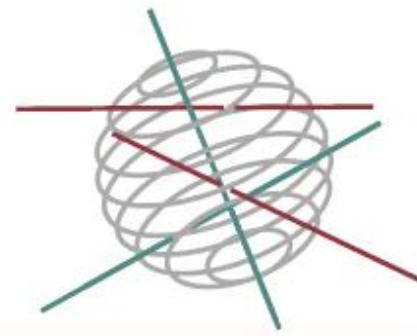# SSD
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Antarctic Microbial Biodiversity: the Importance of Geographical and Ecological factors

"AMBIO"

A. WILMOTTE, W. VYVERMAN, A. WILLEMS, E. VERLEYEN,
K.PEETERS, D. OBBELS, C. SOUFFREAU, P. DE CARVALHO MAALOUF.

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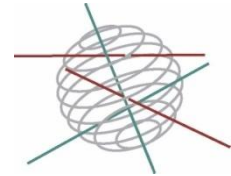
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Antarctica - Biodiversity

FINAL REPORT

Antarctic Microbial Biodiversity: the Importance of Geographical and Ecological factors

"AMBIO"

SD/BA/01

Promotors

Annick Wilmotte
Université de Liège (ULg)

Wim Vyverman
Universiteit Gent (Ugent)

Anne Willems
Universiteit Gent (Ugent)

Authors
Annick Wilmotte, Pedro De Carvalho Maalhouf, (ULg)
Wim Vyverman, Elie Verleyen, Dagmar Obbels, Caroline Souffreau (PAE, UGent)
Anne Willems, Karolien Peeters, (LM-UGent)

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ACRONYMS, ABBREVIATIONS AND UNITS

ANTARIMPACT	BelSPO funded project "Expertisepool on inventarisation and evaluation of the environmental impact of the Antarctic Research Station 'Princess Elisabeth' "
App	Appendix
ARDRA	Amplified Ribosomal DNA Restriction Analysis
BAS	British Antarctic Survey
BB	Belgian Polar Base
BCs	Band Classes
BELDIVA	BelSPO funded project "Belgian microbial DIVersity in Antarctica".
BWASW	BurrowWheeler Aligner – SmithWaterman
CA	Correspondence Analysis
CACHEPEP	Climate and Chemistry (CACHE) : Forcings, Feedbacks and Phasing in the Earth System. Natural climate variability – extending the Americas paleoclimate transect through the Antarctic Peninsula to the Pole
C	cosmopolitan
CCA	Canonical Correspondence Analysis
CEP	Comittee for Environmental Protection (Antarctic Treaty System)
CFU	colony forming units
DCA	Detrended Correspondence Analysis
DGGE	Denaturating Gradient Gel Electrophoresis
DV	Mc Murdo Dry Valleys
EA	East Antarctica
EBA	Evolution and Biodiversity in Antarctica
EC	European Commission
EMBL	European Microbiology Biology Laboratory
Fig.	figure
%GC	percentage of guanine plus cytosine content
HOLANT	BelSPO funded project "Holocene climate variability and ecosystem change in coastal east and maritime Antarctica"
IPY	International Polar Year
ITS	Internal Transcribed Spacer
LAQUAN	previous BelSPO funded project "Late Quaternary climate history of coastal Antarctic environments : a multiproxy approach"
LMG	Laboratorium voor Microbiologie Gent
LSU	Large Subunit
MERGE	Microbiological and Ecological Responses to Global Environmental change
MERLIN	Merging of "REGAL" and "LAQUAN" projects
MICROMAT	previous EC project "Biodiversity of Microbial mats in Antarctica"
N	new (new OTU)
OTU	Operational Taxonomic Unit

PAE / P2	UGent laboratory for Protistology and Aquatic Ecology
PCA	Principal Component Analysis
RDA	Redundancy analysis
RDP	Ribosomal Database Project
REGAL	NIPR project "Research on Ecology and Geohistory of Antarctic Lakes"
repPCR	repetitive extragenic palindromic PCR
RNA	Ribonucleic acid
rRNA/rDNA,	ribosomal RNA/DNA (ribonucleic acid/deoxyribonucleic acid)
SCAR	Scientific Committee on Antarctic Research
SSU	Small Subunit
TM	Transantarctic Mountains
WO	West Ongul (East Antarctica)

SUMMARY

A. Context

Microbial organisms dominate most Antarctic ecosystems and play a crucial role in their functioning and primary productivity. Compared with temperate and tropical regions and despite their ecological importance, little is known about Antarctic microbial diversity and its geographical distribution. This is due the lack of systematic sampling and geographical coverage, and the problems associated with species definition, cryptic diversity and cultivability (e.g. Taton *et al.*, 2003). As a result, we largely lack the 'baseline' data needed to observe possible future changes in microbial diversity and taxonomic composition due to ecosystem change and/or human introductions.

