the lakes Maderos (0.05) and Refugio (0.04). Chica and Limnopolar showed less diverse values with (0.03) and (0.01) respectively. The higher utilization values in Lake Chica are given by a relatively strong growth on Putrescin (A2), which is a compound related to the breakdown of amino acids.

When the results of the carbon utilization for each depth of Lake Limnopolar (Figure 7.4) were analyzed, the most diverse utilization pattern appeared for the deeper sample (3.5m) with AWCD values of 0.83. The surface (0.5m) and the mid sample (2m) showed values of 0.71 and 0.66 respectively. For 3.5m depth sample,

five of the carbon compounds (asparagine AA2, serine AA4, D-cellobiose C1, D-mannitol C6 and D-galacturonic ac. CA3) reached average utilization values of 2 or higher. However, for surface samples, these values were only reached for two carbon sources (D-galactonic acid C10 and D-galacturonic CA3). Additionally, clear differences in the types of metabolized carbon sources with depth were found. Amino acids (especially AA1 and AA2) were mostly used in the 2m and 3.5m depth. Carboxylic acids such as D-galacturonic acid (CA3) were metabolized intensively in practically the entire lake. By contrast, degradation of some polymers such as

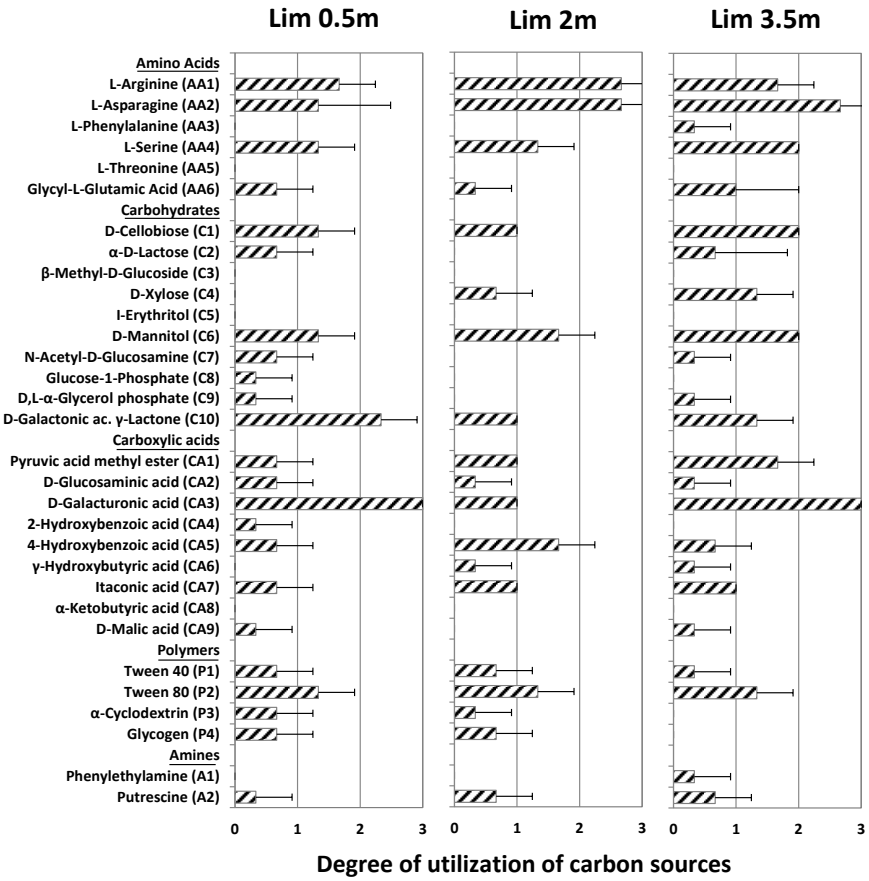


Figure 7.4. Average utilization profiles for the 31 carbon source tested for three depths in Lake Limnopolar.

cyclodextrin (P3) or glycogen (P4) was higher in the middle depth and in the lake surface waters.

The results of a Principal component analysis (PCA) reflected the high environmental heterogeneity of the selected lakes, explaining about 60% of the variation on Axis 1 and to 72% on Axis 2 (Figure 7.5). The plot showed a segregation into three lake groups, when the lower and higher utilization patterns were discriminated through Axis 1. Firstly, lakes Limnopolar and Chica, a second group formed by lakes Refugio and Maderos and finally Lake Somero grouped alone. The grouping of the lakes Limnopolar and Chica was mainly supported by lower usage of most compounds and by the high utilization of D-galacturonic acid (CA3) in both lakes compared with the other studied lakes. The second group (Refugio and Maderos) showed a strong use on most of the amino acids, carbohydrates (particularly the β-methyl-glucoside (C3)) and some of the polymers such as cyclodextrin (P3). Finally, Lake Somero was discriminated from the coastal lakes mainly by Axis 2, linked to the

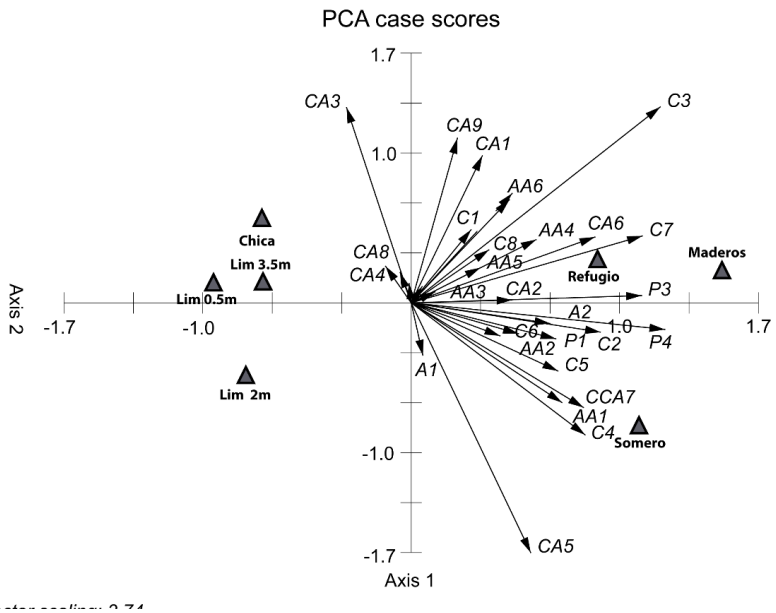


Figure 7.5. Principal component analysis for the five studied lakes in Byers Peninsula showing their grouping according to their carbon source´s utilization degree. Codes used for the different carbon sources are the same reflected in Figures 7.3 and 7.4.

specific utilization of diverse type of compounds like D-xylose (C4), itaconic acid (CA7) and putrescine (A2).

7.5. Discussion

Byers Peninsula displays a relatively great environmental heterogeneity for their different ecosystems, soils, lakes, streams, etc… (Toro et al. 2007, Villaescusa et al. 2010, Kenarova et al. 2013, Rochera et al. 2013). In our case, the five studied lakes of Byers Peninsula showed very different physical and chemical characteristics, as well as heterogeneous trophic status (Table 7.1). These particular limnological features have been commonly associated with two main factors: *lake depth* (closely related with the sediment nutrient recycling and contributions from benthic mosses) and the *external nutrient contributions,* especially supply of organic matter from the marine fauna or nutrient inputs from microbial mats covering the lake proximities (Izaguirre et al. 2003, Schiaffino et al. 2009, Villaescusa et al. 2010).

The limnological heterogeneity in lakes of Byers Peninsula is also related to the origin of the organic carbon, which can be studied by means of the colored dissolved organic matter "CDOM" (Villaescusa et al. 2010). Results in the mentioned study showed that maritime Antarctic lakes present autochthonously-produced organic matter, mainly protein-type CDOM, coming from both the plaktonic (phytoplankton) and benthic (mosses) primary production. However, this carbon pool is supplemented by external inputs of organic matter during the austral summer, mainly humic-CDOM from microbial mats and mosses covering the lakes catchment. These allochthonous compounds mainly comprise algal cryoprotectants, remains of cyanobacteria, microalgae mucilage or exudates and degraded compounds from mosses (Kenarova et al. 2013). Additionally, coastal lakes receive relatively high inputs of allochthonous organic matter, mainly compounds derived from fertilization processes from marine mammals and birds (Hansson 1992, Hansson et al. 1996, Izaguirre et al. 2001, 2003, Gil-Delgado et al. 2013a,b). The existence of this heterogeneity between the studied

lakes supports the study of the physiological activity of the microbial community from a metabolic point of view.

Both regarding the already described heterogeneous composition of organic matter for the studied lakes (Villaescusa et al. 2010) and the physiological diversity values provided by the AWCD index in the present work, the shallow lakes with higher trophic status (Maderos, Refugio and Somero) showed the greater physiological diversity among the studied lakes. However, the organic carbon compounds mainly metabolized in each lake were not homogeneous, showing differences in the physiological diversity. The results of PCA analyses describing this environmental heterogeneity, grouped the five studied lakes in three different clusters according to their average carbon utilization profiles (figure 7.5). First of all there were a group of shallow lakes with high trophic status such as Lake Refugio and Lake Maderos, characterized by the presence of a high bacterial physiological diversity. The second major group included the oligotrophic and deep plateau lakes Limnopolar and Chica with the lower bacterial physiological diversity. Additionally, a third type was discriminated in the analysis for the case of Lake Somero. This lake in spite of being located in the plateau, displayed a higher bacterial physiological diversity compared to the other oligotrophic plateau lakes, in consonance with its higher trophic level (Villaescusa et al., 2010).

For the first group of lakes, Refugio and Maderos (Figure 7.3), some organic compounds are more intensely metabolized than others. Thus, amino acids, such as L-arginine (AA1) and L-asparagine (AA2), that are a common source of carbon, nitrogen and energy derived mainly from primary production in Antarctic freshwater ecosystems (Barbaro et al. 2014), are easily metabolized by the bacterioplankton. Also, like amino acids, carbohydrates that represent an important fraction in the readily used dissolved organic matter in lakes (Hanisch et al. 1996), are easily metabolized in lakes Refugio and Maderos. However, the utilization of β-Methyl-Glucoside (C3) only in these two lakes, discriminate Lake Refugio and Lake Maderos from the rest. This compound is a monosaccharide derived from glucose by an acid

degradation or decomposition in presence of methanol. The high trophic status in these lakes could be the reason of this kind of decomposition reactions, forcing bacteria to assimilate glucose in the form of β-Methyl-Glucoside or other less profitable forms. In addition, the strong use of polymeric substrates like Tween 80 (a polyol derived from bacterial fatty acids and related to oleic acid) reflects the availability in lake water of compounds produced by microbial mats and incorporated to the lake by run-off processes. These organic sources are commonly used in lakes due to their easy assimilation (Chróst et al. 1989, Jørgensen & Jensen 1994, Choi & Dobbs 1999). The diverse metabolic activities displayed in lakes Refugio and Maderos could be related to the presence of heterotrophic bacteria of the genus *Flectobacillus sp.* (Villaescusa et al. 2010, Villaescusa et al. 2013a), that is not present among the other studied lakes. McGuire et al. (1987) described the presence of *Flectobacillus glomeratus* in the Antarctic coastal lake Burton. This bacteria is a psychrophilic heterotrophic organism that displays a wide range of metabolic activities and was also present in the coastal lakes of Byers Peninsula.

The second group in the PCA analysis grouped the lakes Limnopolar and Chica. As already noted, these lakes are situated in the central plateau of Byers Peninsula and are deeper compared to those situated in the coastal area. The main reason to cluster these lakes in the PCA analysis was their less diverse physiological profiles (Figure 7.3) in the Biolog ecoplates assays. This discrimination was also supported by the presence of an intense metabolic activity over the carbon compound D-Galacturonic acid (CA3) that represents an important difference with the others lakes. This compound is an oxidized monosaccharide form of D-galactose that is present in the bacterial mucilage (Nichols et al. 1999) produced by the microbial mats (Bender et al. 1997), which cover part of the catchment of these lakes (Fernández-Valiente et al. 2007). This reflects the close dependence of the carbon cycle of these lakes and the allochthonous organic carbon inputs from the catchment microbial mats.

Lake Somero was appears alone in the PCA biplot. This lake displays comparably high physiological diversity to that of the eutrophic coastal lakes from the

first group. However, the utilization profiles of Lake Somero showed important differences with the coastal lakes. First of all, the absence of metabolic activity over β-Methyl-Glucoside (C3) that was strongly used in the coastal lakes, revealed an important difference in the physiological profile. The less trophic status in this system compared with the coastal ones and the lack of marine mammals and birds could be related with the absence of this compound in this shallow plateau lake. However, the compound that most clearly discriminated Lake Somero from the other lakes was Putrescine (A2). This compound is produced by the degradation of amino acids and is commonly found in the lacustrine sediments. The extreme shallowness and the effect of the wind favor the removal processes and increases the availability of this compound in the water column. Thus, the heterotrophic bacterioplankton in Lake Somero could found in this organic compound a relatively abundant carbon source. Bacteria such as *Sphingomonas sp.*, whose presence have been demonstrated for Lake Somero (Villaescusa et al. 2013a), and are well recognized for their metabolic versatility and ability to degrade various organic compounds derived from biological activity (Dworkin et al. 2006), could be linked to the relatively high putrescine degradation.

All these facts demonstrated that differences in the physiological profiles for the studied lakes can be related with the origin of the organic matter and the relation of each lake with their catchment and the existing flora and fauna. This has also been demonstrated for soil bacterial diversity in Livingston Island (Kenarova et al. 2013), who found that the type of vegetation contributes to the pattern of bacterial carbon utilization rate and the physiological diversity of the bacterial community.

As we have already shown, the results of the principal component analysis revealed that the lakes with the highest trophic status also showed a more diverse physiological activity. This contrasts with the observed results of the comparative study conducted in the same lakes of Byers Peninsula (Villaescusa et al. 2010), where it was revealed that lakes with higher trophic status display lower bacterial taxonomic diversity. Linked both results, eutrophic coastal lakes display a low taxonomic

diversity of bacterial groups but a greater physiological diversity. This can reflect the appearance of non-specialist species with wider-range metabolic activities that fits better under a higher trophic status and a wide presence of organic matter types. On the contrary, oligotrophic lakes situated in the plateau with higher bacterial taxonomic diversity displayed lower physiological diversity. This could imply the existence of non-dominant specialist bacterial species that display a less variety of metabolic capabilities. Additionally, the shallow lakes situated in the plateau such as Lake Somero display an intermediate status compared to coastal lakes and deeper oligotrophic plateau lakes. All these could reflect the existence of an inverse gradient between higher trophic status and physiological diversity.

Though lakes with different trophic status display marked differences in the bacterioplankton physiological diversity, do these differences exist along the water column in the deeper lakes?. Results for the study within the vertical profile of Lake Limnopolar show the existence of clear differences in the metabolic activity and the degree of carbon source´s utilization with depth (Figure 7.4). Some of these differences can be explained by the existence of the so-called "biological stratification in the lake", as was demonstrated by Villaescusa et al. (2013a). This stratification appears as a consequence of the differential organic carbon contributions from the benthic mosses that also creates a physically more stable environment close to the sediment. The greater utilization of D-galacturonic acid (CA3) in Lake Limnopolar surface (0.5m) and bottom (3.5m) samples compared to that of the intermediate sample (2m), reflects the relative influence of the allocthonous non-planktonic sources of organic matter from the microbial mats (surface sample) and the influence of benthic mosses (bottom sample), respectively (Villaescusa et al. 2010). The physiological activity over this compound can be related with the activity of *Sediminibacterium sp.* bacteria, that is commonly associated with sediments. Also some species of *Clostridium sp.*, like *C. psychrophillum*, *C. frigoris* or *C. lacusfryxellense* , that are common users of carboxylic acids, can be responsible of this differential use in surface and bottom samples (Spring et al. 2003, Villaescusa et al. 2013a,b). This result is also supported by the PCA analysis where Lim 2m sample

was slightly separated from the others mainly by a lower utilization of D-galacturonic acid. Therefore, physiological diversity along the water column also reflects the abundance, availability and diversity of the organic carbon compounds.

In conclusion, our study of the dissolved organic carbon utilization in maritime Antarctic lakes allowed a proper description of the bacterioplankton physiological diversity during the austral summer, and is consistent with the patterns of within lakes heterogeneity already demonstrated using other approaches. Also, the knowledge of the organic carbon compounds used in each lake (or within different lake layers) represents a useful tool to understand the bacterioplankton dynamics and the origin of the organic matter that sustains bacterial production in these lakes. In fact, Biolog Ecoplates™ can be used to understand the potential functional capacity of the bacterial community in Antarctic lakes and also help to monitor changes over time (Garland 1997, Preston-Mafham et al. 2002).

8. Response of the planktonic microbial community of Antarctic lakes to resource availability: *in-situ* manipulation experiments

Villaescusa JA, Rochera C, Díazmacip ME, Velázquez D, Quesada A, Camacho A. Response of the planktonic microbial community of Antarctic lakes to resource availability: *in-situ* manipulation experiments (In preparation to be submitted to Microbial Ecology)

8.1. Abstract

Maritime Antarctic lakes present a great development of microbial communities during the austral summer, due to the milder temperatures and the increased inputs of inorganic nutrients and organic matter associated to run-off processes. The relationship between planktonic communities, light availability, inorganic nutrients and organic carbon additions have been simulated and the response studied by means of an *in-situ* manipulation experiment. Our study revealed the effects of inorganic nutrient fertilization on phytoplankton primary production, and its relationship with light availability. The experiment also showed the photoinhibition effect of light over the planktonic production and the phytoplankton response. Glucose additions, however, had a negligible effect on bacterial production, suggesting the existence of specialist bacterial species that perform better using dissolved organic carbon derived from the autochthonous primary production and from allochthonous sources such as exudates from microbial mats.

Keywords: Bacterioplankton, Phytoplankton, "in-situ" bioassay, microbial foodweb

8.2. Introduction

Aquatic systems in Antarctica experience extreme weather conditions, commonly being covered by ice or snow during the whole year. The study of these systems, especially those situated in the McMurdo Dry Valleys area (Hawes et al. 1993, Priscu 1998) has demonstrated that the dynamics and structure of the their biological communities are restricted by a strong control by extreme environmental features. However, lakes situated in the coastal areas of Antarctica, especially those located in the maritime Antarctic region, suffer less severe weather conditions and relatively mild summers (Vincent 2000, Vincent et al. 2008, Convey 2011, Villaescusa et al. 2013b). During the austral summer, the higher temperature and irradiance allow the melting of the ice cover of lakes situated in this area. The melting process is followed by a large development of the planktonic and benthic biological community due to the greater availability of light and nutrients and the higher temperatures. This microbial community plays an important ecological role in the water column driving the system summer dynamics (Ellis Evans 1996, Camacho 2006a, Toro et al. 2007, Vincent et al. 2008). Additionally, during this period, the increase in lake planktonic productivity is supported by external contributions from microbial mats and mosses growing in the lakes catchment and also by organic matter produced by benthic mosses covering the lake bottom (Schiaffino et al. 2009, 2011, Villaescusa et al. 2010, Villaescusa et al. 2013b). These external contributions support the lake´s primary and secondary production and influence the composition and activity of the planktonic communities (Villaescusa et al. 2013a).

In order to study the effect of nutrients and light availability on the planktonic community, an in-situ experiment using microcosms in Lake Limnopolar. This lake, located in the maritime Antarctic region, has been a source of diverse scientific works over the last decade by the Limnopolar research group as summarized in Benayas et al. (2013).

The use of manipulation experiments in freshwater ecosystems has greatly contributed to the understanding of the ecological processes during the last decades (Blumenshine et al. 1998, Dorado-García et al. 2014). These experiments have been conducted at variable scales ranging from small containers to whole lakes (Carpenter et al. 1995). The use of whole-system manipulations is always desired due to their substantial advantages, however, they cannot always be used due to specific ecosystem limitations, and the use of microcosms is a suitable alternative for many ecosystems.

Nutrient addition experiments represent the most frequent type of ecosystem manipulations, as they reveal important features of the ecosystem functioning and are relatively easy and inexpensive. Also, because of the logistic limitation, microcosms experiments are the most suitable in Antarctic lakes allowing to obtain significant results (Sawatrom et al. 2007).

In this experiment several physical and chemical variables were manipulated to simulate different possible scenarios affecting the planktonic communities. The experiment was focused on the manipulation of three environmental features: inorganic nutrient concentration, glucose addition as nutrient for heterotrophic bacterioplankton, and photosynthetic active radiation (PAR) availability. The production rates of the planktonic community were evaluated in the different treatments to study the response of both, the phytoplankton and bacterioplankton. The simulation of different environmental scenarios affecting the planktonic community on Antarctic lakes can be used as a reliable strategy to understand and predict the possible shifts in these systems and the response to these changes of the biological communities.