Most of the earlier diversity studies were carried out with traditional methods such as isolation of bacterial strains and microscopic identifications of cyanobacteria and protists on the basis of morphological features and 'force-fitting' of names of temperate taxa on the Antarctic ones. This approach also lacked stability because of the plasticity of the morphology. Molecular tools enabled studies based on the SSU rRNA gene, and have shown a quite different view of the diversity and the existence of not-yet cultivated genotypes. In contrast to phenotypic markers, the genotypic based approaches have a more fine-grained taxonomic resolution and reflect the evolutionary history of the organisms. Molecular-based approaches also have a considerable potential for the study of the geographical distribution of microorganisms. This is important, because it is still unclear whether geographic isolation is present in microorganisms, and hence whether they exhibit a biogeography at all (Martiny et al. 2006). This 'ubiquity hypothesis' was first formulated by Baas-Becking (1934) and states that 'everything is everywhere, but the environment selects'. It is underlain by the assumption that the vast population sizes of micro-organisms drive ubiquitous dispersal and make local extinction virtually impossible (Finlay et al. 2002). However, various recent studies suggest that micro-organisms, do display restricted geographic ranges (Chao et al 2006; Foissner 2006) and that endemism is possible.

Antarctica is a prime place to investigate **microbial biogeography** and to elucidate the roles of historical processes and contemporary environmental conditions shaping microbial diversity and community structure. This is due to its extreme isolation with respect to the rest of the world, resulting from its geographic setting and the nature of ocean and atmospheric currents as well as of the scattered occurrence of terrestrial oases along the continental margins. Furthermore, organisms inhabiting the continent need to survive in extreme environmental conditions, such as low and extremely fluctuating temperatures, dramatically changing light conditions, high seasonal UV-B loads, and low humidity.

Thus, as a whole, the continent bears wide **environmental gradients** that impose increasing stresses on the biodiversity and community structures of Antarctic environments (Gibson et al. 2006a). In addition, certain habitats offer some protection from the extreme conditions. For example, liquid water in aquatic environments may act as 'thermal buffer' (Vincent and Laybourn-Parry. 2008). Moreover, preliminary data on aerosol diversity in the Antarctic Peninsula showed the potential for wide-range transport of microbial diversity, though much of the aerobiota found was of local origin (Hughes et al. 2004).

B. Objectives

In the present project, we aimed to extend the baseline information of microbial diversity through an integrated and standardized analysis of the microbial diversity of aquatic habitats in terrestrial Antarctic environments. We aimed to use a **polyphasic approach** combining morphologic characterization by microscopy with molecular techniques in order to reveal the diversity of bacteria, cyanobacteria and protists (with special emphasis on green algae and diatoms), which have been identified as interesting focal taxa during our earlier studies. To work in parallel on environmental samples and isolated strains in culture allows us to obtain a more complete image of the diversity.

C. Conclusions

a) Bacteria diversity

Nine samples were used to study the **culturable bacterial diversity** by plating on different types of media and incubation at three relatively low temperatures. A total of 3806 isolates were obtained. They were first characterized by comparison of whole-genome fingerprints (rep-PCR) and this allowed them to be grouped into about 1400 unique rep-types. Very few of these comprised isolates from more than one sample. To identify these organisms, the 16S rRNA gene of a representative of each type was sequenced partially or in full. The diversity recovered belonged to four major phyla, *Actinobacteria*, *Bacteroidetes*, *Proteobacteria* and *Firmicutes.* Isolates belonging to the phylum *Deinococcus-Thermus* were only recovered from samples BB50, BB115, PQ1 and SO6. Many potential new species or new genera were documented among the isolates. Although most genera recovered were reported previously from Antarctica, for 30 out of the 83 genera, this was not the case. Moreover, several isolates belonged to genera that at present contain only one species and even one strain. The additional cultures obtained in this work may give more insight in the diversity present in these genera. Comparison with sequences from public databases indicates that an important number (42.2%) of species recovered seem to be restricted to Antarctica.