8.3. Material and methods

8.3.1. Sampling site

The sampling site is located in the area of Byers Peninsula, situated at the western end of Livingston Island (62º34´35´´ - 62º40´35´´ S and 60º54´14´´ - 61º13´07´´ W), in the South Shetland Islands. Byers Peninsula is one of the largest ice-free areas of the maritime Antarctica. The proximity to the sea favors a less harsh climate compared with Continental Antarctica, having warmer summers with temperature around 1-3ºC, and higher precipitations (Rochera et al. 2010, Villaescusa et al. 2013b). These features allow the appearance of abundant water bodies with characteristic planktonic and benthic microbial communities (Toro et al. 2007, Villaescusa et al. 2010).

The selected lake, Lake Limnopolar, is largely known with respect to its ecological structure and dynamics. This knowdlege, along with its relatively low complex food web, mainly dominated by microorganisms, made this lake a suitable model to further studying ecological interactions by means of manipulation experiments (Camacho 2006a, Toro et al. 2007, Rochera et al. 2010, Villaescusa et al. 2013a,b). Lake Limnopolar is a small lake (2.2 ha) situated in the central plateau of Byers Peninsula, with a relatively shallow depth (4.5m during the austral summer) and a scarcely vegetated drainage watershed covering a surface of 0.582 km^2. This area is mainly covered by microbial mats and moss carpets that contribute with inorganic nutrients and organic matter, as result of their biological activity, to the lake inputs by different run-off flow processes. Also, the benthic population of mosses (*Drepanocladus longifolius*) covering the lake bottom provides organic carbon, increasing their availability in the water column.

8.3.2. Manipulation experiment

During the summer of 2008-09, a sampling was conducted at the middle point of Lake Limnopolar. Water of different depths (0.5, 2 and 3.5m) was collected using a hydrographic bottle (Uwitech) and stored in five liter acid-washed plastic carboys (microcosms). The water of the three different depths was mixed in a single water set and used to develop the microcosm manipulative experiment. Water was used for the experiment was filtered using a 30 µm nytal mesh to eliminate the mesozooplankton, mainly the copepod Boeckella poppei, in order to eliminate the effects of mesozooplankton predation. Then, 8 different manipulation treatments were established combining manipulations of different features: inorganic nutrients addition, organic carbon addition and reduction in the incident light. A treatment without manipulation was used as a control for the experiment.

The mentioned treatments were prepared placing 4.5 l of the filtered water in five liters acid-washed plastic carboys. All, treatments with inorganic nutrient addition were amended to a final concentration of 39 µM of nitrogen and 2.4 µM of phosphorus approximately following the Redfield ratio. For the treatments with glucose addition, 15 ml of glucose was added to a final concentration of 250 µM. At last, a metallic mesh (that absorb approximately 50% of the incident light) was used to cover the treatments where the incident light was reduced. According to these 3 factors (inorganic nutrient addition, glucose addition and light limitation), a total of 8 different combined treatments with 3 replicates each were established as shown in the Figure 8.1.

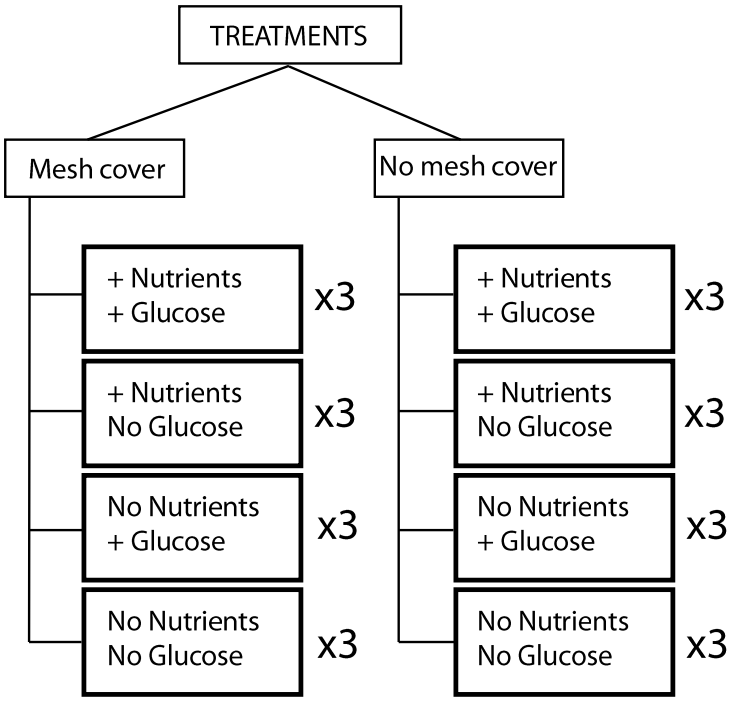


Figure 8.1. Graph showing the 8 different manipulation treatments with their 3 replicates. The different treatments included light availability manipulation (Mesh cover), Glucose addition (+) and Nutrient addition (+).

All 8 treatments and their replicates were incubated in the surface of the lake under natural conditions of light and temperature for a period of six days. At the end of the experiment, the water of each treatment was processed to analyze the phototrophic pigments concentration, as a measure of phytoplankton abundance, as well as of bacterioplankton abundance.

For the quantification of Chlorophyll a concentration, a water fraction of each treatment was quickly filtered at the lake shore through fiber-glass filters (Whatman GF/F) and then stored at -20ºC for further analysis. To estimate the abundance of heterotrophic bacterioplankton, a volume of water of each treatment was fixed with 2% glutaraldehyde and stored at -20ºC for further counts in the laboratory.

8.3.3. Biological enumerations

Photosynthetic pigment analyses were performed by HPLC, as described by Picazo et al. (2013). Chlorophyll-a concentration was used as a marker of the phytoplankton abundance (Jeffrey et al. 1997).

For the heterotrophic bacterioplankton counts, one ml of a water sample previously fixed with 2% glutaraldehyde was incubated at room temperature with SYBR Green-I for 20 minutes. Samples were analyzed using a Beckman Coulter flow cytometer (Cytomics FC 500 MPL) with five fluorescent channels, following the procedures described by Gasol & del Giorgio (2000). Side-angle light scatter (SSC) served as a proxy for bacterial cell size (Trousellier et al. 1999). Fluorescents beads of 0.5 and 1 μm were used as size markers. Clusters of bacteria were counted by logical gating from FL2 vs. SSC histograms. Total heterotrophic bacterioplankton abundance and the relative abundance of bacterioplankton groups LDNA (low DNA) and HDNA (high DNA) were also measured according to Li et al. (1995). The active cell index (ACI) was calculated by dividing the number of HDNA bacterioplankton cells in each sample by the total abundance (Jellet et al. 1996).

8.3.4. Production assays

After the end of the microcosm experiment, some water of each treatment was used to carry out primary and bacterial production measurements. Water of each treatment was placed in 500 ml Nunclon® incubation bottles by duplicate and was incubated, with the same light conditions used for the experiment, for 3 hours in the lake to simulate natural conditions. For the quantification of primary production, 1.5 ml of a solution 1g l^{-1} $NaH^{13}CO_3$ solution was added to each bottle. For bacterial production 250 μl of 13.3 g l^{-1} ^{13}C-Leucine solution was used for incubation. Both samples were filtered in glass fiber filters GF/F and stored at -20ºC.

The samples were analyzed in a mass spectrometer (ICP-MS NexION 300XX Perkin-Elmer) and the results were expressed as percentage of each isotope. Primary production rates were calculated using the equations described in Fernández-Valiente et al. (2007). Specific planktonic primary production rate was referred per chlorophyll-a concentration and time.

Bacterial production was quantified by a variation of the method mentioned in Kirchman et al. (1985), using the stable molecule of ^{13}C-Leucine. In the calculation, it was assumed that the natural concentration of ^{13}C-Leucine in the lake was negligible. The following equation was used:

$$V_c = \frac{\%^{13}C_m - \%^{13}C_b}{\left(\left(100 \cdot \frac{^{13}C_a}{^{13}C_a + LEU}\right) - \%^{13}C_b\right) \cdot T}$$

Where:

Vc: Leucine specific incorporation rate (h^{-1})

% ^{13}Cm: ^{13}C percent in the sample after treatment

% ^{13}Cb: ^{13}C percent in the sample before treatment

^{13}Ca: ^{13}C-Leu concentration added

LEU: ^{13}C-Lue concentration in the lake

T: incubation time (h)

Once the specific leucine incorporation rate was calculated, the carbon incorporation rate for the bacterioplankton was estimated using the factor of 1.5 kg of carbon incorporated x mol of leucine incorporated (Simon & Azam 1989). In addition, we calculated the specific bacterioplankton production rates per cell.

8.3.5. Statistical analysis

To evaluate the possible differences between the assayed treatments, an ANOVA test for the following parameters was done: chlorophyll-a concentration, bacterioplankton

abundance, primary production and bacterial production. Additional post-hoc Tukey test were done to discriminate between the different treatments establishing groups with similar characteristics. SPSS (v.22.0.0.2) was used for these statistical analyses.

8.4. Results

After six days of experiment, final chlorophyll-a concentration (used as a marker of phytoplankton abundance) showed clear differences in the eight assayed treatments (Figure 8.2). The treatments where inorganic nutrients (Nut) were added showed an increase in the chlorophyll-a concentration, reflecting stimulation on phytoplankton growth. Conversely, treatments without inorganic nutrient addition, either with or without, glucose addition, showed no differential response. However, the increase in the chlorophyll-a concentration was only significant higher ($p<0.001$) in the treatments covered with the metallic mesh, with chlorophyll-a values three times higher compared to control treatments. Accordingly, chlorophyll-a concentration in the mesh treatment with the only inorganic nutrient addition reached values of 0.5 µg l^{-1} chlorophyll-a, and for the treatment with nutrients and glucose, values of 0.8 µg l^{-1} chlorophyll-a. All treatments, including controls, not covered by a mesh showed lower Chl-a concentrations than those covered by a mesh.

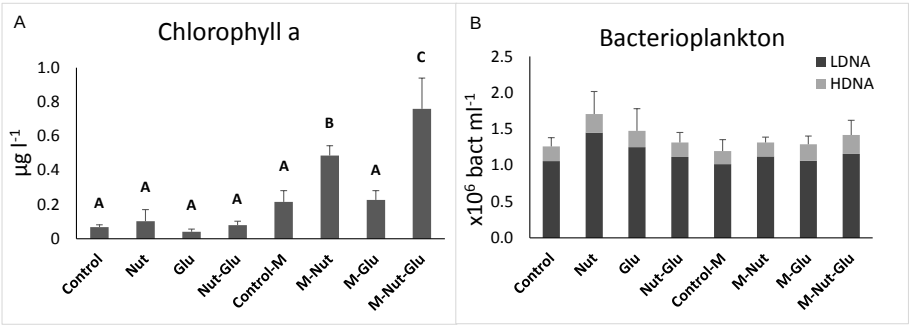


Figure 8.2. A) Final chlorophyll-a concentration values for each of the assayed treatments. Capital letters above the bars represent the three different groups established by the ANOVA analysis due to their significant differences. B) Final bacterioplankton abundance. Grey represents the high DNA bacteria (HDNA). Black represents the low DNA bacteria (LDNA). The ANOVA analysis does not shown significant differences among the different treatments. Nut: nutrient addition, Glu: glucose addition, M: covered with a metallic mesh.

The abundance of heterotrophic bacterioplankton showed final values comprised between 1.2-1.7 x10^6 cell ml^{-1} (Figure 8.2). The different treatments showed no significant differences (p=0.17) in the bacterioplankton abundances. The ratio between high DNA bacteria (HDNA) and total bacteria named as active cell index (ACI) increased slightly in the treatments covered with the metallic mesh and with glucose addition, though these differences were not significant.

As for the concentrations of Chl-a, the results of primary production (Figure 8.3) also showed significantly higher values ($p<0.001$) in the treatments covered by the metallic mesh compared to the uncovered. Among them, the four treatments without the metallic mesh did not show significant differences in the primary production rates, ranging 0.02-0.06 µg C l^{-1} h^{-1}. Contrastingly, primary production rates in the four treatments where the metallic mesh was used showed a significant differeces among the different treatments ($p<0.05$) response. Those with inorganic nutrient addition and covered by the metallic mesh displayed the highest rates, with values of around 0.9-1 µg C l^{-1} h^{-1}. The ratio between primary production and clorophyll-a (reflected as specific PPr) was also higher in the mesh covered treatments, but, among these, treatments amended with glucose showed no significant differences with the control, Meanwhile, mesh covered treatments amended with inorganic nutrients but without glucose showed significantly higher rates.

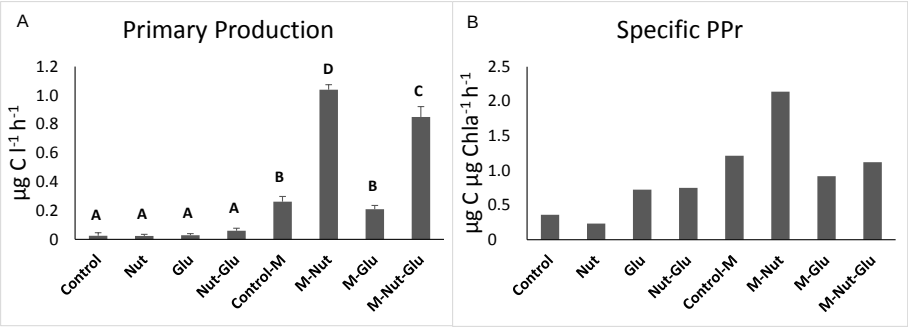


Figure 8.3. A) Primary production (PPr) values for the eight different treatments assayed. Nut: nutrient addition, Glu: glucose addition, M: covered with a metallic mesh. Capital letters above the bars represent the four different groups established by the ANOVA analysis due to their significant differences. B) Specific values of PPr for each treatment.

Bacterial production assays showed values around 0.5-0.8 µg C l^{-1} h^{-1} in seven of the assayed treatments (Figure 8.4). Remarkably, the treatment covered by the metallic mesh and with glucose and nutrient addition displayed significantly higher values ($p<0.001$), around 2.6 µg C l^{-1} h^{-1}.

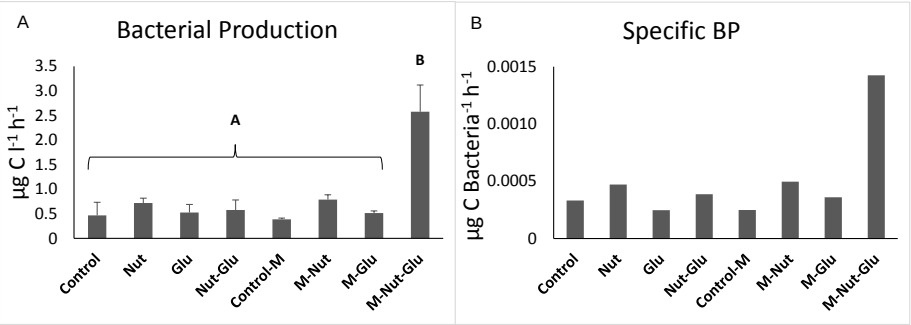


Figure 8.4. A) Bacterial production (BP) final values for the eight different treatments assayed. Nut: nutrient addition, Glu: glucose addition, M: covered with a metallic mesh. Capital letters above the bars represent the two different groups established by the ANOVA analysis due to their significant differences. B) Specific values of BP for each treatment.

8.5. Discussion

The general results of the manipulation in-situ experiments have revealed that the planktonic population of Lake Limnopolar is actively responding to the inorganic nutrient enrichment, and more actively in the treatments where the metallic mesh was used to reduce the incident light on the planktonic organisms. The positive effect of the nutrient addition on the phytoplankton abundance (Figure 8.2), both on chlorophyll-a concentration and on primary production rates (Figure 8.3), reflects a strong nutrient limitation of primary production in these aquatic Antarctic systems, as previously referred (Ellis-Evans 1996, Toro et al. 2007, Villaescusa et al. 2010). However, this response is clearly limited due to the inhibitory effect of light on the phytoplankton, especially in the lake surface layers, as those where incubations were done. This response of the phytoplankton to inorganic nutrient enrichment, derived by the high nutrient limitation, has been previously demonstrated in other Maritime Antarctic lakes (Izaguirre et al. 2003), especially during the first part of the austral

summer. During this period, the increase in temperature allows ice melting and run-off processes in the lake catchment. These features along with the abundant presence of microbial mats and mosses in the catchment (Fernández-Valiente et al. 2007, Villaescusa et al. 2010) increase the inputs of inorganic nutrients and organic matter into the lake.

Our experiment has demonstrated that the nutrient fertilization on Lake Limnopolar´s planktonic community produces an increase in the chlorophyll-a abundance and the primary production rates. However, this response of the phytoplankton was significantly restricted in the treatments where light irradiance was fully affecting the microcosms. This is likely showing that surface plankton communities are photoinhibited during the austral summer. At low latitudes, the harmful effect of light on the planktonic communities is well known, especially in the surface layer of lakes. Alderkamp et al. (2010) described the photodamage that the phytoplankton cells suffer when mixed up to the surface layers of Antarctic systems, reducing their carbon fixation efficiency. The negative effect of excess light has also been well studied on the microbial mats communities that cover the Antarctic lake catchments and wet areas (Fernández-Valiente et al. 2007, Velázquez et al. 2011, Rochera et al. 2013), describing their ecological response and the mechanisms to avoid the photodamage and increase the carbon fixation efficiency.

Accordingly the observed photoinhibition, the treatments where the incident light was reduced by 50% using a metallic mesh, showed higher phytoplankton abundance (Figure 8.2) and carbon fixation rates (Figure 8.3) compared with the treatments without metallic mesh. This increase was higher in the treatments with nutrient fertilization resulting from the already mentioned nutrient limitation (Ellis-Evans 1996, Toro et al. 2007, Villaescusa et al. 2010). Attending to the treatment where both nutrients and glucose were added, the chlorophyll-a concentration showed significant higher values compared to the treatment where only nutrients were added. The significant difference observed between these two treatments, reflected as group B and C in the ANOVA analysis (Figure 8.2), could be related to a high activity of

the bacterioplankton in the first steps of the experiment due to a crossover effect of glucose and inorganic nutrients. Thus, the increase in bacterioplankton production rates could drive in a greater availability of inorganic nutrients due to remineralization processes on the plankton organic remains, result further promoting phytoplankton growth. This effect is produced in spite of the low response of the bacterioplankton against the glucose addition treatments (Figure 8.4), though the possible co-limitation between glucose and inorganic nutrients already reported for oligotrophic systems (Dorado-García et al. 2014). However, this could just be a short-term response that is not reflected in the final primary production rates (Figure 8.3), whereas higher inorganic carbon fixation rates were observed in the treatment exclusively amended with inorganic nutrients fertilization. The treatment with glucose and inorganic nutrients (covered with the metallic mesh), in spite of being fertilized with nutrients, showed lower increase of carbon fixation rates, but instead higher bacterial production rates, which could be related with competition for nutrients between autotrophic and heterotrophic plankton.

The response of bacterial abundance and bacterial production to glucose additions was not significant, except for the bacterial production when both glucose and inorganic nutrients were added and the microcosm were exposed to reduced light. In the later, primary production was also increased as it was not photoinhibited, so the potential effect of glucose amendments on bacterial production was enhanced by the amendment of inorganic nutrients, and by the metabolites excreted by phytoplankton, overall resulting in significantly higher rates of bacterial production. In this treatment phytoplankton could provide bacterioplankton with a more equilibrated nutrient environment, with amino-acids and other organic carbon compounds excreted by these primary producers that enrich the organic carbon pool. This higher production was not reflected on bacterial biomass, suggesting that it was a short term effect or, at the least, it was not producing substantial bacterial population growth during most incubation time. The rest of glucose amended treatments did not result in significant increases neither on bacterial abundance nor in production. This could be showing a certain inorganic nutrient deficiency for bacterioplankton, which could be co-limited

together both by inorganic nutrients and organic carbon. (Dorado-García et al. 2014). Therefore, we can argue that glucose as a sole carbon source seems not to be sufficient to produce a significant effect over bacterioplankton community.

Also, if we attend to the active cell index (ACI) (Figure 8.2), that represents the ratio of active bacteria (high DNA) vs less active bacteria (low DNA) and can be used as a relative indicator of bacterial activity (Gasol & del Giorgio 2000, Jellett et al 1996), the results show a slight increase for the treatment covered with the metallic mesh and fertilized with glucose and inorganic nutrients. This supports the idea that a major productive rate should be related to a higher active bacteria, in our case a major ACI. However, the differences are not significant and these results could not be taken as strong evidence of the bacterial activity in the treatments with glucose addition.

On the other hand, although glucose is a carbohydrate easily metabolized by bacteria and commonly employed in some aquatic ecology experiments (Kamjunke et al. 2008), the non-significant effect of glucose over the bacterial population could be related to the dominance of specialist bacteria with metabolic preference over dissolved organic compounds produced by phytoplankton, benthic mosses or allochthonous microbial mats (Chapter 7). The major source of these organic matter compounds is derived from the authoctonous activity of benthic mosses and the allochthonous activity of microbial mats and mosses covering the catchment of Lake Limnopolar. Thus, the lack of these continuous contribution of organic compounds in our enclosures could result in the lower response observed both in bacterial abundance and production.

Among the dominant bacterial taxa described for Lake Limnopolar are those of genus *Sphingomonas sp.*, (Villaescusa et al., 2010) , an specialist bacterial taxon with metabolic preferences over easily metabolized amino-acids and carboxylic acids compounds (Dworkin et al. 2006) like D-Galacturonic acid. In fact, among several lakes of the area, Lake Limnopolar showed the lower bacterial growth on carbohydrates, as revealed from the physiological pattern has been described using

Ecoplate analyses (Chapter 7), that instead showed the preference of its bacterial community on this kind of compounds., D-Galacturonic acid is an allochthonous compound produced by the bacteria living in the microbial mats as those covering the lake catchment (Bender et al. 1997, Nichols et al. 2005, Fernández-Valiente et al. 2007). These external inputs of organic matter directly affect the lake planktonic community, subsidizing the bacterial production in Lake Limnopolar (Chapter 5) producing an uncoupling process between primary and bacterial production that has been described as previously mentioned.

In conclusion, the results of our in-situ manipulation experiment describe the response of the planktonic community to fertilization processes and the response of phytoplankton over the incident light during the austral summer. The effect of light photoinhibition in these aquatic systems have been clearly demonstrated in the response of the planktonic communities. Additionally, the inorganic nutrient limitation appears as an important factor that drive the productive processes in the planktonic community, both for phytoplankton and for heterotrophic bacterioplankton. Attending to the low response of the bacterial community to glucose addition it can be deduced that the bacterioplankton from Lake Limnopolar is dominated by specialist bacterial taxa that prefer dissolved organic matter compounds derived from phytoplankton activity or other external inputs like microbial mats or mosses, revealing the possible co-limitation between carbon and inorganic nutrients in this oligotrophic system.

9. Zooplankton epibiosis as a response to oligotrophy in Antarctic lakes

Villaescusa JA, Rochera C, Diazmacip ME, Camacho A. Zooplankton epibiosis as a response to oligotrophy in Antarctic lakes (In preparation to be submitted to Polar Biology)

9.1. Abstract

The extreme lifestyle of the organism inhabiting Antarctic lakes requires of different strategies to survive in these harsh ecosystems. In ultraoligotrophic systems as some Antarctic lakes, epibiosis can be a strategy to increase access to resources, especially to organic and inorganic nutrients. Epibiotic algae in these systems can live attached to metazooplankton to take advantage of their high mobility and using their excretions, allowing an improved access to nutrients in these oligotrophic systems. The present study shows the response of algal epibionts, represented as diatoms and euglenophytes, living on copepods (*Boeckella poppei*) to variations in nutrient concentrations using manipulative experiments performed in the Antarctic Lake Limnopolar. Our results showed an increase in algae epibionts as response to a reduction in inorganic nutrient concentration. This can be associated with a nutritional response of the algae epibionts that in this case, could take advantage of the copepods to increase their inorganic nutrient availability.

Keywords: Epibiosis, Copepods, Plankton, Antarctic lakes

9.2. Introduction

The living conditions in Antarctic lakes are extreme, with low temperatures, limited light availability and scarcity of nutrients. Still, lakes such as those situated in the maritime Antarctic region, display a marked development of planktonic and benthic microbial communities during the austral summer (Ellis-Evans 1996; Toro et al. 2007; Schiaffino et al. 2009; Villaescusa et al. 2010). Despite the common ultra-oligotrophic conditions of these lakes, microbial communities successfully grow, forming simple food webs that can be further controlled by the grazing activity of metazooplankton (Camacho 2006a). Zooplankton can hold algal epibionts (Ikeda 1977; Gibson 1979; Threlkeld et al. 1993; Carman and Doobs 1997; Fernández-Leborans 2010), although the ecological role of this non-parasitic relationship is unclear.

Epibiosis in crustacean zooplankton has been studied for a long time in aquatic systems (e.g. Threlkeld et al. 1993; Carman and Dobbs 1997; Barea-Arco et al. 2001; Gárate-Lizárraga & Muñetón-Gómez 2009; Fernández-Leborans 2010), being considered from different points of view: ecological, physiological and evolutionary. Some hypotheses have been raised to explain these relationships. In Antarctic lakes where the nutrient availability is an important issue, one of the main hypothesis is that epibiosis is a response to oligotrophic conditions. Epibiotic algae live attached to metazooplankton to take advantage of their high mobility and using their excretions, allowing an improved access to the nutrients in these nutrient-poor lakes. The present study experimentally assesses the occurrence of algae epibionts on the dominant copepod *Boeckella poppei* at different levels of nutrient availability in maritime Antarctic lakes.

9.3. Material and methods

9.3.1. Study area

Our study was developed in Byers Peninsula, Livingston Island, South Shetland Islands (62º34´35´´ - 62º40´35´´ S and 60º54´14´´ - 61º13´07´´ W). Byers Peninsula is one of the largest ice-free areas of the maritime Antarctica, situated at the western end of Livingston Island. The climate in this part of the Maritime Antarctica is less extreme than in Continental Antarctica, with mean summer temperatures of about 1-3ºC (Bañón 2001; Rochera et al. 2010). These summer temperatures contribute to the presence of diverse ice-free water bodies where planktonic and benthic microbial communities are profusely settled (Toro et al. 2007; Villaescusa et al. 2010).

Lake Limnopolar is a small (2.2 ha), relatively shallow (4.5 m maximum depth during the summer ice free period) water body located in the central plateau of Byers Peninsula. The lake has a scarcely vegetated drainage watershed of 0.582 km^2 covered mainly by microbial mats and moss carpets where most of the catchment run-off flows into the lake through several small streams. The lake displays a marked oligotrophic status with low inorganic nutrient concentration and low phytoplankton abundances (Toro et al. 2007; Villaescusa et al. 2010). The planktonic food web has low complexity, being mainly dominated by microorganisms (Camacho et al. 2006a; Toro et al. 2007; Rochera et al. 2010). Crustacean zooplankton dominates the microbial food web as main predators, and is primarily composed by the calanoid copepod *Boeckella poppei* and the fairy shrimp *Branchinecta gaini* (Paggi 1987; Toro et al. 2007).

9.3.2. Sampling and experimental setup

The influence of environmental nutrient concentration on the relative abundance of epibiont algae on the copepod *Boeckella poppei* (Figure 9.1) in Lake Limnopolar was studied by means of a microcosm experiment. The water used for the experiment was

collected in the mid part of the lake and filtered using a 50µm mesh net to remove metazoan. This comprised the setup of transparent PVC carboys (5 L) showing increasing nutrient concentrations. First, a treatment consisted in the direct dilution of lake water by 5 times, using nutrient-free Milli-Q distilled water. Another treatment was maintained without manipulation and used as a control (1x). The other four treatments, conducted by triplicate, consisted in an increasing nutrient addition as follows: 2x, 10x, 100x and 300x, from a starting nutrient stock of 3 µM NH_4NO_3 and 0.4 µM NaH_2PO_4. Each carboy was then filled with 60 copepods and these were incubated at the site for 14 d. After whose, all copepods from each carboy were captured and starved for 24 h to clean up their guts from ingested algae. Subsequently they were stored at -20ºC. The remaining water was then filtered through GF/F Whatman filters and stored at -20ºC. Once in the lab, the chlorophyll-a content in the copepods (corresponding to the epibiotic algae) and in the seston was extracted overnight in pure acetone and quantified by HPLC as described by Picazo et al. (2013).

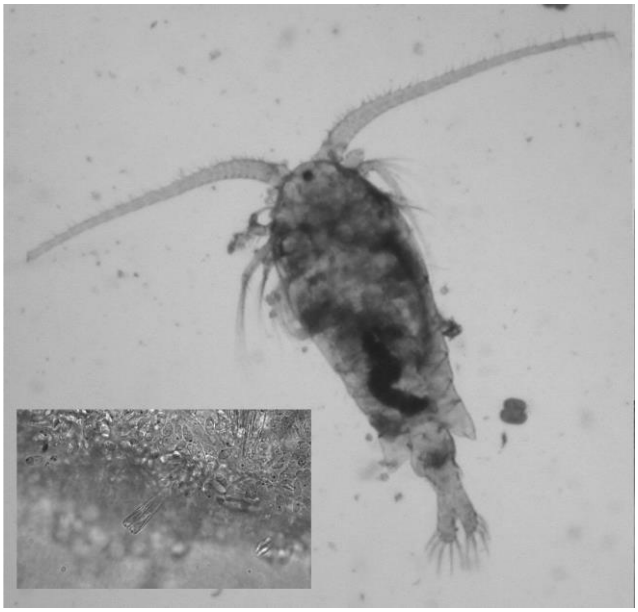


Figure 9.1. Algae epibionts on the surface of *Boeckella poppei*

9.4. Results

The concentrations of chlorophyll-a (Chl-a) were evaluated separately in the copepods and seston at each treatment at the end of the experiment (Figure 9.2a). The Chl-a concentrations in the controls of both compartments were in average 0.011 µg Chl-a Cop^{-1} and 0.026 µg Chla l^{-1} respectively. In contrast, the Chl-a content in copepods was higher in the five-fold diluted treatment because the notable reduction observed in the seston concentrations (<0.01 µg Chla l^{-1}). In fertilized samples, Chl-a concentrations increased gradually in both epibiotic and plankton compartments as nutrient additions were higher until the treatment 10x, getting in this later similar mean values of 0.042 µg Chl-a Cop^{-1} and 0.041 µg Chla l^{-1} respectively. On the other hand, concentrations decreased notably in the containers submitted to higher nutrients concentrations (100x and 300x), particularly in treatment 300x, where epibiotic Chl-a was even undetectable.

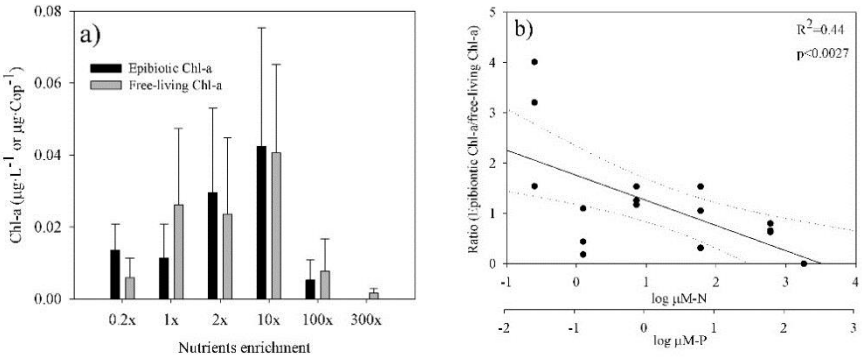


Figure 9.2. a) Algal epibiotic Chl-a concentration (black) and free-living Chl-a concentration (grey) in each nutrient manipulation treatment. b) Relation between epibiont Chla concentration and free-living Chla concentration as a function of the concentration of nutrients.

The epibiont algae Chl-a concentration was divided by phytoplankton Chl-a concentration to be used as an estimator of the relative increase in algae epibionts, mainly represented as diatoms and euglenophytes. (Figure 9.2b). From the previous mentioned estimator, ratios were calculated for all treatments except for 300x since

epibiotic Chl-a was undetectable in this case. The values of this relationship and nutrients content in samples significantly correlated, being lower as fertilization augmented (Fig. 9.2b). The average of these ratios varied from 2.3 in the treatment five-fold diluted to 0.2 in the treatment enriched 100 times.

Beyond the significance of the Spearman correlation, pairwise analyses also showed significant differences among some treatments. Thus, the Tukey post hoc analysis showed this ratio to be significantly higher in five-fold diluted treatment compared to treatments 1x (p-value=0.011), 10x (p-value=0.032) and 100x (p-value=0.015). On the other hand, differences between other treatments were not significant.

9.5. Discussion

Studies of epibiosis on copepods around the world, have revealed that diatoms are commonly the main epibiotic microalgae on marine systems (Carman & Dobbs 1997). However, in low salinity waters and epicontinental systems, epibiotic diatoms are not the dominant fraction, with other photoautotrophic microalgae like euglenophytes also living as epibionts (Shelton 1974; Møhlenberg & Kaas 1990). Some hypotheses have been made to explain the appearance of this kind of relationship between copepods and microalgae. Different authors (Ikeda 1977; Threlkeld et al. 1993; Carman and Doobs 1997; Fernández-Leborans 2010) suggested that epibiosis can be explained as a mobility function or mating success. However, some authors already considered the epibiosis from a nutritional point of view (Ikeda 1977, Gibson 1979).

Results obtained with our manipulation experiments agree with the hypothesis that considers epibiosis as a nutritional strategy. Thus, results of epibiont Chl-a and planktonic Chl-a ratio (Figure 9.2b) display an increase in the relative algae epibiont abundance (expressed as epibiont Chl-a concentration) when the inorganic concentration was diluted. As previously mentioned, these results could be explained by the appearance of a nutritional strategy by the algae epibionts in response to an

extreme oligotrophia. In our case, the algal epibionts take advantage of the copepod nutrient excretions to increase access to inorganic nutrient in such an oligotrophioc environment.

Thus, the nutritional epibiontic strategy mentioned above is also explainned in the inorganic enriched treatments, showing a decrease in the algae epibiont ratio as response to an increase in the inorganic nutrient availability. In this case, epibiosis could be a less profitable strategy for epibionts that prefer to live separated from their host. However, the extreme inorganic nutrient lack or enchicment could derive in a dramatic episode for algae epibionts, reflecting a stress situation without nutrients of with toxic concentrations of them in the case of an extreme fertilization.

To conclude, our results demonstrate the existence of epibiosis as an efficient strategy for some algal groups, like diatoms and euglenophytes, to increase the access to inorganic nutrients in ultra-oligotrophic environments such as Antarctic lakes. Therefore, crustacean zooplankton exerts a commensalism relationship with the studied algae epibionts, being an inorganic nutrient pool for these algal groups.

10. Carbon dynamics modelization and biological community sensitivity to temperature in an oligotrophic freshwater Antarctic lake

Villaescusa JA, Jørgensen SE, Rochera C, Velázquez D, Quesada A, Camacho A (In press) Carbon dynamics modelization and biological community sensitivity to temperature in an oligotrophic freshwater Antarctic lake. Ecological Modelling

10.1. Abstract

Lake Limnopolar, located in one of the areas on Earth experiencing the strongest local warming, has been studied as a maritime Antarctic lake model by the Limnopolar Research Team during the last decade. Data collected during this period revealed the existence of an important meteorological interannual variability in the area of Byers Peninsula. With the aim of increasing the knowledge of this ecosystem and its sensibility to climate change as a model ecosystem, as well as to calibrate the extent of the interannual variability, a carbon flow model was developed partly describing its microbial food web. This preliminary model aims to describe part of the carbon dynamics, especially for bacterioplankton and associated factors, in this maritime Antarctic lake highly affected by temperature increase linked to regional warming. To describe the system, the effects of the variation of different forcing functions influencing the carbon flow within the microbial community, like temperature and irradiance, were studied. Among the studied factors, the sensitivity analysis showed the strongest response of the model to temperature changes. Consumption rates of organic matter by bacterioplankton, and therefore its abundance in lake water, greatly increased when temperature rise was higher. However, the highly variable meteorology over years in such an extreme environment causes that our model may fit well for some years, but fails to describe the system in years with contrasting meteorological conditions. Despite this assumption, the model reveals that maritime Antarctic lakes are very sensitive to temperature changes. This response can be monitored using eco-exergy, which allows a description of the system complexity. Due to this temperature sensitivity, the warming occurring in this area would lead to significant changes in the carbon flow, and consequently on the abundance of plankton in these systems.

Keywords: Maritime Antarctic lakes, Ecological model, Carbon flow, Bacterioplankton, Temperature sensitivity, Climate change

10.2. Introduction

During the last decades, empirical evidences have led the scientific community to the search for clues and indicators of the nature of climate change (IPCC 2007). Maritime Antarctica, which includes the west Antarctic Peninsula and surrounding islands, is probably one of the most affected areas by the rising temperatures (Clarke et al. 2007, Russell and McGregor 2010). The climate in maritime Antarctica is less extreme than in continental Antarctica, with mean temperatures between 1-3ºC during summer (Toro et al. 2007; Bañón et al. 2013). The Maritime Antarctica is rich in water bodies that become ice-free during the Austral summer. Among them some representative lakes are good candidates as indicators of global change, particularly those located in Antarctic Special Protected Areas (ASPA) with almost no direct impact caused by human interference. Some of these protected areas, such as Byers Peninsula, located in Livingston Island (South Shetland Islands), have been the subject of extensive limnological studies since 2001 by the Limnopolar Research Team (Quesada et al. 2013). These studies have shown most ecological features and dynamics of the lakes therein, especially Lake Limnopolar that was selected as a model lake by our research team (e.g. Camacho 2006a, Fernández-Valiente et al. 2007; Toro et al. 2007; Quesada et al. 2009; Rochera et al. 2010, 2013; Villaescusa et al. 2010, 2013a, 2013b; Velázquez et al. 2011, 2013, Benayas et al. 2013). Antarctic freshwater lakes are characterized by the presence of short food webs dominated by microorganisms (Frenot et al. 2005). However, lakes situated in maritime Antarctica and nearby regions present slightly more complex food webs compared to those situated in continental Antarctica (Ellis Evans 1996). In this sense, they are somewhat similar to other lakes located at high mountain sites of lower latitudes (Carrillo et al. 2006, Catalan et al. 2006), though lacking fish. In spite of the existence of enough information about the ecology of Antarctic lakes, attempts to model aspects of the ecological functioning of these lakes have not been accomplished so far.

Many lake models have been developed during the last forty years, but as far as we know our study presents the first preliminary model of the biological functioning

of an Antarctic lake. Based upon a few years' observations and the analysis of samples collected during the Antarctic summer, it has been possible to develop a model covering the carbon flow and the microbial components of the biological community of an Antarctic lake. However, the modelling process revealed some problems related to the system high sensitivity to within years differences in environmental conditions that impede the generalization of the model without taking into account of these conditions. The presumably strong effect of the rising temperatures and the simplicity of Antarctic aquatic systems (Quayle et al. 2002) when compared with those situated in temperate climates, make Antarctic lakes excellent candidates for the development of ecological models mainly based on temperature (Reid and Crout 2008). Thus, the use of models can help us to forecast changes in the structure and dynamics of the biological communities of Antarctic lakes in response to the local warming affecting maritime Antarctica and nearby areas. Specifically, the development of a model based on temperature and carbon dynamics in the well-studied Lake Limnopolar, let us the opportunity to describe in more detail the response of the biological community of this type of systems to changes in temperature.

The calculation of system eco-exergy in Antarctic lakes during the austral summer can be used as a useful tool to describe the system complexity and the working capacity of the microbial community and its response against temperature changes. Eco-exergy, described in Jørgensen and Svirezhev (2004), developed by Jørgensen et al. (2010) and revised by Silow and Mokry (2010), is defined as the distance between the present system state and its state if it was in thermodynamic equilibrium with the environment, measured in energy units. Thus, eco-exergy provides a numeric value that measures the changes in complexity of the planktonic community as a response to temperature change.

Taking into account the above mentioned statements, the model developed for Lake Limnopolar was focused on a detailed description of the carbon and energy sources driving lake's functioning, as well as on an accurate selection of the main state variables likely controlling the system, paying especial attention to the

bacterioplankton community. Unlike temperate lakes where phytoplankton play a prominent role, nutrient fluxes in Lake Limnopolar flow mainly through the bacterioplankton community, and its abundance and activity is linked to the availability of dissolved organic carbon (DOC) in the water column. However, prognoses of our model should be taken with care due to their characteristic properties and the problems caused by the high interannual meteorological variability in the area, especially linked to how climate warming could affect the performance of each component of the biological community and the interactions among them, which is quite variable from year to year, and thus should be considered as a preliminary model that could be further improved when data from more field seasons are available. In any case, as demonstrated by our model, the strong warming in the area of the maritime Antarctica and the observed lake community response makes temperature a key sensitive forcing function to monitor and detect changes on several components of the microbial community and lacustrine processes driven by climate change.