However, it is known that only about 5% of all bacterial species are currently present in databases and this number may therefore come down in the future. It does suggest that in Antarctica both cosmopolitan taxa as well as taxa with limited dispersal and which evolved in isolation occur.

A selection of **Flavobacterium** isolates recovered was studied in more detail using phylogeny of the 16S rRNA gene and the *gyrB* gene, as well as biochemical and chemotaxonomic approaches. The data revealed new strains from the Antarctic *Flavobacterium micromati* as well as twelve potential new species among our isolates. These will be studied further to describe and name them. To investigate the distribution of two of these potential new Antarctic *Flavobacterium* species, a PCR test using specific 16S rDNA primers was developed and used to detect these species in the community DNA of 32 Antarctic samples. This test can be used in the future to investigate the distribution of these species in environmental samples.

b) Cyanobacterial diversity

Different molecular methods were used to study the **cyanobacterial diversity** in strains (5) and environmental samples (95).

Five **cyanobacterial strains** from four continental samples were isolated. They belonged to the Oscillatoriaceae. Sequence analysis of these strains allowed the finding of 2 OTUs, not revealed by other molecular techniques (clone libraries and DGGE) stressing the importance of a polyphasic approach to unveil the microbial diversity of an environmental sample.

The **uncultivated diversity** was studied using clone libraries and DGGE. Clone libraries gave quite a large range of richness depending on the samples, from 2 to 12 OTUs (OTUs defined at a threshold of 98.5 % 16S rRNA similarity). The comparison with the sequences in public databases showed the existence of a majority of cosmopolitan OTUs, but also OTUs restricted to the cold biosphere (Arctic and/or alpine), and a minority of potentially endemic OTUs.

A Detrended Correspondence Analysis (DCA) was run with data from clone libraries from 20 samples of Prydz Bay, the Transantarctic Mountains, Shackelton Range and the Antarctic Peninsula and revealed that the OTU composition is geographically structured as each region has a more or less unique flora. The differences might be underlain by several reasons, such as differences in limnological properties between regions or rather the result from dispersal limitation among cyanobacteria. We can also observe that saline samples are grouped.

The DGGE band pattern analysis for 95 samples representing the 3 biogeographic provinces showed no clear community structure, probably due to mixed band classes.

The DGGE bands from a subset of 56 samples were sequenced and grouped into OTUs. We obtained a total of 33 OTUs for which the distribution was investigated. This showed different patterns of distribution: most of them (60.6%) were geographically and ecologically widespread. The rest (39.4%) of them seemed to be restricted to the "cold biosphere" (polar and alpine habitats). Among the latter, 5 OTUs seem to be endemic to Antarctica.

These sequence analyses point towards the existence of environmental and geographic limitations on the distribution of the cyanobacterial OTUs. Thus, both cosmopolitan and potentially endemic distributions were observed.

c) Microalgal diversity

The **cultivable diversity of coccal green algae** was studied in samples from 33 lakes in maritime and continental Antarctica. The 14 distinct chlorophycean and trebouxiophycean lineages observed were compared with the sequences present in GenBank and point to a wide phylogenetic diversity of apparently endemic Antarctic lineages at different taxonomic levels. Two taxa were detected in most regions, suggesting that they are widely dispersed over Antarctica. Most of the studied taxa (10 out of 14) however were only retrieved from one ice-free region. A molecular clock was applied and calibrated using absolute ages estimated by setting the split of Chlorophyta and Streptophyta at 700 and 1500 Ma. On this basis, the majority (16/26) of the lineages have estimated ages between 17 and 84 Ma and likely diverged from their closest relatives around the time of the opening of Drake Passage, while some lineages with longer branch lengths have estimated ages (330 to 708 Ma), that precede the break-up of Gondwana. The variation in branch length points to several independent but rare colonisation events.

In **diatoms**, the **cultivable diversity** was studied in the globally distributed species complex *Pinnularia borealis*. The time-calibrated molecular phylogeny based on concatenated *rbcL* and LSU (D1-D2 region) sequence data showed a divergence of the Continental Antarctic lineages from a western European lineage around 7.67 Ma (14.77-1.95 Ma, 95% Highest Posterior Probability interval (HPD)). Combined, the findings in **green algae** and **diatoms** are in agreement with patterns found in multicellular organisms and they support the 'glacial refugia hypothesis', which states that long-term survival took place which resulted in a specific Antarctic flora and fauna.

d) Geographic patterns in Antarctic microbial diversity

A comparison of the **uncultured diversity** in 41 samples revealed that conductivity and variables related to salinity significantly explain differences in the community structure of **diatoms, green algae, and cyanobacteria**.

The Denaturing Gradient Gel Electrophoresis using a universal prokaryote or cyanobacterial primers resulted in a relatively large amount of mixed bands, which prevented a multivariate analysis. A variation partitioning analysis of 41 samples in which all microbial groups were studied revealed that geographical variables were more important in the eukaryotic microorganisms compared with the prokaryotes. If these differences between the different taxonomic groups are real, the contrasting patterns observed between prokaryotes and eukaryotes are likely related to life cycle characteristics (e.g. formation of spores, resting stage, sexual versus asexual phase). Hence, we hypothesize that findings from one particular microbial group cannot be generalized to microbes as a whole. A 454 pyrosequencing analyses will enable us to test further this hypothesis. What we already found is that **cultivation and culture-independent approaches are complementary in exploring the diversity of a particular habitat**, because some cultivated taxa were not detected using the 454 pyrosequencing analysis.

D. Scientific support to a sustainable development policy

Our biodiversity analyses have revealed a considerable diversity. Depending on the microbial group (bacteria, cyanobacteria or microalgae), and based mostly on SSU rRNA sequences, a number of species new to science and possibly unique to Antarctica were identified. These findings demonstrate the large value of Antarctica as a relatively unexplored territory that represents an immense resource for biotechnological, biomedical and environmental and applications. The discovery that Antarctic lakes are dominated by endemic microbial organisms has important implications for the conservation of these ecosystems. More in particular, the identification of Antarctic Special Protected Areas (ASPAs) was traditionally based on the diversity/presence of multicellular organisms. Because microorganisms, together with a few mosses, lichens, two flowering plants and a number of small invertebrates, are the only permanent inhabitants, they should be an additional criterion for the delineation of ASPAs. For example, an endemic, as yet unidentified diatom species occurs in a few lakes in the Larsemann Hills. The presence of this taxon is likely related to the fact that some of the lakes acted as glacial refugia during past glacial maxima (Hodgson et al. 2001). The protection of this region should thus be a priority.

It is also apparent that we have only just caught 'the tip of the iceberg' of the biodiversity that inhabits Antarctica. Further studies are needed and will undoubtedly yield even more novel organisms and insights. In view of the anticipated increased effects of global warming (such as rising temperature, increased desiccation, changes in UV radiation and snow/ice cover), it seems urgent to further assess particularly the impact of global change on Antarctic biota. Indeed, in addition to endemics, the total microbial biota is important for the ecosystem functioning and might be impacted by future climate change effects.

Moreover, particular care should be taken to avoid the introduction of alien species from outside Antarctica, and also between different regions of the continent and the sub-Antarctic islands. This is one of the priorities for the Committee for Environmental Protection (CEP).

E. Keywords

Microbial diversity, Antarctica, biogeography, bacteria, cyanobacteria, microalgae, endemism, cosmopolitanism

I. INTRODUCTION

High latitude ecosystems are particularly **sensitive** to climate change (e.g. Quayle et al. 2002) and direct human activity (pollution, physical damage, introduction of alien species; Robinson et al. 2003). **Microbial organisms dominate** most Antarctic ecosystems (including coastal and inland lakes, meltwater streams, cryoconites, …) and play a crucial role in their functioning; they form the base of the food web, are the main actors in the biogeochemical cycles, and mediate bioerosion (Vincent 2000; Friedmann 1993). Moreover, their fossil remains and biogeochemical markers provide a sensitive testimony of past environmental change (Verleyen et al. 2004a,b; Hodgson et al. 2005a,b).