10.3. Material and methods

10.3.1. Study area

Lake Limnopolar is located on Byers Peninsula (Livingston Island, South Shetland Islands, Antarctica; 62º34´35´´- 62º40´35´´ S and 60º54´14´´- 61º13´07´´W) (Figure 10.1). The lake is a small (2.2 ha) and shallow (4.5 m maximum depth during the summer) water body located in the central plateau of the peninsula. It has a scarcely vegetated drainage watershed of 0.582 km^2 mainly covered by microbial mats and moss carpets. Byers Peninsula is one of the largest ice-free areas on the Maritime Antarctica (SCAR 2003). It is characterized by the presence of a great amount of water bodies, where the melting of their ice caps during the austral summer allows for a great development of microbial communities, both planktonic and benthic (Toro et al. 2007, Villaescusa et al. 2010). Lake Limnopolar was selected to develop an ecological

model due to its highly representativeness of inland maritime Antarctic lakes, and also to higher knowledge on their ecological features.

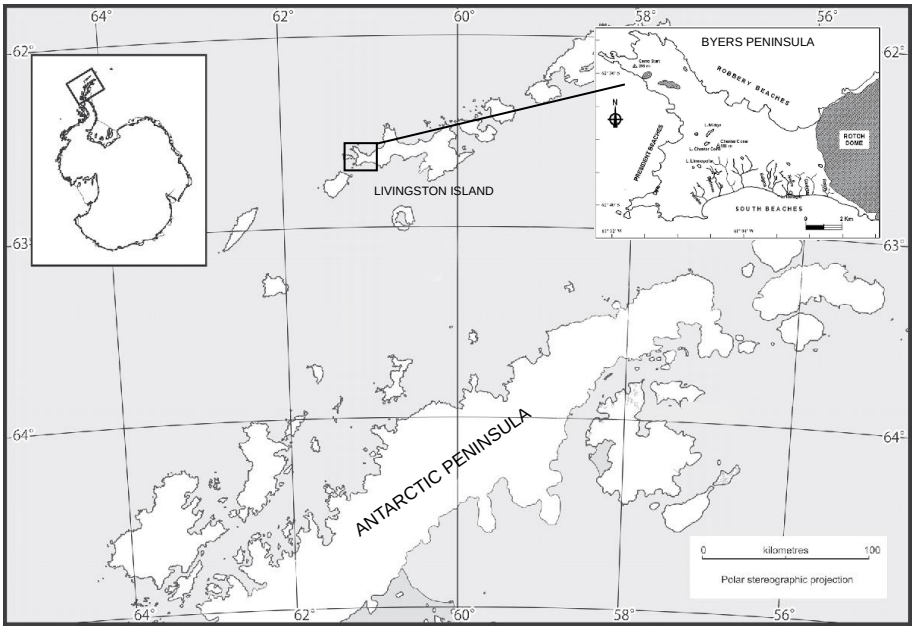


Figure 10.1. Figure 1. Map showing the Byers Peninsula, situated in the west part of Livingston Island (Antarctica). Lake Limnopolar appears in the mid part of the Byers Peninsula plateau.

10.3.2. Ecosystem features

Lake Limnopolar is completely covered by ice during most of the year (Rochera et al. 2010). During the austral summer, however, the ice cover melts keeping the lake without ice for a variable period. For the studied years 2001 to 2012, the summer ice free period in Lake Limnopolar ranged from a minimum of 41 for up to 116 days, depending on the year (Rochera et al. 2010, Villaescusa et al. 2013b). Temperature and solar radiation during the spring and early summer are the main physical factors, together with the amount of snowfall accumulated in the catchment, forcing the length of the ice-free period. Light extinction in ice-covered lakes is higher when the ice cover is present (Hawes and Schwarz 2001), but when the ice layer melts light penetration increases and activity of primary producers rises, and even more when the

ice cap disappears. A wide range of trophic status can be seen in different lakes of Byers Peninsula (Villaescusa et al. 2010). Among them, Lake Limnopolar, as a representative example of non-coastal maritime Antarctic lakes, shows an ultraoligotrophic status with low concentrations of inorganic nutrients (dissolved inorganic nitrogen below 2 µM and soluble reactive phosphorus mostly below 0.03 µM). As typical for non-coastal maritime Antarctic lakes, supply of inorganic nutrients and organic carbon to the lake is influenced by the microbial mats growing within the lake's catchment, being even more important during the first days of summer by the intense run-off processes produced when the snow melts.

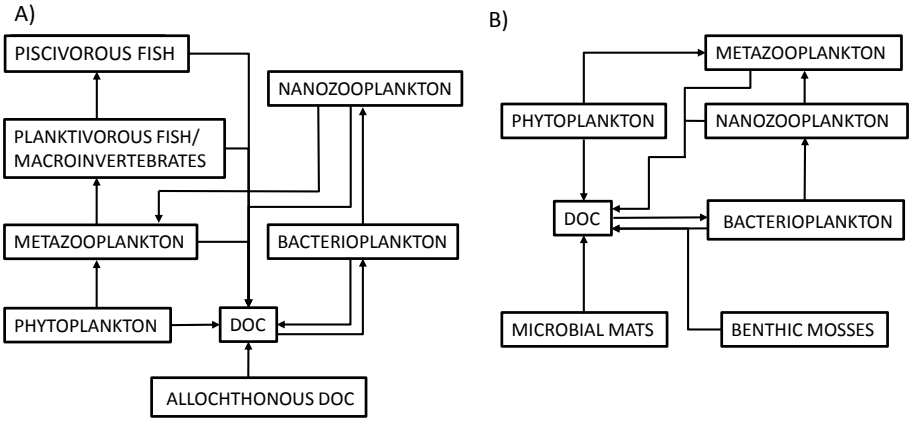


Figure 10.2. Conceptual diagrams of A) a classical planktonic food web. B) microbial food web of Lake Limnopolar.

Planktonic food webs of temperate lakes show greater complexity than those found in Antarctic lakes, showing the classic food web, including bacterioplankton, phytoplankton, zooplankton, macroinvertebrates/planktivorous fish and piscivorous fish (Figure 10.2A). In these systems, inputs of inorganic nutrients, mainly nitrogen and phosphorus, allow an important phytoplankton development, which mostly supports the rest of the plankton community. Contrastingly, the planktonic food web of maritime Antarctic lakes shows a lower complexity (Figure 10.2B), and is mainly composed of microorganisms, (Ellis-Evans 1996; Hansson et al. 1996; Wynn-Williams 1996, Laybourn-Parry et al. 2001, Sommer and Sommer 2006). Due to these particular characteristics, energy transfer between planktonic organisms, mostly

circulates through the microbial loop (Azam et al. 1983; Laybourn-Parry 1997, Camacho 2006a). In addition to other components, the phytoplankton community in the lake is mainly composed by Prasinophytes, Chrysophytes, and nektobenthonic diatoms (Camacho 2006a, Toro et al. 2007, Quesada et al. 2009), as well as a small fraction of unicellular picocyanobacteria (Toro et al. 2007, Rochera et al. 2010). The influence of subsidized supplies of allochthonous carbon sources in the lake is reflected by a high abundance of heterotrophic bacterioplankton, showing higher values than those expected for such oligotrophic conditions in a cold environment, comprised between $1x10^6$ to $4x10^6$ cell ml^{-1} (Toro et al. 2007, Villaescusa et al. 2010). The high abundance of bacterioplankton is likely supported mostly by the inputs of organic carbon from the microbial mats, lichens and mosses that cover part of the lake water catchment area (Camacho 2006a, Fernández-Valiente et al. 2007, Velázquez et al. 2013), as well as by the organic carbon released by the aquatic mosses that cover the lake Limnopolar bottom (Camacho 2006a, Toro et al. 2007), as it occurs in other Antarctic lakes (Imura et al. 2003). The conspicuous development of bacterioplankton confers an important trophic role to this microbial planktonic component during the austral summer, supporting the presence of planktonic predators such as crustacean zooplankton, heterotrophic nanoflagelates and ciliates (Petz et al. 2005, Toro et al. 2007).

Table 10.1. State variables and forcing functions of the model, including abbreviations and units.

State Variables	Abbreviations	Units
Particulated Organic Carbon	POC	gC m^{-3}
Dissolved Organic Carbon	DOC	gC m^{-3}
Bacterioplankton	BACT	gC m^{-3}
Phytoplankton	PHYT	gC m^{-3}
Aquatic Mosses	MOSS	gC m^{-2}
Microbial Mats	MATS	gC m^{-2}
Soluble Reactive Phosphorus	SRP	g m^{-3}
Forcing Functions	**Name**	**Units**
Photosynthetic Active Radiation	PAR	µE m^{-2} day^{-1}
Temperature	Temperature	ºC
Lake Inflow	Inflow	gC m^{-3} day^{-1}
Lake Outflow	Outflow	gC m^{-3} day^{-1}
Volume	Vol	m^3

10.3.3. Model development

The observed data on Lake Limnopolar during the last 12 years revealed that bacterioplankton appears as a key component of the food web. Accordingly, a carbon flow model was developed to study the effects of temperature on the bacterioplankton community, mainly focusing on the abundance of bacterioplankton and the inputs of organic matter from phytoplankton, from the catchment's microbial mats, and from the benthic mosses covering the lake bottom. Considering the previous observations on Lake Limnopolar and other similar lakes of Byers Peninsula (Toro et al. 2007, Villaescusa et al. 2010), seven state variables (Table 10.1) were chosen to describe the main compartments in the lake. Four state variables described carbon dynamics within several lake compartments, namely: *phytoplankton abundance*, grouping all the planktonic primary producers in the lake, *bacterioplankton abundance*, which directly profits from the available organic carbon, *dissolved organic carbon concentration* (DOC) and *particulate organic carbon concentration* (POC). Also *soluble reactive phosphorus* (SRP) was included as state variable to represent the

inorganic nutrient limitation in the lake. Additionally, two state variables were included to reflect key elements such as the *benthic aquatic mosses* and *allochthonous carbon inputs from the microbial mats* due to their importance as suppliers of dissolved organic carbon (Camacho 2006a, Toro el al. 2007). The main forcing functions included (Table 10.1) for the ice free period were photosynthetic active radiation (PAR) and temperature (ºC). Also, the hydrological features of the lake were included as forcing functions, represented by the total lake volume, lake water inflow and lake water outflow. Model processes were described by specific equations (Table 10.2) according to Jørgensen and Bendoricchio (2001) using Ecotox: Ecological modeling and Ecotoxicology software. This program works like a complete database that was used to obtain the equations and the better initial parameters for the model. After this selection, the equations were calibrated adjusting the Ecotox parameters to get the best fit with the observed values on the lake. This software was also used to find initial parameters for the calibration of model equations. The model flow diagram obtained using Stella (modeling & simulation software) is shown in Figure 10.3. This diagram shows the different state variables and all the main processes that connect them along with the carbon flow in the system, as well as the referred equations (Table 10.2) used for simulations.

Table 10.2. Equations used in the model to describe the main lacustrine processes related to the carbon cycle. Initial values of the parameter's development were obtained using Ecotox (Ecological Modeling and Ecotoxicology) software (Jørgensen and Bendoricchio, 2001).

Process	ID	Description	Equation
Plankton			
DOC input	1	External input of dissolved organic carbon	(Mats DOC*(Inflow /1000)/Vol)+(Moss DOC/Vol)
DOC rec	2	Internal input of dissolved organic carbon	Bact R+Phyt ex
DOC out	3	Lake output of dissolved organic carbon	DOC*R Outflow
DOC dep	4	Dissolved organic carbon deposition	0.15*DOC
POC input	5	External input of particulated organic carbon	0.15*DOC input
POC deg	6	Degradation of particulated organic carbon	0.2*POC
POC rec	7	Internal input of particulated organic carbon	Bact G+Bact M+Phyt M
Bact upt C	8	Bacterial uptake of dissolved organic carbon	0.28*BACT* ((DOC-0.1)/(DOC +0.1)) * 1.065^(Temperature-12)
Bact G	9	Bacterial grazing efficience	0.15*Bact upt C
Bact R	10	Bacterial respiration	0.01*BACT
Bact M	11	Bacterial mortality	0.04*BACT
Bact out	12	Lake output of bacteria	BACT*R Outflow
Bact graz	13	Zooplankton grazing over bacterioplankton	0.18*((BACT-0.03)/(BACT+1.2))*1.08^(Temperature-20)
Phyt upt C	14	Phytoplankton carbon fixation	PHYT*43.7*((SRP)/(SRP+0.01)*((PAR*0.615)/((PAR*0.615)+0.17)) + 2*((PAR*0.368)/((PAR*0.368)+0.17))+((PAR*0.265)/((PAR*0.265) +0.17))) * 1.08^(Temperature-20)
Phyt M	15	Phytoplankton mortality	0.6*PHYT
Phyt ex	16	Phytoplankton excretion	0.35*Phyt upt C
Phyt out	17	Lake output of phytoplankton	PHYT*R Outflow
Phyt graz	18	Zooplankton grazing over phytoplankton	0.18*((PHYT-0.0004)/(PHYT+0.12))*1.08^(Temperature-20)
R Inflow	19	Relative inflow	Inflow /Vol
R Outflow	20	Relative outflow	Outflow /Vol
SRP input	21	Input of soluble reactive phosphorus	(R Inflow*Mats ex/166)+(Moss ex/166)+ (Bact R/43.7)
SRP out	22	Lake output of soluble reactive phosphorus	SRP*R_Outflow
SRP upt	23	Phytoplankton SRP uptake	Phyt upt C/43.7
Benthos			
Moss upt C	24	Mosses carbon uptake	MOSS*0.05*((PAR*0.265)/((PAR*0.265)+0.1))*1.06^(Temperature-12)
Moss ex	25	Mosses excretion	(0.0014*MOSS)*1.08^(Temperature-12)
Moss DOC	26	Imput of Doc from aquatical mosses	Moss ex*15225
Lake Catchment			
Mats upt C	27	Microbial mats carbon uptake	MATS*0.008*(PAR/(PAR+0.1))*1.065^(Temperature-16)
Mats ex	28	Microbial mats excretion	0.0025*MATS*1.09^(Temperature-12)
Mats Surface	29	Surface covered by microbial mats	MATS*5742/276.09
Mats DOC	30	Imput of DOC from microbial mats	Mats ex*Mats Surface

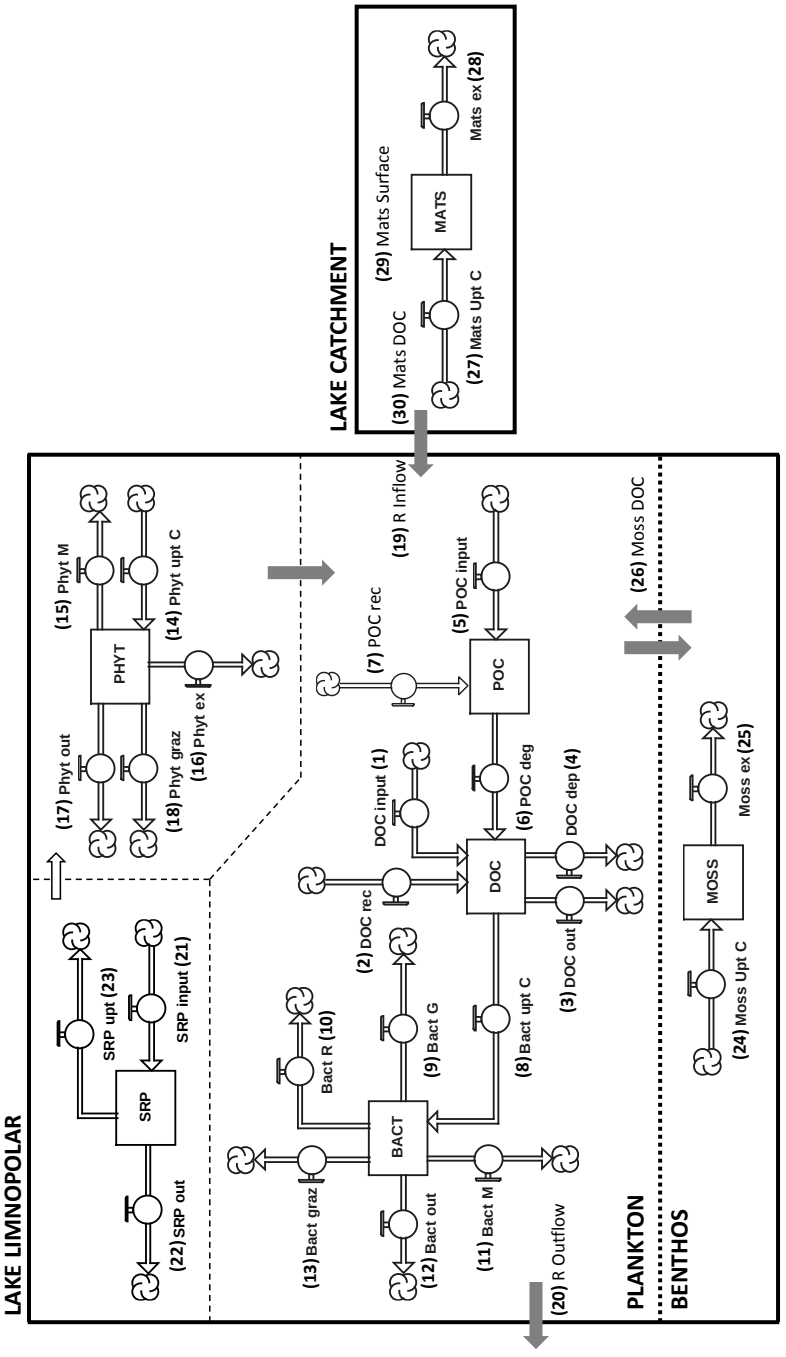


Figure 10.3. Flow diagram of the Lake Limnopolar model developed in Stella software. The squares show the state variables and arrows the main processes in the lake. The grey arrows show the flow lines of the carbon dynamic between the different compartments in the system and the white arrow show the SRP flow to phytoplankton. Not all the connections are shown, but can be extracted from the model equations, which are described by a number and shown in Table 2.

10.4. Results

10.4.1. Sensitivity analysis

A sensitivity analysis was performed to identify the main variables in the model that are the most affected by the forcing functions. The model was tested for a period of 53 days using different possible scenarios for the main forcing functions affecting the system. First of all, the temperature was assayed using four possible scenarios where temperature increased by 1 degree in each one, for a total increase of 4ºC, using the initial temperature of 5ºC as initial temperature value. When the temperature increased, a strong response by the bacterioplankton and phytoplankton community was shown (Figure 10.4). Temperature rises were followed by strong increases in the average bacterioplankton abundance, reaching values around 2.5 times higher for the maximum increase in temperature. The rise in the bacterioplankton abundance was also linked to an increase in the amount of particulate organic carbon (POC), mostly due to bacterioplankton biomass. However, the simulated concentration of dissolved organic carbon (DOC) in lake waters increases only slightly due to the strong consumption exerted by the bacterioplankton, in spite of the DOC contributions from benthic mosses and microbial mats, whose main augments result in more DOC increases and not so much as biomass production. Similarly to bacterioplankton, phytoplankton abundance was enhanced by temperature rises, but in this case the abundance increased only by 1.5 times when the temperature was increased by 4ºC.

Additionally, different scenarios of incident light (PAR) were assayed, by increasing the initial observed available PAR (0.3 µE m^{-2} s^{-1}) by 25 and 50% (which is somewhat realistic when considering that earlier removal of the ice cover associated to warming would drive to earlier increases in light availability during the annual cycle). The sensitivity analysis showed a very low influence of PAR on the bacterioplankton abundances, DOC and POC concentration. In the model, despite of the increase in the incident light, the carbon accumulation of the photosynthetic organisms (phytoplankton, benthic mosses and microbial mats) increased only

slightly, reaching values between 1 to 5% higher compared with the normal values of incident light. The carbon values for phytoplankton increased from 0.0078 gC m^{-3} to 0.0080 gC m^{-3} and 0.0082 gC m^{-3} for the 25% and 50% PAR increase, respectively. Benthic mosses showed a similar pattern with initial values of 13.8 gC m^{-2} that increased to 14.4 gC m^{-2} and 15 gC m^{-2} for the 25% and 50% PAR rises, respectively. At last, the microbial mats biomass showed slightly increased value from 284.3 gC m^{-2} to 285.7 gC m^{-2} and 286.8 gC m^{-2} for the 25% and 50% PAR increases, respectively. According to these results, the low effect of the PAR availability on the bacterioplankton abundance and the much more significant effect of temperature over the plankton communities, shows the higher importance of temperature over light availability as factor controlling the carbon dynamics of the modeled lake.