Compared with temperate and tropical microbial diversity and despite their ecological importance, **little is known** about Antarctic microbial diversity and its geographical distribution (Sabbe et al. 2003; Gibson et al., 2006a; Taton et al. 2006a; Hughes et al. 2004). This is underlain by various causes, in particular the lack of systematic sampling and geographical coverage, and the problems associated with species definition, cryptic diversity and cultivability (Sabbe et al., 2003; Taton et al. 2003, Van Trappen et al., 2002). As a result, we largely lack the **'baseline' data** needed to understand the contribution of various processes that are responsible for the geographical patterns in microbial diversity and composition and to observe possible future changes in microbial diversity and taxonomic composition due to ecosystem change and/or human introductions (Cowan & Tow 2004).

A great deal of the earlier diversity studies were carried out with **traditional** methods such as isolation of bacterial strains and microscopic identifications of cyanobacteria and protists on the basis of morphological features and 'force-fitting' of names of temperate taxa on the Antarctic ones. The latter bias gave an impression of cosmopolitanism of these taxonomic groups. Since the mid-eighties, **molecular taxonomic markers** have been increasingly used for genotypic characterizations of strains but also to retrieve directly the microbial diversity in environmental samples. The latter, largely based on ribosomal RNA operon sequences (mostly SSU rRNA, but recently also on the Internally Transcribed Spacer between SSU and LSU rRNA genes), have shown quite a different view of diversity and the existence of not-yet cultivated genotypes. In contrast to phenotypic markers, the genotypic ones are comparable, stable in different environmental conditions and reflect the evolutionary history of the organisms. However, it has been shown that molecular methods can sometimes introduce biases (e.g. Speksnijder et al., 2001); it is therefore important to combine both culture based studies and different molecular community analyses. By doing so, they also have a considerable potential for the study of the geographical distribution of microorganisms (Amann et al. 1995; Loisel et al. 2006).

This is important, because it is still unclear whether geographic isolation is present in microorganisms, and hence whether they exhibit a **biogeography** at all (Martiny et al. 2006). This 'ubiquity hypothesis' was first formulated by Baas-Becking (1934) and states that 'everything is everywhere, but the environment selects'. It is underlain by the assumption that the vast population sizes of micro-organisms drive ubiquitous dispersal and make local extinction virtually impossible (Finlay et al. 2002). However, various recent studies suggest that micro-organisms, do display restricted geographic ranges (Chao et al 2006; Foissner 2006) and that endemism is possible.

Antarctica is a prime place to investigate **microbial biogeography** and to elucidate the roles of historical processes and contemporary environmental conditions shaping microbial diversity and community structure (cf. Martiny et al. 2006), by virtue of its extreme isolation with respect to the rest of the world, resulting from its geographic position and the nature of ocean and atmospheric currents as well as the scattered occurrence of terrestrial oases along the margins of the continent. Furthermore, organisms inhabiting the continent need to survive in extreme environmental conditions, such as low and extremely fluctuating temperatures, dramatically changing light conditions, high seasonal UV-B loads, and low humidity. Thus, as a whole, the continent bears wide **environmental gradients** that impose increasing stresses on biodiversity and community structures (Gibson et al. 2006a). In addition, certain habitats offer some protection from the extreme conditions. For example, liquid water in aquatic environments may act as 'thermal buffer' (Gibson et al. 2006b). Moreover, preliminary data on aerosol diversity in the Antarctic Peninsula showed the potential for wide-range transport of microbial diversity, though much of the aerobiota found was of local origin (Hughes et al. 2004).

The present project builds on our combined expertise developed within the EC MICROMAT project and aims to extend the baseline information of microbial diversity through an integrated and standardized analysis of the microbial diversity of lacustrine and terrestrial habitats of Continental, Maritime and Sub-Antarctic regions. We aimed to use a combination of approaches, based on isolation and characterization of strains, sequencing of molecular taxonomic markers and application of probes in bacteria (with special emphasis on Proteobacteria), and protists (with special emphasis on green algae and diatoms), which have been identified as interesting focal taxa. Using a community fingerprinting approach, we aimed to construct a dataset covering different geographical regions, and spanning broad ecological gradients. Our objective was, for the first time, assess the relative importance of ecological versus historical factors in explaining the geographical distribution of microbial communities in Antarctica and sub-Antarctica.

The **outcomes** of this project can be articulated as follows:

1.   We have expanded the existing database of rRNA gene sequences of bacteria, cyanobacteria and microalgae with new samples from Maritime, Continental Antarctica, as well as from the Sub-Antarctic (only cyanobacteria) based upon isolates, clone libraries and DGGE of environmental DNA. As the DGGE method has revealed to be unsatisfactory for several groups, in the last year of the project, we have started to use the 454 pyrosequencing technique, which was not foreseen originally,

2.   We have enlarged the existing collections of Antarctic bacteria, cyanobacteria, green algae and diatoms, with new documented isolates,

3.   We have studied the microbial diversity in lacustrine and terrestrial habitats in Sub-, Maritime and Continental Antarctica,

4.   We have studied the community turnover among comparable habitats along ecological and geographical gradients to analyze the congruence and disparity in patterns of diversity and turnover observed for different taxa,

6.   We have contributed to identify regions of unique microbial diversity that deserve to be protected, like the Larsemann Hills.

7.   We have disseminated new information *via* peer-reviewed publications, presentations at scientific meetings, but also to a wide public. We have also designed a project website (www.ambio.ulg.ac.be). A final international workshop has been organized at the University of Liège on 31 May 2011, with the title 'Antarctic biodiversity: status and trends'. It has involved both talks and videoconferences to decrease the carbon footprint and allow more colleagues to attend the meeting (http://www.cip.ulg.ac.be/newsite/pages/workshop_antarctic_2011.php).

At the international level, AMBIO has contributed to the SCAR program EBA (Evolution and Biodiversity in the Antarctic) and the MERGE IPY program. MERGE aims to study microbiological and ecological responses to global environmental changes in Polar Regions. The AMBIO project has benefited from these international networks, to obtain new samples and present its activities in scientific fora. The project has also ensured that the Belgian expertise in microbial diversity could be maintained and further developed, and aided Belgium to fulfil its international obligations to carry out Antarctic research in the frame of the Antarctic Treaty System. A better knowledge of the lakes' microbial biodiversity and biogeography is useful to determine priorities for the environmental management.

II. METHODOLOGY AND RESULTS

II.1. MATERIALS AND METHODS

II.1.1. SAMPLES

We obtained a large set of samples from previous research collaborations, exchange with international partners, and during the MERLIN sampling campaign organized in January-February 2007 (in collaboration with the Japanese REGAL project, the British Antarctic Survey project CACHE-PEP and the BELSPO-project HOLANT). Currently we have more than 267 samples (excluding duplicates and different conservation methods) available from 13 different ice-free regions in Antarctica (Fig. 1).
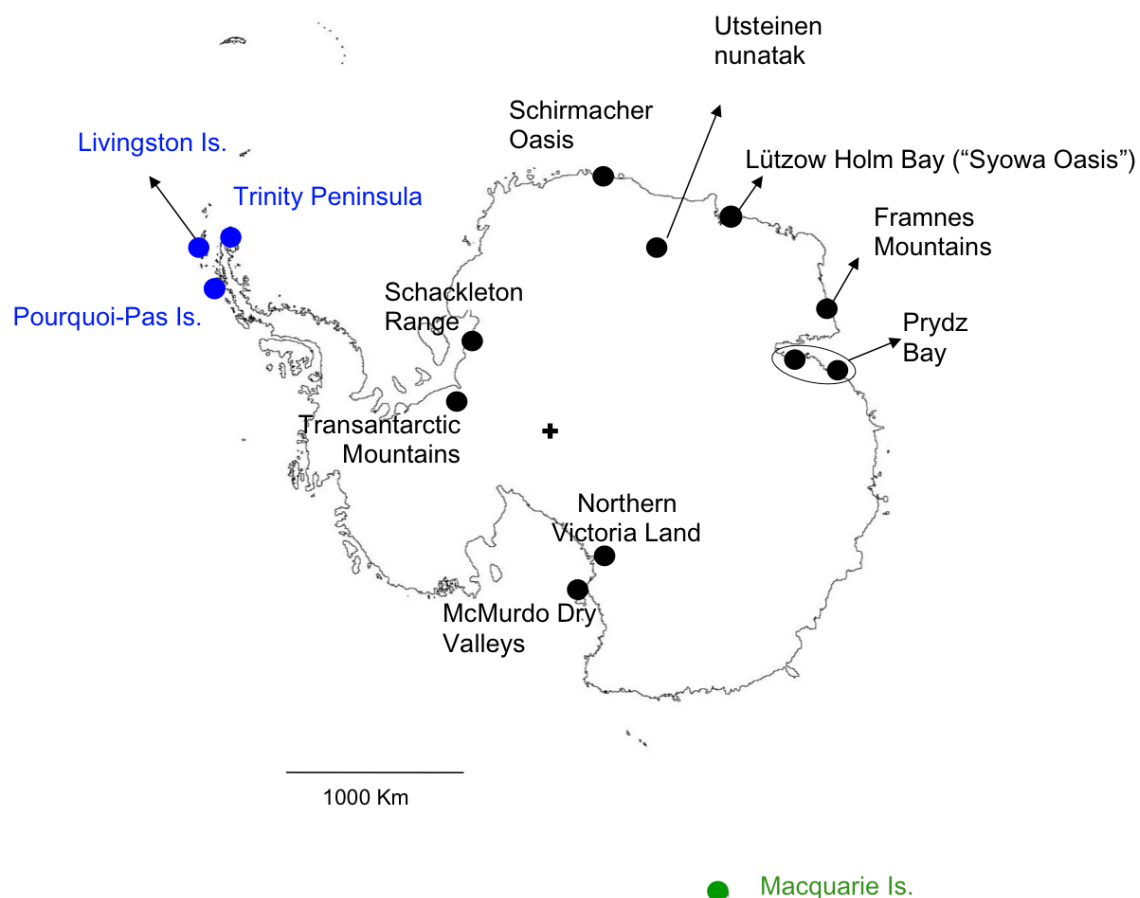
Fig.**1**: Map showing the locations of the samples studied. In black are samples from Continental Antarctica, blue are from Maritime Antarctica and in green from the Sub-Antarctic Islands.