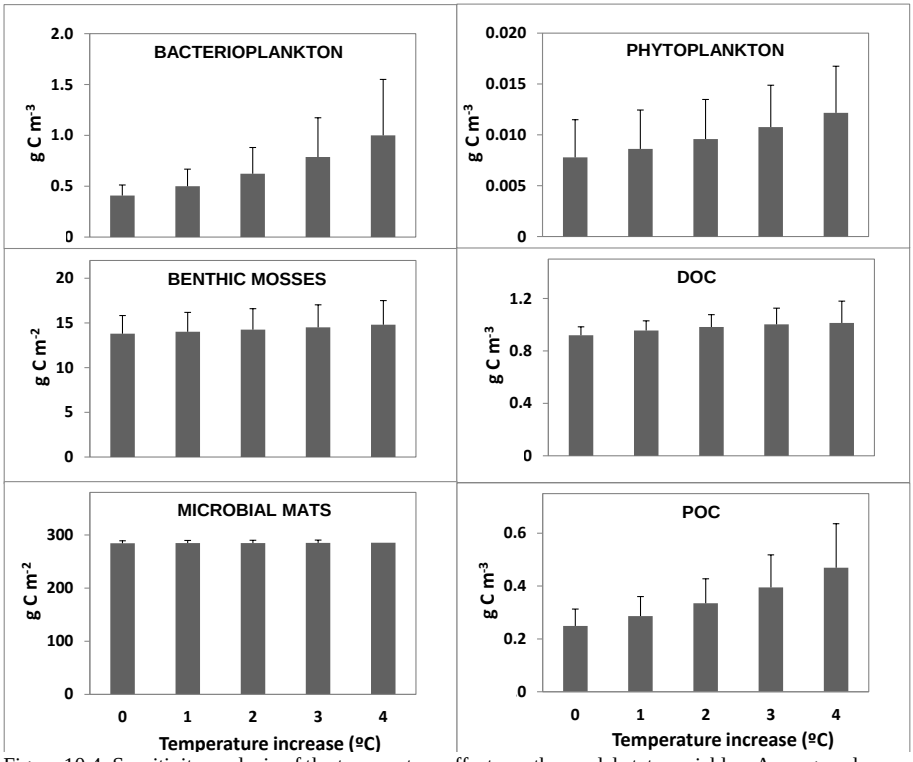


Figure 10.4. Sensitivity analysis of the temperature effects on the model state variables. Average values for the simulation period are shown.

System complexity was measured as an energy value represented as eco-exergy. The density equation designed to estimate eco-exergy was calculated using the Beta values given by Jørgensen et al. (2010) for each of the state variables used in our model. Thus, eco-exergy was calculated by multiplying the value of the different model state variables (in carbon) by their corresponding Beta values. The results show an increase in the eco-exergy as the temperature increase in the system, from values of 95098 to 97521 KJ/m^3 when the temperature was increased by 4ºC. This increase in the eco-exergy values can be interpreted as a potential increase in the system complexity with the rise in temperature.

10.4.2. Calibration

After the selection of the variables and the design of the preliminary equations, the model calibration process was performed using the data set of the 2008/09 summer period. The data covering this period are the most complete along with the 2006/07 data set, and they were used for the calibration and validation respectively. The different state variables used in the calibration were measured during a period of 53 days from 13th December to 3rd February of 2008/09 summer. The lake was completely ice free during the whole sampling period. This survey was conducted during five sampling dates at three different depths, the inputs and outputs to/from the lake were also measured in each sampling event. Also, for the case of photosynthetic active radiation (PAR), a continuous record of the surface irradiance during the ice-free period was obtained. The PAR values were used to estimate the energy input for photosynthetic carbon uptake of the phototrophic organisms in the model. To correct the percentage of surface irradiance reaching each depth, three different values were applied in the model equation (0.615, 0.365 and 0.265 for 0.5, 2 and 3 m depth, respectively). These 3 values were obtained from an in-situ light profile performed during each of the sampling events, evaluating the portion of light reaching each depth. The calibration procedure started by comparing the data observed in the field for each of the state variables against the results obtained after running the model. Each parameter obtained by the Ecotox software was assayed and modified to find the

best fit with values observed in the field during the austral summer 2008-09. The equations related to the aquatic mosses and microbial mats processes, like primary production and carbon excretion, were calibrated first. The process followed to a fitting of the carbon assimilation values, either organic by the bacterioplankton or inorganic by phytoplankton. Simultaneously, the grazing rates over the bacterioplankton and phytoplankton community were adjusted to reflect the field observed values. All the grazing parameters were obtained using the Ecotox software and modified for the best fit in the calibration process. After multiple trial-error tests and changes in the parameters, the final equations were obtained (Table 10.2) describing the best fitting for the dynamics of the plankton community in the lake, terms of carbon, as well as the that of organic carbon provided by microbial mats and benthic aquatic mosses. After running the model, the different estimated state variables were obtained (Figure 10.5), showing a similar pattern as the observed for the 2008/09 summer. The model showed an acceptable average deviation for the mean state variables values compared with the observed data of around 10%. Bacterioplankton abundances results were acceptable with average observed values of 0.408 gC m^{-3} and estimated average values of 0.454 gC m^{-3}. DOC and POC concentration displayed observed average values of 0.959 gC m^{-3} and 0.209 gC m^{-3} respectively. Meanwhile, the estimated average values of DOC and POC were 0.919 gC m^{-3} and 0.249 gC m^{-3} respectively, showing a relatively good calibration. Despite this acceptable fit, the calibration for the phytoplankton was less accurate, with observed average values of 0.005 gC m^{-3} and estimated average values of 0.008 gC m^{-3}. This deviation is mainly due to the strong initial growth of the phytoplankton community just after the ice melts, responding to the sudden increase in light availability and the contributions of nutrients, for which the model is not sensitive enough.

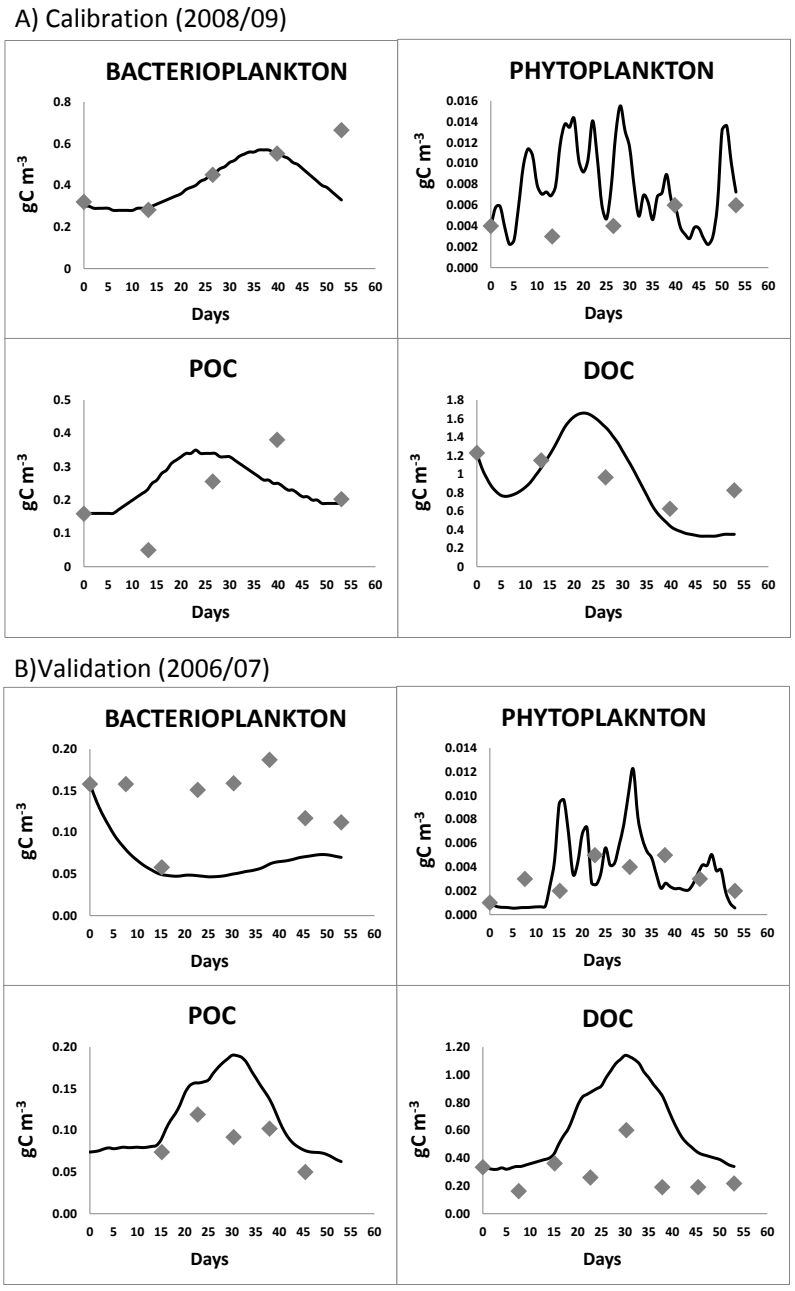


Figure 10.5. A) State variable dynamics described by the developed model (solid line) and in-situ measured values (grey markers) for the austral summer 2008-09 on Lake Limnopolar (data used for model calibration). B) Annual interpolation and attempts of validation of the state variable dynamics described by the developed model (solid line) and in-situ measured values (black markers) for the austral summer 2006-07 on Lake Limnopolar (data used for model validation).

10.4.3. Annual comparison and validation

The obtained model was tested using data collected during the Antarctic campaign 2006/07 by our research group. The data set was composed by a collection of physical (temperature and irradiance), and chemical (inorganic nutrients, dissolved organic carbon and particulate organic carbon concentration) variables along with biological variables like bacterioplankton and phytoplankton abundance. This simulation belongs to a period of three months, including December, January and February. It is important to note that the initial steps of the model (around 10 days) represented a period with a low incident light (PAR) caused by the presence of a partial ice cover on the lake in 2006/07, compared to the calibration period of 2008/09 when the ice cap disappeared previously to data collection. According to this, and after changing the forcing function values in the system and the initial values of each state variable, the model was run.

Validation results (Figure 10.5) were not completely satisfactory and were unable to grant an accurate description of part of the planktonic food web features of the 06-07 summer. The observed average values for DOC and POC concentration were 0.290 gC m^{-3} and 0.087 gC m^{-3} respectively. The estimated average values showed a clear deviation with values of 0.623 gC m^{-3} and 0.113 gC m^{-3} for DOC and POC respectively. However, phytoplankton abundance showed lesser differences between the observed and simulated average values. Observed average value for phytoplankton was 0.003 gC m^{-3} and the simulated average value was 0.004 gC m^{-3}. Although the validation process was relatively acceptable for the above mentioned variables, bacterioplankton abundances showed, however, a high error sometimes as high as 40-50%, with observed average values of 0.138 gC m^{-3} and simulated average values of 0.067 gC m^{-3}.

10.5. Discussion

The results obtained by the model are acceptable when knowing the high interannual variability in the lake features (Rochera et al., 2010). However, the limited availability of data, only five sampling dates during the 2008/09 austral summer, due to the common logistic and sampling difficulties associated with work in polar areas, raised some difficulties in the elaboration of the carbon model, which was developed for the first time for any polar lake. As mentioned, maritime Antarctica and particularly Byers Peninsula displays very unstable and different meteorological conditions among years (Bañón et al. 2013), causing huge differences in the summer dynamics of the lake and its biological communities (Rochera et al. 2010; Villaescusa et al. 2013a,b). Although the summer of 2008/09 only showed a slightly higher average air temperature, approximately 0.2ºC warmer than 2006/07, the average maximum and minimum temperatures in 2008/09 summer showed higher and lower values respectively. These characteristics reflect a more extreme 2008/09 summer in comparison to 2006/07, but also the winter and the spring were different for these years (Bañón et al. 2013), driving to even more differences in the environmental features for the lake and its catchment when both years are compared. One of the most relevant differences deals with the quite different amounts of snow cover both in the lake and its catchment, which greatly influences lake dynamics (Toro et al., 2007; Velazquez et al., 2013). For the year used for calibration (2008/09 summer), the ice cap melted completely during November and the lake remained ice free for a longer period (Villaescusa et al. 2013b). However, for the year used for validation (2006/07 summer) the ice cap did not totally melted until mid December. Therefore, during all the period used for the calibration the lake was ice-free, which did not occur for the initial phase of the validation period. Moreover, temperature during these two years was warmer compared to the summer periods of 2001 through 2004, resulting in a longer ice-free period compared to the 2001-2004 summer periods, which implies further difficulties in the generalization of the model performance for the whole studied period in Lake Limnopolar. The dynamics of the Lake Limnopolar for the modeled sampling seasons are detailed in Rochera et al. (2010) and Villaescusa et al. (2013b).

The meteorological heterogeneity in Byers Peninsula has been previously described (Rochera et al. 2010) using data from the meteorological station situated in Lake Limnopolar. Due to this feature, our model can be used to describe with precision a year with similar characteristics that those observed in the calibration year 2008/09. Nonetheless, the partially unsatisfactory validation results (Figure 10.5) performed with 2006/07 data show that we must act with care when try to extrapolate our model for years with different climatic characteristics. Due to this issue, the quite different values of the forcing functions of the year used for validation compared to that year used to calibrate the model can be affecting these values and promote the high error for predicted state variables such as the bacterioplankton abundance.

Despite the possible mentioned limitations due to the interannual variability, our model is useful to make prognoses on the possible effects of variations in relevant forcing factors which are likely to shift by the effects of climate change, such as temperature, which is strongly increasing within the area (Quayle et al. 2002). Sensitivity analysis reveals the strong effect of temperature on the planktonic community, especially over bacterioplankton abundance that significantly increases with the relatively small increments of temperature (Figure 10.4). Additionally to this significant response of the bacterioplankton against temperature changes, the sensitivity analysis also shows a slight positive effect of the incident light on the photosynthetic communities on the lake, especially on phytoplankton and, to a lesser extent, on benthic mosses. This response is likely to increase as a consequence of an earlier melting of the ice crust covering the lake if the climate becomes less harsh. Yet, although the absence of the lake ice cover after the melting could potentially exert an important influence on the phytoplankton and benthic mosses production, the low response of the plankton community against light, as shown in the sensitivity analysis, can be explained because the main source of DOC for the bacterioplankton is external (released by microbial mats and mosses within the catchment) and thus it is not directly related to the lake ice cover.

All these results reveal the relevance of the physical variables on the microorganisms in Antarctic systems, as previously argued (Fountain et al. 1999).

Additionally to these results showing the importance of the physical control for the biological communities, other changes due to the interactions among the modeled state variables (bacterioplankton, phytoplankton, etc) with other components of the food web can also be important in controlling the dynamics of the biological community. This occurs since it is known that the phagotrophic organisms, mainly composed by metazooplankton (*Boeckella poppei*), heterotrophic ciliates (with only one planktonic species, *Balanion planctonicum*), (Petz et al. 2005) and several species of nanoflagellates, may exert a strong top-down control on the bacterioplankton of Lake Limnopolar (Camacho 2006a). These interactions, however, were not included in our model as the available information for both the calibration and the validation was scarce.

In response to an increase in temperature, the system gains eco-exergy, which allows the calculation of the system working capacity and therefore the activity of organisms expressed on the distance from thermodynamic equilibrium (Jørgensen et al. 2010). This gain in the eco-exergy parameter also reflects an increase in the system complexity, strongly driven by the increase in bacterioplankton density. Thus, bacterioplankton is the state variable that more strongly responds to the simulated increases in temperatures, but this response is mediated by changes in other variables that influence bacterial growth. As shown in the sensitivity analysis, the increase in temperature does not show a direct increase in the DOC abundance in lake water, however, it allows higher production rates of the microbial mats and benthic mosses that imply greater subsidies of organic matter (mainly DOC and POC) to the lake, consequently providing carbon sources for bacterioplankton growth. Therefore, changes in these parameters will affect model complexity and also the eco-exergy, which can be used as evidence of a change in the activity of the microbial community.

The strong phytoplankton growth in the initial steps of the simulation, although not totally fitted, reflects quite well a common process occurring in the lake when ice melting occurs, with a pulse of inorganic nutrients resulting from nutrients accumulated on the ice covering the lake and the snow cover on the catchment. This

occurs simultaneously with an increase in light availability and temperature during the early summer due to ice melting, producing an initial explosive growth of phytoplankton immediately below the ice cover, which is partly simulated by the model. This is a well known phenomenon in Antarctic lakes, commonly associated with the presence of different groups of *Prasinophyceae* (*Chlorophyta*) (Bell and Laybourn-Parry 1999), which are able to take advantage of this favorable period. Although our collected samples did not reflect the existence of this phytoplankton bloom in the selected year for calibration (2008-2009) because of sampling limitations, many of our data from other years showed that this is a common phenomenon that repeats every year in Lake Limnopolar and other maritime Antarctic lakes. In the case of Lake Limnopolar, subsurface chlorophyll maxima develop immediately below the ice cover when the melting process is quite advanced and they remain during the initial phase of the ice-free period (Rochera et al. 2010; Villaescusa et al. 2013b). In addition to the data on taxa-specific photosynthetic pigments and microscopic observations, a large proportion of ssDNA viruses have been detected during late spring and early summer, most of which are related to eukaryotic organisms and specifically to the *Prasinophyceae* blooms found under the ice cover before the total ice melting (López-Bueno et al. 2009).

As a result of our model, the environmental response of the microbial community in these aquatic polar systems may have shown to be different compared to lakes from temperate environments, where typically macroorganisms, such as fish, have a more important role in the system. Thus, the increase in temperature an light availability in Antarctic systems during the austral summer (reflecting milder weather conditions) would produce a strong response of the microbial community, mainly granted by the higher contributions of inorganic nutrients and organic matter due to the more active biological activity of microbial mats and mosses within the catchment. Since under milder environmental conditions biological interactions could exert a deeper control on the functioning of the Antarctic lake ecosystems (Ellis-Evans 1996, Camacho 2006a), changes in the forcing factors of the system that are primarily identified as physical abiotic variables and can be linked to climate warming, such as temperature

and light availability, are also likely to influence the strength of biological interactions, thus producing a feedback effect on ecosystem functioning. Thus, lake modeling can be used as a useful tool to predict changes in the microbial communities and ecosystem functioning in these aquatic maritime Antarctic systems. However, ecological studies in these areas and intensive monitoring of warming and climate changes will be necessary to elaborate more precise and accurate models that may also include other biotic interactions, then allowing to make fine prognoses for the future of these sensitive ecosystems.

10.6. Conclusions

In conclusion, the results of this preliminary model show that bacterioplankton abundance and the supply of dissolved organic carbon (DOC), which are mainly controlled by the temperature patterns, play a key role in the dynamics of maritime Antarctic lacustrine planktonic communities, and especially on bacterioplankton. In this way, the model sensitivity analysis showed the most important features of the studied system and its relationship with climate warming that represents an important concern in this Antarctic area. Although development of models, such as that of the Lake Limnopolar, can provide with an additional tool to improve the knowledge of limnetic Antarctic systems, in our case interpretation and extrapolation of its results should be taken with care due to the high observed annual meteorological heterogeneity in this area. The model describes very well the austral summer of 2008-2009 and could be used to describe a summer with similar meteorological features. However, due to the meteorological heterogeneity and the poor data, the model fails to describe with precision years with completely different meteorological features like the summer of 2006-2007. Thus, more complete data sets for more than two years could provide the model with more reliability against years with different meteorological features. Ultimately, the model general prognosis about the strong ecological effect of temperature increase such as those occurring in this region of the Earth, can undoubtedly be accepted.

Our preliminary model represents an advance in the modeling of polar ecosystems and allows a better understanding of temperature effects on the planktonic and benthic communities, especially with the existing regional warming in this area of Antarctica that is directly affecting the structure and dynamics of these microbial communities. Therefore, modeling tools such as the described in the present work could be very useful to predict future changes in these ecosystems which are strongly affected by the regional warming.

11. General discussion

11.1. General discussion

Byers Peninsula is situated in the west part of Livingston Island (South Shetland Islands) and represents an excellent example of a maritime Antarctic habitat not directly affected by the anthropic activity. This area, one of the largest ice-free areas in the maritime Antarctica, displays an important number of lakes and streams (Toro et al. 2007). Also, its status of Antarctic Special Protected Area (ASPA) has allowed the conservation of relatively pristine characteristics, displaying a huge number of diverse and unaltered aquatic ecosystems.

The occurrence of many ice free water bodies (lakes, ponds, pool and streams) during the austral summer is favored by the less extreme climate compared to that of continental Antarctica (Vincent 2000, Bañón et al. 2001, Convey 2011). Additionally, the great biological and ecological interest in this area has been widely demonstrated in a lot of recent studies (summarized in Quesada et al. 2009, 2013, and Benayas et al. 2013), that have proposed Byers as a reference site for limnological studies in maritime Antarctica.

The first parts of this thesis were focused on the study of the environmental heterogeneity of the water bodies of Byers Peninsula (Chapter 3). The evaluation of the chemical and biological features of these lakes indicates a contrasting trophic status among them, clearly influenced by the external inputs of inorganic nutrients and organic matter but also related to the nutrient removal processes from the lake sediment, being these features closely linked to the lakes' morphologies and the ice dynamics in their basins.

Coastal lakes in Byers Peninsula, such as Lake Refugio, display a clearly marked eutrophic status due to the great inputs of organic matter and inorganic nutrients from marine fauna (Hansson 1992, Hansson et al. 1996, Izaguirre et al. 2001, 2003, Mataloni et al. 1998). Also, the shallowness of Lake Refugio (0.5m) greatly contributes to increase its trophic status due to nutrient removal from the sediment.

On the other hand, lakes situated in the central plateau of Byers Peninsula, like Limnopolar, Midge, Chester, Chica and Turbio, show quite different trophic status. These lakes commonly display a higher depth compared to the coastal lakes (between 4-5 m) which, together with the absence of marine fauna, causes a marked oligotrophic status. In these lakes, the effect of the sediment is less important and the external inputs of nutrients and organic matter become a relevant factor that drives the system. However, some lakes situated in the central plateau as Lake Somero, due to their sallowness (0.5m), display a higher trophic status in spite of being located in this area. The close relationship with the sediment and the important nutrient removal favored by the wind after the melting of the ice cover, allows a higher abundance of phytoplankton and bacterioplankton in Lake Somero compared to the other lakes in this area.

According to the above features mentioned and using an ordination analysis (PCA), three groups of lakes were discriminated. The first group included the deep oligotrophic lakes situated in the plateau (Limnopolar, Chester, Midge, Chica, and Turbio). The second group includes the shallow lake Somero situated in the plateau, and the last group the eutrophic coastal lakes such as Lake Refugio. The main discrimination feature on the PCA analysis is related to the trophic status on the lakes. Thus, lakes with a close relationship with the sediment, such as Chica and Turbio, are more similar to the shallow Lake Somero, however the clear difference in their depths was a strong parameter to segregate Lake Somero in another group. Finally, Lake Refugio appears completely separated from the rest due to their clearly marked eutrophic status. This trophic pattern observed in the case of Byers Peninsula has been also described in other maritime Antarctic Lakes and other waterbodies situated in the Antarctic Peninsula (Izaguirre et al. 2001, 2003, Schiaffino et al. 2009, 2011).

The study of the bacterioplankton composition using DGGE analyses described a high number of dominant bacterial groups. Our analyses showed a higher bacterial richness in the deep and oligotrophic plateau lakes compared to the shallow lakes situated in both, the plateau and the coast. These results show that lakes with higher

trophic status and bacterial abundance display the lower bacterioplankton group diversity. Pearce (2005) found a similar pattern of reduced bacterioplankton diversity with increasing trophic status in lakes from the nearby Signy Island, although other studies performed in lakes of the maritime Antarctica failed to find a clear pattern (Schiaffino et al. 2009).

The sequencing of the dominant bands obtained by DGGE revealed a high correspondence with non-cultivated bacterial groups. The majority of the dominant OTUs detected were affiliated with α-Proteobacteria and Bacteroidetes, both of which are dominant in other maritime Antarctic lakes, together with β-Proteobacteria and Actinobacteria (Pearce 2005, Pearce et al. 2007). The cluster and correspondence analysis, using the band presence-absence in the DGGE fingerprinting, displayed a similar three group pattern than the previous PCA analysis using environmental variables as main features. As a result, these data reveal that the composition of the bacterioplankton assemblages displays a marked heterogeneity in the studied lakes, matching up very well with the environmental diversity among the studied lakes. These results reflect the close relationship among the environmental and biological features in maritime Antarctic systems, also evidencing the marked heterogeneity of the studied lakes.

To understand the displayed environmental heterogeneity along with the observed bacterioplankton diversity in Byers Peninsula lakes, we conducted a in-depth study of the capabilities of using different types of carbon sources by each lake's microbiota, and its relationship with the autochthonous bacterial groups (Chapter 7). Using the carbon ecoplates (Biolog) in each of the studied lakes, we evidenced differences in their carbon physiological profiles that can be related to the origin of the organic matter and the relation of each lake with their catchment, flora and fauna. This was already been demonstrated in the case of soil bacterial diversity in Livingston Island (Kenarova et al. 2013), but no any of this types of studies was never performed for inland aquatic ecosystems in Antarctica. Our results revealed a repetitive pattern by which the lakes with the highest trophic status also showed the more diverse physiological activity. This idea contrasts with the observed DGGE

results in the Chapter 3 where the lakes with higher trophic status displayed the lower bacterial taxonomic diversity (described using their DGGE fingerprinting), in such a way that eutrophic coastal lakes displayed a low taxonomic diversity of bacterial groups, but a greater physiological diversity. This can reflect the existence in these lakes of non-specialist species with a wide-range of metabolic capabilities that respond to the higher trophic status and a wide presence of organic matter types. On the contrary, oligotrophic lakes situated in the plateau showing higher bacterial taxonomic diversity displayed lower physiological diversity. This feature represents the occurrence of non-dominant specialist bacterial species that display a lower variety of metabolic capabilities. Additionally, the shallow lakes situated in the plateau such as Lake Somero display an intermediate status compared to coastal lakes and deeper oligotrophic plateau lakes. These results could reflect the existence of a gradient between higher trophic status and the physiological diversity in these systems.

After describing the environmental heterogeneity of the lakes on Byers peninsula, our study focused its attention on the deep Lake Limnopolar located in the central plateau of Byers Peninsula. This lake was previously selected as a "model lake" and a lot of descriptive and experimental work has been already done there. Our current study of Lake Limnopolar was centered in two Antarctic campaigns where most of the research activity was developed, coinciding with the austral summers of 2006/07 and 2008/09. During these years, the meteorological station situated close to Lake Limnopolar revealed warmer summers compared to the previously monitored summers on 2001-2004 (Rochera et al. 2010). These results demonstrate the high variability on the meteorological features in this area and how this influences the length of the ice-free period in the lakes, as well as the length of the productive season.

During both years (2006/07 and 2008/09), the low inorganic nutrients and chlorophyll-a concentrations measured in the lake clearly showed the marked ultra-oligotrophic status previously described for Lake Limnopolar. Such nutrient scarcity has also been already reported in the others lakes located in the central plateau of

Byers Peninsula (Chapter 3), as well as for other inland maritime Antarctic lakes (Drago 1989, Izaguirre et al. 2003).

During this period, we focused our attention mainly on the study of the bacterioplankton dynamics in the Lake Limnopolar (Chapter 4), showing changing dynamics along the studied periods that were quite different for both years (2006/07-2008/09). The clear difference in the bacterioplankton abundances between the two studied summers could be directly related to the differential availability of organic carbon and of the length of the productive period. On the other hand, the results on the use of the dissolved organic carbon by the bacterioplankton (Chapter 7) revealed a statistically significant correlation between the bacterioplankton abundance for the different studied depths and the availability of the different types of dissolved organic carbon. Thus, the bacterioplankton community composition seems to be related to the origin of the organic matter which can be allochthonous or autochthonous. In this way, the variation in the length of the ice-free period between years could be affecting the external contributions of organic carbon by run-off processes and, at the end, affect the bacterioplankton composition during the austral summer. As a result, the environmental features of each year could induce important changes in Lake Limnopolar planktonic composition. These results show the potential relevance of the warming occurring in this area (Quayle et al. 2002) that can directly affect the bacterioplankton composition in these aquatic systems.

Additionally, we evaluated the phytoplankton and bacterioplankton production rates in Lake Limnopolar and their relationship with the autochthonous and allochthonous supply of organic matter (Chapter 5). Our results on the lake production assays showed an uncoupling between primary and bacterial production, revealing the importance of external contributions of organic matter from the catchment. The allochthonous carbon subsidizes the planktonic production in the lake allowing the appearance of the mentioned uncoupling between the primary and bacterial planktonic productivity.

This uncoupling between phytoplankton and bacterial production is mainly explained attending to the non-planktonic compartments that can be autochthonous, like benthic mosses, or allochthonous, like microbial mats growing in the lake catchment. The presence of benthic mosses communities in maritime Antarctic systems has already been described in lakes of King George Island (Montecino et al. 1991) and in continental Antarctic lakes (Imura et al. 2003, Moorhead et al. 2005, Ask et al. 2009). On this way, benthic mosses in Lake Limnopolar represent a total carbon production three times higher compared to the observed for the phytoplankton. Thus, the benthic mosses community and its activity act as an additional source of organic matter for the planktonic bacterial production.

However, the carbon contributions by benthic mosses seems not to be the main source of carbon that subsidize the planktonic production. As stated in Chapter 3, microbial mats growing in the catchment of Lake Limnopolar represent an important source of carbon for the bacterioplankton in this system. Nevertheless, microbial mats grow in the catchment of Byers' lakes (Fernández-Valiente et al. 2007) and the contributions of organic carbon from these mats communities to the lakes largely depend on the existence of runoff processes. Due to this feature, the greater contributions of organic matter occur after the snow melting, preceding the higher planktonic bacterial production rates in the lake.

Summarizing, the allochthonous contributions of organic carbon from the microbial mats, along with the autochthonous contributions of organic carbon from the benthic mosses, subsidize the bacterial production in Lake Limnopolar and support the observed uncoupling between primary and bacterial production. This feature could seem strange in Antarctic systems that have been seen as mainly based on the internal carbon supply (Dore & Priscu 2001) but as said before, our system receives external inputs of allochthonous organic matter from the catchment that can subsidize the lake bacterial production as has been described in other systems (Priscu et al.1998, Kritzberg et al. 2004).

On the other hand, the study of the bacterial diversity in Lake Limnopolar (Chapter 6) analyzing 177 extracted DNA sequences, revealed the existence of a vertical stratification in terms of taxonomic composition between surface and bottom waters. Accordingly, it is not unreasonable to think in this vertical segregation of the bacterial assemblages as a biological stratification, even though a clear physical vertical gradient is not displayed. The analysis of the retrieved sequences with >97% identity, and the origin of the closest matches found in GenBank, demonstrate the importance of the catchment inflow (soil bacteria), birds (enteric bacteria from bird faeces), and sea spray (airborne bacteria) in explaining the taxonomic composition of the bacterial community in surface waters. Contrastingly, bacterioplankton composition of deep waters is much more similar to that commonly described for other cold freshwater environments. This appears to be the case even though no physical stability of the water column is displayed, meaning that a more stable environment could be provided by the dense carpet of mosses covering the bottom of the lake, which confers a higher resilience to this bacterioplankton community. In addition, if we compare the observed bacterial diversity vertical stratification with the carbon source´s utilization for each depth, a similar pattern arises. This "taxonomic and physiological stratification" appears because of the organic carbon contributions from the benthic mosses that also create a more stable environment near the sediment.

Additionally, if we focus our attention on the general results of the in-situ manipulative experiment (Chapter 8), where both the inorganic nutrient and the organic carbon concentration were manipulated along with the availability of light, we can state that the planktonic population of Lake Limnopolar is responding actively to the treatments with inorganic nutrient addition, and especially to those treatments where a metallic mesh was used to reduce the incident light over the planktonic organisms. The positive response of the phytoplankton (referred as chlorophyll-a concentration) to the nutrient addition reflects the high nutrient limitation in Lake Limnopolar, that can be extrapolated to other Antarctic systems. Our experiment has demonstrated the response of the planktonic community to fertilization processes and the close relationship of the phytoplankton with the incident light during the austral summer. The light photoinhibition effect on the planktonic community and the

inorganic nutrient limitation, appear as the main factors that drive the productive processes in the planktonic community. The observed harmful effect of radiation over the planktonic communities and its effect over phytoplankton abundances have already been studied for other maritime Antarctic systems (Alderkamp et al. 2010) and in particular over the microbial mat communities that are present in Antarctic lake catchments and wet areas (Fernández-Valiente et al. 2007, Velázquez et al. 2011, Rochera et al. 2013).

The effect of the manipulations on the bacterioplankton abundance and bacterial production showed a low response specifically in the glucose addition treatment. Although glucose is a carbohydrate easily metabolized by bacteria and commonly used in some aquatic ecology experiments (Kamjunke 2008), the response of bacterioplankton measured as bacterial production seems not to be significant in our experiment. The lack of significant effect of glucose over the bacterial population could be related to the existence of specialist bacterial species that prefer dissolved organic matter compounds derived from phytoplankton activity or other external inputs like microbial mats or mosses, revealing the possible co-limitation between carbon and inorganic nutrients for the bacterioplankton in this oligotrophic system.

Manipulation experiments on copepods' algal epibionts (Chapter 9) in Lake Limnopolar have demonstrated the existence of this behavior as a nutritional strategy. In our case, the algal epibionts take advantage of the higher mobility and the debris and excretions of zooplankton, thus increasing its inorganic nutrient availability. Therefore, the observed response in our experiment reflects a positive response of copepod´s microalgae epibionts against nutrient limitation. In this situation, it seems that epibiosis act as efficient strategy when the nutrient availability is low. Therefore, the zooplankton exert a commensalism relationship with their epibionts offering a higher inorganic nutrient availability due to their, excretions, grazing remnants and their higher mobility.

As conclusion of this thesis, and using the global obtained data, a bacterioplankton carbon utilization model was developed for Lake Limnopolar with

the objective of using it as a model for inland lakes of the maritime Antarctica. The results obtained by the model are acceptable assuming the high interannual environmental variability in Byers Peninsula as demonstrated in Chapter 4 and in previous work (Rochera et al. 2010, Bañón et al. 2013). Our model clearly shows the control exerted by the temperature on the bacterioplankton dynamics and the availability of carbon sources, mainly dissolved organic carbon (DOC). This temperature control is demonstrated very clearly by the model, showing the changes that can be expected from global warming, as reflected in the sensitivity analysis. Although the development of ecological models represents an important tool to improve the knowledge of limnetic Antarctic systems, the interpretation and extrapolation of our results should be taken with care due to the high annual meteorological heterogeneity in Byers Peninsula. Thus, the present model can be used as an accurate tool to describe a year with similar meteorological characteristics than 2008-2009, but should be treated with care on the extrapolation for years with clearly different environmental features. Anyway, its general prognosis on the strong ecological effect of temperature increases such as those occurring in this region of the Earth can undoubtedly be accepted. To conclude, the present model represents an advance in ecological modeling on polar ecosystems, being the first attempt to model such kind of aquatic ecosystem. Also, it is a profitable tool to study the effects of climate change, especially the climate warming affecting the region of maritime Antarctica.

This thesis represents a great improvement in the scientific knowledge on Byers Peninsula, greatly contributing to the ecological knowledge of the maritime Antarctic Lakes and the description of their planktonic communities, especially on the bacterioplankton. Moreover, it is set as a starting point of future studies that implies longer data sets to study the environmental heterogeneity in this area of Antarctica, especially for the development of better ecological models based on the carbon dynamics.

12. Conclusions

12.1. Conclusions

1. Lakes situated in Byers Peninsula display a contrasting trophic status clearly dependent on the influence of external inputs from marine fauna and run-off processes in the catchment. However, the nutrient recycling within the lake basin and the lake´s morphology along as with the ice dynamics clearly influence their trophic status.

2. Lakes in Byers Peninsula display an important environmental heterogeneity that directly affects their trophic status. In the present work, a close relationship between bacterioplankton diversity and the trophic status on each lake has been demonstrated. Therefore, bacterial diversity in maritime Antarctic lakes can be used as an indicator of the lake features.

3. Byers Peninsula displays a high meteorological variability between years. During the austral summer, this feature directly affects the biological structure and function on Byers' lakes due to the large influence that the meteorological conditions exert over the duration of the ice-free period and the length of the productive season.

4. The planktonic community of Lake Limnopolar is clearly subsidized by the external inputs of organic matter and nutrients mainly derived from the microbial mats covering their catchment. These allochthonous inputs subsidize the planktonic production, allowing the appearance of an uncoupling between primary and bacterial production.

5. Lake Limnopolar display a marked vertical heterogeneity in their bacterial group composition. This feature is directly related to the allochthonous inputs of organic matter and the occurrence of a more stable habitat in the benthic layer of the lake, closely related to the presence of benthic mosses. Therefore,

Lake Limnopolar display a bacterioplankton vertical stratification in spite of the inexistence of physical stratification.

6. The trophic status of Byers lakes is directly related to the physiological diversity of the bacterioplankton. This fact contrast with the observed pattern by which lakes with higher trophic status display the lesser taxonomic diversity. This feature could be explained by the occurrence of non-specialist species with wide-range metabolic activities that respond to the higher trophic status in coastal lakes.

7. Phytoplankton community in Lake Limnopolar actively responds to inorganic nutrient additions in manipulative experiments. This response reflects a high nutrient limitation. Also, the availability of light, in spite of being a key resource for the phytoplankton production in this systems, produces a photoinhibition effect in the surface layers of Lake Limnopolar when excessive.

8. Copepod´s microalgal epibionts actively respond to nutrient limitation in oligotrophic maritime Antarctic lakes by attaching to their commensals. In this way, epibiosis acts as an efficient strategy when the nutrient availability is low. Thus, zooplankton exert a commensalism relationship with their algal epibionts, offering a greater access to inorganic resources.

9. Lake Limnopolar ecological model clearly shows the important effects that temperature exert on the planktonic community of maritime Antarctic lakes. Carbon flow models act as a profitable tool to understand the direct and indirect effects of temperature associated with the regional warming affecting this area of Antarctica.

12.2. Conclusiones

1. Los lagos situados en la Peninsula Byers presentan características tróficas muy dispares, claramente influenciadas por los aportes externos debidos a la excorrentia en la cuenca y a la fauna marina. Sin embargo, los procesos de reciclado en el sedimento de los lagos y su morfología, junto con la dinámica del hielo afectan de forma evidente el estado trófico de los mismos.

2. Los lagos de la Peninsula Byers presentan una marcada heterogenidad ambiental, la cual afecta de forma directa a su estado trófico. En el presente trabajo, se ha demostrado la extrecha relación entre la diversidad del bacterioplancton y el estado trófico de los lagos de la Península Byers. De esta forma, la diversidad bacteriana en los lagos de la Antártida marítima puede ser usada como un indicador de las características de estos lagos.

3. La Península Byers presenta una elevada variabilidad meteorológica interanual. Durante el verano austral, esta característica afecta directamente a la estructura biológica en los lagos debido a la influencia que las condiciones meteorológicas ejercen durante el periodo libre de hielo, afectando directamente a la duración del periodo productivo en los lagos.

4. La comunidad planctónica en el Lago Limnopolar esta claramente regulada por las contribuciones externas de materia orgánica derivada principalmente de los aportes de los tapetes microbianos que cubren la cuenca del lago. Estos aportes externos contribuyen a la producción planctónica fomentando la aparición de un desacoplamiento entre la producción primaria y la producción bacteriana.

5. El Lago Limnopolar presenta una marcada heterogeneidad vertical en cuanto a la composición de grupos bacterianos. Esta característica esta directamente relacionada con los aportes externos de materia orgánica y con la aparición

de un hábitat estable en la parte profunda del lago, relaciondo directamente con la presencia de musgos bentónicos. Podemos decir por tanto, que el Lago Limnopolar presenta una estratificación vertical del bacterioplancton a pesar de no existir una estratificación física o química.

6. El estado trófico en los lagos de la Península Byers esta directamente relacionado con la diversidad fisiológica del bacterioplancton. Esta idea contrasta con el patrón observado en los mismos, donde lagos con un elevado caracater trófico presentaban una baja diversidad taxonómica. Esta característica puede explicarse por la aparición de especies no especialistas con un amplio rango de actividades metabólicas en los lagos costeros eutróficos.

7. La comunidad del fitoplancton en el Lago Limnopolar responde activamente a los experimentos de fertilización con nutrientes inorgánicos. Esta respuesta refleja la existencia de una fuerte limitación por nutrientes en los lagos de la Antártida marítima. Además, la disponibilidad de luz, a pesar de ser un recurso clave para la producción primaria en estos sistemas, puede producir un efecto de fotoinhibición en las capas superficiales de estos sistemas.

8. Las algas epibiontes de copépodos responden activamente a la limitación por nutrientes en los lagos de la Antártida marítima. De esta forma, la epibiosis funciona como una estrategia eficiente cuando existe una fuerte limitación por nutrientes. Por lo tanto, los copépodos ejercen una relación comensalista con las algas epibiontes, ofreciendoles un mayor acceso a los nutrientes inorgánicos.