II.1.1.1. Sources of samples
- Samples from the Prydz Bay area (Larsemann Hills, Vestfold Hills, Rauer islands and Bølingen islands), McMurdo Dry Valleys, Trinity Peninsula (Beak

Island and View Point), and Livingston Island were obtained during field sampling campaigns/projects in which one of the project partners was involved.

- Samples from the Transantarctic Mountains (Davis Valley and Shackleton Range), and Pourquoi-Pas Island were obtained through the exchange of samples with BAS (December 2006).

- Samples from the Lützow Holm Bay area ("Syowa Oasis", Skarvsness Langhovde Glacier, East and West Ongul) and Schirmacher Oasis were obtained during MERLIN (see below).

- Samples from the Framnes Mountains (Chapman Ridge and Stillwell Hills) were obtained through collaboration with Dr. John Gibson (University of Tasmania, Australia).

- Samples from the Northern Victoria Land were obtained through collaboration with Francesca Borghini and Roberto Bargagli (University of Siena, Italy)

- Samples from Amsterdam Island were collected by Bart Van De Vijver (Botanical Garden of Belgium, Belgium) in December 2007. Only fresh samples for the isolation of microalgae were obtained as the biotopes did not contain microbial mats.

- Samples from the Utsteinen nunatak (site of the Belgian Princess Elisabeth station) were obtained by Dr Damien Ertz (January 2007) and currently studied by the BELSPO ANTAR-IMPACT project.

- During AMBIO, new samples were obtained from the Sør Rondane Mountains (large region including the Utsteinen nunatak) during the BELSPO BELDIVA sampling campaign

- Samples from Macquarie Islands were obtained in 2009 through collaboration with Dr. Dana Bergström (Australian Antarctic Division)

II.1.1.2. Sampling procedures
Benthic microbial mats were sampled using a UWITEC glew corer) in the deepest part in deep lakes and using a spatula in terrestrial habitats and in the littoral zone of deep lakes and shallow lakes (lake depth <2m).

II.1.1.3. Sample storage
The majority of the samples consist of sediment samples transported and stored at -20°C. A few samples were preserved in ethanol. Some recent samples were also kept cool for the isolation of micro-algae.

II.1.1.4. Supporting environmental data
In addition to the samples, we obtained the available environmental and geographical data from the sampling locations from related research projects or through collaboration with our partners and incorporated these data in our sample database.

During the sampling campaigns organized by AMBIO members, temperature, conductivity, pH, salinity, and oxygen concentration were measured in the field using a YSI 600 water quality meter. Water samples for the analysis of nutrients and photosynthetic pigments were collected in acid-washed Nalgene bottles and frozen until analysis.

II.1.1.5. Overview of samples used for different analyses

Although we aimed to work on the same samples for the analysis of both cultivated and uncultivated diversity of bacteria, cyanobacteria and microalgae, this was not always possible for practical reasons.