9. El modelo ecológico desarrollado para el Lago Limnopolar muestra claramente el efecto que la temperatura ejerce sobre las comunidades planctónica de los lagos de la Antártida marítima. Este tipo de modelos

basados en el carbono pueden emplearse como una herramienta útil para comprender el efecto directo e indirecto de la temperatura y su relación con el calentamiento regional que afecta esta área de la Antártida.

13. References

References

1. Alderkamp AC, de Baar HJW, Visser RJW, Arrigo KR (2010) Can photoinhibition control phytoplankton abundance in deeply mixed water columns of the Southern Ocean?. Limnol Oceanogr 55:1248-1264
2. Agogué H, Casamayor EO, Bourrain M, Obernosterer I, Joux F, Lebaron P (2005) A survey on bacteria inhabiting the sea surface microlayer of coastal ecosystems. FES Microbial Ecol 54:269-280
3. Aguilera A, Souza-Egipsy V, González-Toril E, Rendueles O, Amils R (2010) Eukaryotic microbial diversity of phototrophic microbial mats in two Icelandic geothermal hot springs. Int Microbiol 13:21-32
4. APHA, AWWA, WEF (1992) Standard Methods for the Examination of Water and Wastewater, 18th ed. American Public Health Association, American Water Works Association and Water Environment Federation, Washington D.C., 1100 pp.
5. Altschul SF, Madden TL, Schaeffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389-3402
6. Ask J, Karlsson J, Persson L, Ask P, Byström P, Jansson M (2009) Terrestrial organic matter and light penetration: Effects on bacterial and primary production in lakes. Limnol Oceanogr 54:2034-2040
7. Azam F, Fenchel T, Field JG, Grey JS, Meyer-Reil KA, Thingstad F (1983) The ecological role of water-column microbes in the sea. Mar Ecol Prog Ser 10:257-263
8. Azam F, Smith DC, Hollibaugh JT (1991) The role of the microbial loop in Antarctic pelagic ecosystems. In: Polar Research 10, Sakshaug E, Hopkins CCE, Oritsland NA (eds.):pp. 239-243. P Pro Mare Symp Polar Mar Ecol. Trondheim
9. Bañón M (2001) Observaciones meteorológicas en la Base Antártica Española Juan Carlos I. Monografía A-151, Instituto Nacional de Meteorología, Ministerio de Medio Ambiente. Madrid

10. Bañón M, Justel A, Velázquez D, Quesada A (2013) Regional weather survey on Byers Peninsula, Livingston Island, South Shetland Islands, Antarctica. Antarc Sci 25:146-156
11. Barbaro E, Zangrando R, Vecchiato M, Turetta C, Barbante C, Gambaro A (2014) D- and L- amino acids in Antarctic lakes: assessment of a very sensitive HPLC-MS method. Anal Bioanal Chem 406:5259-5270
12. Barberá NA, Casamayor EO (2010) Global phylogenetic community structure and beta-diversity patterns of surface bacterioplankton metacommunities. Aquat Microb Ecol 59:1-10
13. Barea-Arco J, Perez-Martinez C, Morales-Baquero R (2001) Evidence of mutualistic relationship between an algal epibiont and its host, Daphnia pulicaria. Limnol Oceanogr 46:871-881
14. Barberá NA, Casamayor EO (2011) Euxinic freshwater hypolimnia promote bacterial endemicity in continental areas. Microb Ecol 61:465-472
15. Bell EM, Laybourn-Parry J (1999) Annual plankton dynamics in an Antarctic saline lake. Freshwater Biol 41:507-519
16. Benayas J, Pertierra L, Tejedo P, Lara F, Bermúdez O, Hughes KA, Quesada A (2013) A review of scientific research trends within ASPA No. 126 Byers Peninsula, South Shetland Islands, Antarctica. Antarc Sci 25:128-145
17. Bender J, Rodriguez-Eaton S, Ekanemesang UM, Phillips P (1994) Characterization of metal-binding bioflocculants produced by the cyanobacterial component of mixed microbial mats. Appl Environ Microbiol 60:2311-2315
18. Berlanga M, Aas JA, Paster BJ, Boumenna T, Dewhirst FE, Guerrero R (2008) Phylogenetic diversity and temporal variation in the Spirochaeta populations from two Mediterranean microbial mats. Int Microbiol 11:267-274
19. Blumenshine SC, Vandeboncoeur Y, Lodge DM, Cottingham KL, Knight SE (1997) Benthic pelagic links: Responses of benthos to water-column nutrients. J N Am Benthol Soc 16:466-479
20. Bosshard PP, Santini Y, Grüter D, Stettler R, Bachofen R (1999) Bacterial diversity and community composition in the chemocline of the meromictic

alpine Lake Cadagno as revealed by 16S rRNA gene analysis. FEMS Microbiol Ecol 31:173-182

21. Bradley WC, Owen TL (2007) Multiple carbon substrate utilization by bacteria at the sediment-water interface: seasonal patterns in a stratified eutrophic reservoir. Hydrobiologia 586:43-56
22. Butler HG (1999) Seasonal dynamics of the plankton microbial community in a maritime Antarctic lake undergoing eutrophication. J Plankton Res 20:1501-1525
23. Butler H, Atkinson A, Gordon M (2005) Omnivory and predation impact of the calanoid copepod *Boeckella popei* in a Maritime Antarctic lake. Polar Biol 28 815-821
24. Camacho A (2006a) Planktonic microbial assemblages and the potential effects of metazooplankton predation on the food web of lakes from the Maritime Antarctica and sub-Antarctic islands. Rev Environ Sci Biotechnol 5:167-185
25. Camacho A (2006b) On the occurrence and ecological features of deep chlorophyll maxima (DCM) in Spanish stratified lakes. Limnetica 25:453-478
26. Camacho A, Villaescusa JA, Rochera C, Jørgensen SE (2014) Modeling the response of the planktonic microbial community to warming effects in maritime Antarctic Lakes: Ecological implications. In Chang NB, Xu FL, Jørgensen SE (Eds.) Developments in environmental modelling. Elsevier, Amsterdam, pp. 231-250
27. Cammack WKL, Kalff J, Prairie YT, Smith EM (2004) Fluorescent dissolved organic matter in lakes: Relationships with heterotrophic metabolism. Limnol Oceanogr 49:2034-2045
28. Carman KR, Dobbs FC (1997) Epibiotic microorganisms on copepods and other marine crustaceans. Microsc Res Techniq 37:116-135
29. Carpenter SR, Chisholm SW, Krebs CJ, Schindler DW, Wright RE (1995) Ecosystem experiments. Science 269:324-327

30. Carrillo P, Medina-Sánchez JM, Villar- Argaiz M, Delgado- Molina JA, Bullejos FJ (2006) Complex interactions in microbial food webs: Stoichiometric and functional approaches. Limnetica 25:189-204
31. Casamayor EO, Schäfer H, Bañeras L, Pedrós-Alió C, Muyzer G (2000). Identification of and spatio-temporal differences between microbial assemblages from two neighboring sulfurous lakes: comparison by microscopy and denaturing gradient gel electrophoresis. Appl Environ Microbiol 66:499-508
32. Casamayor EO, Muyzer G, Pedrós-Alió C (2001) Composition and temporal dynamics of planktonic archaeal assemblages from anaerobic sulfurous environments studied by 16S rDNA Denaturing Gradient Gel Electrophoresis and sequencing. Aquat Microb Ecol 25:237-246
33. Casamayor EO, Pedrós-Alió C, Muyzer G, Amann R (2002) Microheterogeneity in 16S rDNA-defined bacterial populations from a stratified planktonic environment is related to temporal succession and to ecological adaptations. Appl Environ Microbiol 68:1706-1714
34. Catalán J, Camarero L, Felip M, Pla S, Ventura M, Buchaca T, Bartumeus F, De Mendoza G, Miró A, Casamayor EO, Medina- Sánchez JM, Bacardit M, Altuna M, Bartrons M, Díaz de Quijano D (2006) High mountain lakes: extreme habitats and witnesses of environmental change. Limnetica 25:551-584
35. Choi KH, Dobbs FC (1999) Comparison of two kinds of Biolog microplates (GN and Eco) in their ability to distinguish among aquatic microbial communities. J Microbiol Meth 36:203-213
36. Chróst RJ, Münster U, Rai H, Albrecht D, Witzel P, Overbeck J (1989) Photosynthetic production and exoenzymatic degradation of organic matter in the euphotic zone of a eutrophic lake. J Plankton Res 11:223-242
37. Clarke A, Murphy EJ, Meredith MP, King JC, Peck LS, Barnes DKA, Smith RC (2007) Climate change and the marine ecosystem of the western Antarctic Peninsula. Phil. Trans R Soc Lond B Biol Sci 362:149-166
38. Coble PG (1996) Characterization of marine and terrestrial DOM in seawater using excitation-emission matrix spectroscopy. Mar Chem 51:325-346

39. Convey P (2011) Antarctic terrestrial biodiversity in a changing world. Polar Biol 34:1629-1641
40. Cottrell MT, Kirchman DL (2003) Contribution of major bacterial groups to bacterial biomass production (thymidine and leucine incorporation) in the Delaware estuary. Limnol Oceanogr 40:168-178
41. Decho AW (1994) Exopolymers in microbial mats: Assessing their adaptive role. In: Stal LJ, Caumette P (eds) Microbial mats. Springer-Verlag, Berlin, p 215-219
42. Díez B, Pedrós-Alió C, Marsh TL, Massana R (2001) Application of Denaturing gradient gel electrophoresis (DGGE) to study the diversity of marine picoeukaryotic assemblages and comparison of DGGE with other molecular techniques. Appl Environ Microbiol 67:2942-2951
43. Dorado-García I, Medina-Sanchez JM, Herrera G, Cabrerizo MJ, Carrillo P (2014) Quantification of Carbon and Phosphorus Co-Limitation in Bacterioplankton: New Insights on an Old Topic. PlosOne 9:e99288
44. Dore JE, Priscu JC (2001) Phytoplankton phosphorus deficiency and alkaline phosphatise activity in the McMurdo Dry Valley lakes, Antarctica. Limnol Oceanogr 46:1331-1346
45. Duarte CM, Agustí S, Varqué D, Agawin N, Felipe J, Casamayor EO, Gasol JM (2005) Experimental test of bacteria-phytoplankton coupling in the Southern Ocean. Limnol Oceanogr 50:1844-1854
46. Dumestre JF, Casamayor EO, Massana R, Pedrós-Alió C (2002) Changes in bacterial and archaeal assemblages in an equatorial river induced by the water eutrophication of Petit Saut dam reservoir (French Guiana). Aquat Microb Ecol 26:209-221
47. Drago EC (1989) Thermal summer characteristics of lakes and ponds on Deception Island, Antarctica. Hydrobiologia 184:51-60
48. Dworkin M, Falkow S, Rosenberg E, Schleifer KH, Stackebrandt E (Eds.) (2006) The Prokaryotes. 3rd edition. Springer. 7:602-604
49. Eiler A, Bertilsson S (2004) Composition of freshwater bacterial communities associated with cyanobacterial blooms in four Swedish lakes. Environ Microbiol 6:1228-1243

50. Ellis-Evans JC (1996) Microbial diversity and function in Antarctic freshwater ecosystems. Biodiv Conserv 5:1395-1431
51. Ellis-Evans JC (1996) Biological and chemical features of lakes and streams. In: Geomorphological map of Byers Peninsula, Livingston Island. López-Martínez J, Thomson MRA, Thomson JW (eds.): 20-22. BAS GEOMAP Series, Sheet 5-A. British Antarctic Survey, Cambridge
52. Ellis-Evans JC, Laybourn-Parry J, Bayliss PR, Perriss SJ (1998) Physical, chemical and microbial community characteristics of lakes of the Lasermann Hills, Continental Antarctica. Arch Hydrobiol 141:209-230
53. Fernández-Leborans G (2010) Epibiosis in crustacea: an overview. Crustaceana 83:549-640
54. Fernández-Valiente E, Quesada A, Howard-Williams C, Hawes I (2001) N2 fixation in cyanobacterial mats from ponds on the McMurdo Ice Shelf, Antarctica. Microbial Ecol 42:338-349
55. Fernández-Valiente E, Camacho A, Rochera C, Rico E, Vincent WF, Quesada A (2007) Community structure and physiological characterization of microbial mats in Byers Peninsula. Livingston Island (South Shetland Islands, Antarctica). FEMS Microbiol. Ecol 59:377-385
56. Fouilland E, Mostajir B (2010) Revisited phytoplanktonic carbon dependency of heterotrophic bacteria in freshwaters, transitional, coastal and oceanic waters. FEMS Microbiol Ecol 73:419-429
57. Fountain AG, Berry Lyons W, Burkins M.D, Dana GL, Doran PT, Lewis KJ, McKnight DM, Moorhead DLL, Parsons AN, Priscu JC, Wall DH, Wharton RA, Ross VA (1999) Physical control on the Taylor Valley Ecosystem, Antarctica. Bioscience 40:961-971
58. Fourcans A, de Oteyza TG, Wieland A, Sole A, Diestra E, van Bleijswijk J, Grimalt JO, Kuhl M, Esteve I, Muyzer G, Caumette P, Duran R (2004) Characterization of functional bacterial groups in a hypersaline microbial mat community (Salins-de-Giraud, Camargue, France). FEMS Microbiol Ecol 51:55-70

59. Frenot Y, Chown SL, Whinam J, Selkirk PM, Convey P, Skotnicki M, Bergstrom DM (2005) Biological invasions in the Antarctic: extent, impact and implications. Biol Rev 80:45-72
60. Fritsen CH, Priscu JC (1998) Cyanobacterial assemblages in permanent ice covers on Antarctic lakes: distribution, growth rate and temperature response of photosynthesis. J Phycol 10:587-597
61. Gárate-Lizárraga I, Muñetón-Gómez M (2009) Primer registro de la diatomea epibionte Pseudohimantidium pacificum y de otras asociaciones simbióticas en el golfo de california. Acta Bot Mex 88:31-45
62. García-Jurado F, Jiménez-Gómez F, Guerrero F (2011) Effects of a dry period on the limnological characteristics of a Mediterranean high mountain lake. Limnetica 30:5-16. GOOD IJ (1953). The population frequencies of species and the estimation of the population parameters. Biometrika 40:237-264
63. Garland JL (1997) Analysis and interpretation of community-level physiological profiles in microbial ecology. FEMS Microbiol Ecol 24:289-300
64. Garland JL, Mills AL (1991) Classification and characterization of heterotrophic microbial communities on the basis of patterns of community level sole-carbon-source utilization. Appl Environ Microbiol 57:2351-2359
65. Gasol JM, del Giorgio P (2000) Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities. Sci Mar 64:197-224
66. Gibson RA (1979) Protoraphis atlantica sp. nov., a new marineepizoic diatom. Bacillaria 2:109-125
67. Gil-Delgado JA, González-Solís J, Barbosa A (2013a) Populations of breeding birds in Byers Peninsula, Livingston Island, South Shetland Islands. Antarct Sci 25:303-306
68. Gil-Delgado JA, Villaescusa JA, Diazmacip ME, Velázquez D, Rico E, Toro M, Quesada A, Camacho A (2013b) Minimum population size demonstrate an increase in southern elephant seals (*Mirounga leonine*) on Livingston Island, maritime Antarctica. Polar Biol 36:607-610

69. Guillemete F, del Giorgio PA (2012) Simultaneous consumption and production of fluorescent dissolved organic matter by lake bacterioplankton. Environ Microbiol 14:1432-1443
70. Hanisch K, Schweitzer B, Simon M (1996). Use of Dissolved Carbohydrates by Planktonic Bacteria in a Mesotrophic lake. Microb Ecol 31:41-55
71. Hansson LA (1992) The role of food-chain composition and nutrient availability in shaping algal biomass development. Ecology 73:241-247
72. Hansson LA, Dartnall HJG, Ellis-Evans JC, MacAlister H, Tranvik LJ (1996) Variation in physical, chemical and biological components in the sub-Antarctic lakes of South Georgia. Ecography 19:393-403
73. Hawes I (1993) Photosynthesis in thick cyanobacterial films: a comparison of annual and perennial Antarctic mat communities. Hydrobiologia 252:203-209
74. Hawes I, Howard-Williams C, Pridmore RD (1993) Environmental control of microbial communities in the ponds of the McMurdo Ice Shelf, Antarctica. Arch Hydrobiol 127:271-287
75. Hawes I, Schwarz AMJ (2001) Absorption and utilization of irradiance by cyanobacterial mats in two ice-covered Antarctic lakes with contrasting light climate. J Phycol 37:5-15
76. Heywood RB (1967) Antarctic ecosystems. The freshwater lakes of Signy Island and their fauna. Phil Trans Royal Soc London B 261:347-362
77. Heywood RB (1968) Ecology of the fresh-water lakes of Signy Island, South Orkney Islands: II. Physical and chemical properties of the lakes. Brit Antarct Sur Bull 18:11-44
78. Heywood RB (1970) Ecology of the fresh-water lakes of Signy Island, South Orkeney Islands: III. Biology of the copepod Pseudoboeckella silvestri Daday (Calanoida, Centropaghidae). Br Antarct Sur Bull 23:1-18
79. Herbei R, Lyons WB, Laybourn-Parry J, Gardner C, Priscu JC, McKnight DM (2010) Physiochemical properties influencing biomass abundance and primary production in Lake Hoare, Antarctica. Ecol Model 221:1184-1193

80. Hervàs A, Casamayor EO (2009) High similarity between bacterioneuston and airborne bacterial community compositions in a high mountain lake area. FEMS Microbiol Ecol 67:219-228
81. Ikeda T (1977) A pelagic marine copepod associated with diatoms. Bull Plank Soc Japan 24:39-42
82. Imura S, Bando T, Saito S, Seto K, Kanda H (2003) Benthic moss pillars in Antarctic lakes. Polar Biol 22:137-140
83. IPCC (2007) Climate Change 2007: Synthesis Report. Contribution of Working Groups I, II and III to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change [Core Writing Team, Pachauri RK and Reisinger A (eds.)]. IPCC, Geneva, Switzerland, 104 pp
84. Izaguirre I, Mataloni G, Allende L, Vinocur A (2001) Summer fluctuations of microbial planktonic communities in a eutrophic lake Cierva Point, Antarctica. J Plankton Res 23:1095-1109
85. Izaguirre I, Allende I, Marinone MC (2003) Comparative study of the planktonic communities of three lakes of contrasting trophic status at Hope Bay (Antarctic Peninsula). J Plankton Res 25:1079-1097
86. Izaguirre I, Haydée P, Allende L, Unrein F, Rodríguez P, Marinone MC, Tell G (2012) Responses of a maritime Antarctic lake to a catastrophic draining event under a climate change scenario. Polar Biol 35:231-239
87. Jeffrey SW, Mantoura RFC, Wright SW (1997) Phytoplankton pigments in oceanography: guidelines to modern methods. UNESCO, Paris, 681 pp
88. Jellett JF, Li WKW, Dickie PM, Boraie A, Kepkay PE (1996) Metabolic activity of bacterioplankton communities assessed by flow cytometry and single carbon substrate utilization. Mar Ecol Prog Ser 136:213-225
89. Jørgensen NOG, Jensen RE (1994) Microbial fluxes of free monosaccharides and total carbohydrates in freshwater determined by PAD-HPLC. FEMS Microbiol Ecol 14:79-94
90. Jørgensen SE, Bendoricchio G (2001) Fundamentals of Ecological Modeling. Elsevier, Amsterdam, p. 530
91. Jørgensen SE, Svirezhev Y (2004) Toward a thermodynamic theory for ecological systems. Elsevier, Amsterdam, p. 230

92. Jørgensen SE, Ludovisi A, Nielsen SN (2010) The free energy and information embodied in the amino acid chains of organisms. Ecological Modeling. 221:2388-2392
93. Kamjunke N, Kohler B, Wannicke N (2008) Algae as competitors for glucose with heterotrophic bacteria. J Phycol 44:616-623
94. Kenarova A, Encheva M, Chipeva V, Chipev N, Hristova P, Moncheva P (2013) Physiological diversity of bacterial communities from different soil locations on Livingston Island, South Shetland archipelago, Antarctica. Polar Biol 36:223-233
95. Kirchman D, K´Nees E, Hodson R (1985) Leucine incorporation and its potencial as a measure of protein synthesis by bacterial in natural aquatic systems. Appl Environ Microbiol 49:599-607
96. Kritzberg EC, Cole JJ, Pace ML, Graneli W, Bade DL (2004) Autochthonous versus allochthonous carbon sources of bacteria: Results from whole-lake C-13 addition experiments. Limnol Oceanogr 49:588-596
97. Laybourn-Parry J (1997) The microbial loop in Antarctic lakes. In: Howards-Williams C, Lyons WB, Hawes I (eds). Ecosystem Processes in Antarctic Ice-free Landscapes. Balkema, Rotterdam. 231-240
98. Laybourn-Parry J (2002) Survival mechanisms in Antarctic lakes. Philos T the R Soc B 357:863-869
99. Laybourn-Parry J (2009) No place too cold. Science 324:1521-1522
100. Laybourn-Parry J, Marchant HJ, Brown PE (1991) The plankton of a large freshwater Antarctic lake. J Plan Res 13:1137-1150
101. Laybourn-Parry J, Bayliss P, Ellis-Evans JC (1995) The dynamics of heterotrophic nanoflagellates and bacterioplankton in a large ultra-oligotrophic Antarctic lake. J Plankton Res 17:1834-1850
102. Laybourn-Parry J, Quayle WC, Henshaw T, Ruddell A, Marchant HJ (2001) Life on the edge: the plankton and chemistry of Beaver lake, an ultra-oligotrophic epishelf lake, Antarctica. Freshwater Biol 46:1205-1217
103. Laybourn-Parry J, Henshaw J, Jones DJ, Quayle W (2004) Bacterioplankton production in freshwater Antarctic lakes. Freshwater Biol 49: 735-744

104. Laybourn-Parry J, Madan NJ, Marshal WA, Marchant HJ, Wright SW (2006) Carbon dynamics in an ultra-oligotrophic epishelf lake (Beaver Lake, Antarctica) in summer. Freshwater Biol 51:1116-1130
105. Laybourn-Parry J, Pearce D (2007) The biodiversity and ecology of Antarctic lakes: models for evolution. Philos T Royal Soc B 362:2273-228
106. Lewis WM Jr (2011) Global primary production of lakes: 19th Baldi Memorial Lecture. Island Waters 1:1-28
107. Li S, Ochyra R, Wu P, Seppelt RD, Cai M, Wang H, Li C (2009) *Depranocladus longifolius* (Amblystegiaceae), an addition to the moss flora of King George Island, South Shetland Islands, with a review of Antarctic benthic mosses. Polar Biol 32:1415-1425
108. Li WKW, Jellett JF, Dickie PM (1995) Flow cytometric analysis of marine bacteria stained with TOTO or TO-PRO. Limnol Oceanogr 40:1485-1495
109. Llorens-Marès T, Auguet JC, Casamayor EO (2012) Winter to spring changes in bacterial community composition in the slush layer of a high-mountain lake (Lake Redon, Pyrenees). Environ Microbiol Reports 4:50-56
110. López-Bueno A, Tamames J, Velázquez D, Andrés M, Quesada A, Alcami A (2009) High diversity of the viral community from an Antarctic lake. Science 326:585
111. Lovejoy C, Vincent WF, Bonilla S, Roy S, Martineau MJ, Terrado R, Potvin M, Massana R, Pedrós-Alió C (2007) Distribution, phylogeny, and growth of cold-adapted picoprasinophytes in arctic seas. J Phycol 43:78-89
112. Manahan SE (2000) Environmental Chemistry, Seventh Edition. CRC Press, 876 pp.
113. Mataloni G, Tesolin G, Tell G (1998) Characterization of a small eutrophic Antarctic lake (Otero Lake, Cierva Point) on the basis of algal assemblages and water chemistry. Polar Biol 19:107-114
114. McGuire AJ, Franzmann PD, McMeekin TA (1987) *Flectobacillus glomeratus* sp. nov., a curved, nonmotile, pigmented bacterium isolated from Antarctic marine environments. Syst Appl Microbiol 9(3):265-72

115. Mcknight DM, Howes BL, Taylor CD, Goehringer DD (2000) Phytoplankton dynamics in a stably stratified Antarctic lake during winter darkness. J Phycol 36:852-861
116. Meredith MP, King JC (2005) Rapid climate change in the ocean west of the Antarctic Peninsula during the second half of the 20th century. Geophys Res Lett 32, L19604, http://dx.doi.org/10.1029/2005GL024042
117. Michaud L, Caruso C, Mangano S, Interdonato F, Bruni V, Logiudice A (2012) Predominance of *Flavobacterium*, *Pseudomonas* and *Polaromonas* within the prokaryotic community of freshwater shallow lakes in the northern Victoria Land, East Antarctica. FEMS Microbiol Ecol 82(2):391-404
118. Montecino V, Pizarro G, Cabrera S, Contreras M (1991) Spatial and temporal photosynthetic compartments during summer in Antarctic Lake Kitiesh. Polar Biol 11:371-377
119. Moorhead D, Schmeling J, Hawes I (2005) Modelling the contribution of benthic microbial mats to net primary production in Lake Hoare, McMurdo Dry Valleys. Ant Sci 17:33-45
120. Morales-Baquero R, Carrillo P, Cruz-Pizarro L, Sánchez Castillo P (1992) southernmost high mountain lakes in Europe (Sierra Nevada) as reference sites for polution and climate change monitoring. Limnetica 8:39-47
121. Mosier AC, Murray AE, Frisen CH (2007) Microbiota within the perennial ice cover of Lake Vida, Antarctica. FEMS Microbiol Ecol 59:274-288
122. Møhlenberg F, Kaas H (1990) Colacium vesiculosum Ehrenberg (Euglenophyceae), infestation of planktonic copepods in the Western Baltic. Ophelia 31:125-132
123. Navarro MB, Modenutti B, Callieri C, Bertoni R, Balseiro E (2009) Balance between primary and bacterial production in North Patagonian shallow lakes. Aquat Ecol 43:867-878
124. Nichols DS, Bowman JP, Sanderson K, Mancuso-Nichols C, Lewis TE, Mc Meekin TA, Nichols PD (1999) Development with Antarctic microorganisms: culture collections, bioactivity screening, taxonomy, PUFA production and cold-adapter enzyme. Curr Opin Biotech 10:240-246

125. Noble RT, Fuhrman JA (1998) Use of SYBR Green I for rapid epifluorescence counts of marine viruses and bacteria. Aquat Microb Ecol. 14:113-118
126. Park JW, Yoo JS, Roh DH (2006) Identification of novel psychrotolerant bacterial strain and production of beta-galactosidase. Misainmurhag Hoiji 42:40-46
127. Pearce DA (2003). Bacterioplankton community structure in a maritime Antarctic oligrotrophic lake during a period of holomixis, as determined by denaturing gradient gel electrophoresis (DGGE) and fluorescence in situ hybridization (FISH). Microb Ecol 46:92-105
128. Pearce DA (2005) The structure and stability of the bacterioplankton community in Antarctic freshwater lakes, subject to extremely rapid environmental change. FEMS Microbiol Ecol 53:61-72
129. Pearce DA, Butler HG (2002) Short-term stability of the microbial community structure in a Maritime Antarctic lake. Polar Biol 25:479-487
130. Pearce DA, van der Gast CJ, Lawley B, Ellis-Evans JC (2003) Bacterioplankton community diversity in a Maritime Antarctic lake, determined by culture-dependent and culture-independent techniques. FEMS Microbiol Ecol 45:59-70
131. Pearce DA, Cockell CS, Lindström ES, Tranvik LJ (2007) First evidence for a bipolar distribution of dominant freshwater lake bacterioplankton. Antarct Sci 19:245-252
132. Pearce DA, Galand PE (2008) Microbial biodiversity and biogeography. In: Vincent W, Laybourn-Parry J (eds) Polar lakes and rivers, limnology of Arctic and Antarctic aquatic ecosystems. Oxford University Press, New York, pp 213-230
133. Pedrós-Alió C (2006) Marine microbial diversity: can it be determined? Trends Microbiol 14:257-263
134. Personnic S, Domaizon I, Dorigo U, Berdjeb L, Jacquet S (2009) Seasonal and spatial variability of virio, bacterio, and picophytoplanktonic abundances in three peri-alpine lakes. Hydrobiologia 627:99-116

135. Petz W, Valbonesi A, Quesada A (2005) Ciliate biodiversity in freshwater habitats of the maritime and continental Antarctica. Terra Antarctica 11:43-50.

136. Picazo A, Rochera C, Vicente E, Miracle MR, Camacho A (2013) Spectrophotometric methods for the determination of the photosynthetic pigments in stratified lakes: a critical analysis based on comparisons with HPLC determinations in a model lake. Limnética 32:139-158

137. Pimenov NV, Zyakun AM, Prusakova TS, Lunina ON, Ivanov MV (2008) Application of ^{13}C mineral carbon for assessment of the primary production of organic matter in aquatic environments. Microbiology 77:224-227

138. Pinckney JL, Millie DF, Howe KE, Paerl HW, Hurley JP (1996) Flow scintillation counting of 14C-labeled microalgal photosynthetic pigments. J Plankton Res 18:1867-1880

139. Preston-Mafham J, Boddy L, Randerson PF (2002) Analysis of microbial community functional diversity using sole-carbon-source utilixation profiles- a critique. FEMS Microbiol Ecol 42:1-14

140. Priscu JC (ed) (1998) Ecosystem dynamics in a polar desert: the McMurdo Dry Valleys, Antarctica. Antarc Res Series 72, American Geophysical Union, Washington DC, 369 pp

141. Priscu JC, Fritsen CH, Adams EE, Giovannoni SJ, Paerl HW, McKay CP, Doran PT, Gordon DA, Lanoil BD, Pinckney JL (1998) Perennial Antarctic lake ice: an oasis for life in a polar desert. Science 280:2095-2098

142. Quayle WC, Peck LS, Peat H, Ellis-Evans JC, Harrigan PR (2002) Extreme responses to climate change in Antarctic lakes. Science 295:645

143. Qu JH, Yuan HL (2008) *Sediminibacterium salmoneum gen. nov. sp. nov.*, a member of the phylum *Bacteroidetes* isolated from sediment of a eutrophic reservoir. Int J Syst Evol Micr 58:2191-2194

144. Quesada A, Vincent WF (1997) Strategies of adaptation by Antarctic cyanobacteria to ultraviolet radiation. Eur J Phycol 32:335-342

145. Quesada A, Camacho A, Rochera C, Velázquez D (2009) Byers Peninsula: A reference site for coastal, terrestrial and limnetic ecosystem studies in maritime Antarctica. Polar Sci 3:181-187

146. Quesada A, Camacho A, Lyons WB (2013) Multidisciplinary research on Byers Peninsula, Livingston Island: a future benchmark for change in maritime Antarctica. Antarct Sci 25:123-127
147. Reche I, Pulido-Villena E, Morales-Baquero R, Casamayor EO (2005) Does ecosystem size determine aquatic bacterial richness? Ecology 86:1715-1722
148. Reid T, Crout N (2008) A thermodynamic model of freshwater Antarctic lake ice. Ecol model 210:221-241
149. Riis T, Christoffersen KS, Baattrup-Pedersen A (2014) Effects of warming on annual production and nutrient-use efficiency of aquatic mosses in a high Arctic lake. Freshwater Biol 58:1622-1632
150. Rochera C, Justel A, Fernández-Valiente E, Bañón M, Rico E, Toro M, Camacho A, Quesada A (2010) Interannual meteorological variability and its effects on a lake from maritime Antarctica. Polar Biol 33:1615-1628
151. Rochera C, Villaescusa JA, Velázquez D, Fernández-Valiente E, Quesada A, Camacho A. (2013) Vertical structure of bi-layered microbial mats from Byers Peninsula, Maritime Antarctica. Antarct Sci 25:270-276
152. Rodríguez P, Rico E (2008) A new freshwater oligochaete species (Clitellata: Enchytraeidae) from Livingston Island, Antarctica. Polar Biol 31:1267-1279
153. Russell A, McGregor GR (2010) Southern hemisphere atmospheric circulation: impacts on Antarctic climate and reconstructions from Antarctic ice core data. Climatic Change 99:155-192
154. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406-425
155. Salomo S, Muench C, Roeske I (2009) Evaluation of the metabolic diversity of microbial communities in four different filter layers of a constructed wetland with vertical flow by Biolog™ analysis. Water Res 43:4569-4578
156. Sawatrom C, Laybourn-Parry J, Graneli W, Anesio AM (2007) Heterotrophic bacterial and viral dynamics in Artic freswaters: results from a field study and nutrient-temperature manipulation experiments. Polar Biol 30:1407-1415

157. SCAR (2003) Management Plan for Antarctic Specially Protected Area No. 126 Byers Peninsula, Livingston Island, South Shetland Islands. SCAR Bulletin 150, July
158. Schiaffino MR, Unrein F, Gasol JM, Farias ME, Estevez C, Balagué V, Izaguirre I (2009) Comparative analysis of bacterioplankton assemblages from maritime Antarctic freshwater lakes with contrasting trophic status. Polar Biol 32:923-936
159. Schiaffino MR, Unrein F, Gasol JM, Ramon M, Balagué V, Izaguirre I (2011) Bacterial community structure in a latitudinal gradient of lakes: the roles of spatial versus environmental factors. Freshwater Biol 56:1973-1991
160. Serrano E, Martínez de Pisón E, López-Martínez J (1996) Periglacial and nival landforms and deposits. En: López-Martínez J., Thomson M.R.A. y Thomson, J.W. (Eds.). Geomorphological map of Byers Peninsula, Livingston Island. BAS GEOMAP Series, Sheet 5-A, 28-34. Cambridge, British Antarctic Survey
161. Shelton RGJ (1974) Observations on the occurrence of an epizooic, blue-green alga on the chemoreceptor setae of the brown shrimp, Crangon crangon (L.). J Mar Biol Assoc UK 54:301-307
162. Silow E Mokry A (2010) Exergy as a tool for ecosystem health assessment. Entropy 12:902-925
163. Simon M, Azam F (1989) Protein content and protein synthesis rates of planktonic marine bacteria. Mar Ecol Prog Ser 51:201-213
164. Singleton DR, Furlong MA, Rathbun SL, Whitman WB (2001) Quantitative comparisons of 16S rRNA gene sequence libraries from environmental samples. Appl Environ Microbiol 67:4374-4376
165. Sommer U, Sommer F (2006) Cladocerans versus copepods: the cause of contrasting top-down controls on freshwater and marine phytoplankton. Oecología 147:183-194
166. Spring S, Merkhoffer B, Weiss N, Kroppenstedt RM, Hippe H, Stackebrandt E (2003) Characterization of novel psychrophilic clostridia from an Antarctic microbial mat: description of *Clostridium frigoris* sp. nov., *Clostridium lacusfryxellense* sp. nov., *Clostridium bowmanii* sp. nov. and *Clostridium*

psychrophilum sp. nov. and reclassification of *Clostridium laramiense* as *Clostridium estertheticum* subsp. *laramiense* subsp. nov. Int J Syst Evol Micr 53:1019-1029

167. Stedmond CA (2005) Resolving the variability in dissolved organic matter fluorescence in a temperate estuary and its catchment using PARAFAC analysis. Limnol Oceanogr 50:686-697
168. Stefanowicz A (2006) The Biolog Plates Technique as a Tool in Ecological Studies of Microbial Communities. Pol J Environ Stud 15:669-676
169. Steig E, Schneider D, Rutherford S, Mann M, Comiso J, Shindell D (2009) Warming of the Antarctic ice-sheet surface since the 1957 International Geophysical Year. Nature 457:459-463
170. Tamames J, Abellan JJ, Pignatelli M, Camacho A, Moya A (2010) Environmental distribution of prokaryotic taxa. BMC Microbiol, 10.1186/1471-2180-10-85
171. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731-2739
172. Threlkeld ST, Chiavelli DA, Willey RL (1993) The organization of zooplankton epibiont communities. Trends Ecol Evol 8:317-321
173. Thingstad TF (2000) Control of bacterial growth in idealized food webs. In: Microbial Ecology of the Oceans. Kirchman DL (ed.): 261-288. John Wiley and Sons, New York
174. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673-4680
175. Toro M, Camacho A, Rochera C, Rico E, Bañón M, Fernandéz-Valiente E, Marco E, Justel A, Avedaño MC, Ariosa Y, Vincent WF, Quesada A (2007) Limnological characteristics of the freshwater ecosystems of Byers Peninsula, Livingston Island, in maritime Antarctica. Polar Biol 30:635-649

176. Thomson MRA, López-Martínez J (1996) Introduction to the geomorphology quaternary geology of Byers Península. En: *Supplementary text of the Geomorphological Map of Byers Peninsula*. BAS Geomap Series, 5-A, 1-4. Cambridge: British Antarctic Survey
177. Troussellier M, Courties C, Lebaron P, Servais P (1999) Flow cytometric discrimination of bacterial populations in seawater based on SYTO 13 staining of nucleic acids. FEMS Microbiology Ecology 29:319-330
178. Unrein F, Izaguirre I, Massana R, Balagué V, Gasol JM (2005) Nanoplankton assemblages in Maritime Antarctic lakes: characterization and molecular fingerprinting comparison. Aquat Microb Ecol 40:269-282
179. Vadeboncoeur Y, Peterson G, Vander Zanden MJ, Kalff J (2008) Benthic algal production across lake size gradients: Interactions among morphometry, nutrients and light. Ecology 88:2542-2552
180. Van Heukelem L, Lewitus AJ, Kana TM (1992) High-performance liquid chromatography of phytoplankton pigments using polymeric reversed-phase C_{18} column. Journal of Phycology 28:867-872
181. Van Trappen S, Mergaert J, van Eygen S, Dawynt P, Cnockaert MC, Swings J (2002). Diversity of 746 heterotrophic bacteria isolated from microbial mats from ten Antarctic lakes. Syst Appl Microbiol 25:603-610
182. Velázquez D, Rochera C, Camacho A, Quesada A (2011) Temperature effects on carbon and nitrogen metabolism in some Maritime Antarctic freshwater phototrophic communities. Polar Biol 34:1045-1055
183. Velázquez D, Lezcano MA, Frias A, Quesada A (2013) Ecological relationships and stoichiometry within a Maritime Antarctic watershed. Ant Sci 25: 191-197
184. Verdouw HC, van Echteld JA, Dekkers T (1978) Ammonia determination based on indophenol formation with sodium salicylate. Wal Res 13:339-402
185. Villaescusa JA, Casamayor EO, Rochera C, Velázquez D, Chicote, Quesada A, Camacho A (2010) A close link between bacterial community composition and environmental heterogeneity in maritime Antarctic lakes. Int Microbiol 13:67-77

186. Villaescusa JA, Casamayor EO, Rochera C, Quesada A, Michaud L, Camacho A (2013a) Heterogeneous vertical structure of the bacterioplankton community in a nonstratified Antarctic lake. Antarct Sci 25:229-238
187. Villaescusa JA, Rochera C, Velázquez D, Rico E, Quesada A, Camacho A (2013b) Bacterioplankton summer dynamics in a maritime Antarctic lake. Limnética 32:253-268
188. Vincent WF (2000) Microbial ecosystems of Antarctica (studies in polar research). Cambridge University Press, Cambridge
189. Vincent WF, Downes MT, Castenholz RW, Howard-Williams C (1993) Community structure and pigment organization of cyanobacteria-dominated microbial mats in Antarctica. Eur J Phycol 28:213-221
190. Vincent WF, Hobbie JE, Laybourn-Parry J (2008) Introduction to the limnology of high-latitude lake and river ecosystems. In: Vincent W, Laybourn-Parry J (eds) Polar lakes and rivers, limnology of arctic and Antarctic aquatic ecosystems. Oxford University Press, New York, pp 1-23
191. Wagner M, Amman R, Lemmer H, Schleifer H (1993) Probing activated sludge with oligonucleotides specific for proteobacteria: inadequacy of culture-dependent methods for de- scribing microbial community structure. Appl Environ Microbiol 59:1520-1525
192. Wang Y, Ouyang Z, Zheng H, et al. (2011) Carbon metabolism of soil microbial communities of restored forest in southern China. J Soils Sed 11(5):789-799
193. Wetzel RG, Likens GE (1991) Limnological Analyses. 2nd Ed. Springer-Verlag. 391 pp
194. Wu X, Xi W, Ye W, Yang H (2007) Bacterial community composition of a shallow hypertrophic freshwater lake in China, revealed by 16S rRNA gene sequences. FEMS Microbiol Ecol 61:85-96
195. Wynn-Williams DD (1996) Antarctic microbial diversity: The basis of polar ecosystem processes. Biodiv Conserv 5:1271-1923
196. Xing P, Hahn MW, Wu LQ (2009) Low taxon richness of bacterioplankton in high altitude lakes of the eastern Tibetan plateau, with a predominance of

Bacteroidetes and *Synechococcus* spp. Appl Environ Microbiol 75:7017-7025