The molecular techniques such as DGGE allowed us to analyze the largest set of samples. From the samples that were initially available, we made a selection of 83 samples (see II.1.1.6). Samples for other analyses are generally a subset of the samples used for this uncultivated diversity analysis. The samples from Macquarie Islands arrived quite late and therefore, they were used only for cyanobacterial diversity analyses (Table 1).

For **bacterial cultures**, a subset of 9 frozen samples was selected based on the ordination analysis of both environmental data as well as DGGE data for bacteria. We processed one sample at a time because of the large number of plates involved. The samples used include 2 samples from the Transantarctic Mountains: TM2 from a cyanobacterial mat at the bottom of Forlidas Pond littoral (Pensacola Mountains) and TM4 a littoral sample from Lundström Lake (Shackleton Range); a littoral sample PQ1 from Narrows Lake (Pourquoi-Pas Island); samples LA3 (Langhovde Peninsula), SK5 (Skarvsness Peninsula) and WO10 (West Ongul Island) were taken from 3 lakes in Lützow-Holm Bay, Syowa and sample SO6 originated from the Schirmacher Oasis. Two samples from the Belgian Base site at Utsteinen (Dronning Maud Land): BB50 "gravel and green microbial/algal mat" from the Nunatak Utsteinen and BB115 "black mat on gravel and rock debris" from a frozen lake on the south side of the nunatak Utsteinen were analyzed using the same working method in the frame of the ANTAR-IMPACT project. Their results were included here to allow comparison with those of the other seven samples. **Pyrosequencing** analysis was also performed on these two samples.

For the **cyanobacterial cultured** diversity, we focused on 4 samples from the Transantarctic Mountains and Shackelton Range, 4 from East Antarctica (Lützow Holm bay, Schirmacher Oasis and Larsemann Hills) and 4 from Macquarie Island. For the **uncultivated diversity** analysis, we performed clone libraries on 6 samples, 4 from the Transantarctic Mountains and Shackelton Range, 1 from West Ongul (Lützow Holm Bay) and 1 bi-laminated sample from Triangular Lake (Byers Peninsula, Maritime Antarctica). We analysed 74 of the 83 selected samples (see

below) by DGGE, plus 12 samples from Macquarie Island (Sub-Antarctica). Out of these 86 samples, the bands of 56 (45 continental, 3 maritime and 8 sub-Antarctic) ones were sequenced for phylogenetic and distribution analyses.

For the **cultivation of microalgae**, we relied on the availability of fresh, cooled samples. For **green algae**, we analysed samples from lakes in the Rauer Islands and Vestfold Hills (Prydz Bay, Princess Elizabeth Land), the McMurdo Dry Valleys (Victoria Land), View Point and Beak Island (Prince Gustav Channel, Antarctic Peninsula), Schirmacher Oasis (Dronning Maud Land) and East Ongul, West Ongul, Langhovde and Skarvsness in the Lützow Holm Bay area (Dronning Maud Land). **Diatoms** were isolated in samples from View Point and Beak Island (Prince Gustav Channel, Antarctic Peninsula), Schirmacher Oasis (Dronning Maud Land) and East Ongul, West Ongul, Langhovde and Skarvsness in the Lützow Holm Bay area (Enderby Land).

Table 1: Overview of the samples studied showing the number of samples studied for the different tasks. (): studied during earlier/other project(s), but will be included in AMBIO results for comparison. *: see Taton *et al.* 2003, 2006*a* and 2006*b*.

Region	Available samples	DGGE selection (all)	Biogeogr. selection uncul. div.	add. DGGE cyano	Clone libs. cyano	Bact isol.	Cyan isol.	Microalg. isol.	Remarks
S. Georgia	5+	0	0	0	0	0	0	0	Currently no frozen samples, new samples expected
Livingston Isl.	1+	0	0	0	2	0	0	0	Lake mat sample
Pourquoi-Pas Isl.	9	3	0	3	0	1	0	0	
Trinity Peninsula	65	0	0	0	0	0	0	9	Currently only fresh and ethanol samples available
Transantarctic Mtns.+ Shackelton Range	18	4	4	4	4	2	3	0	
Schirmacher Oasis	11	3	0	2	0	1	0	3	
Utsteinen	52+	(24)	2	0	0	(2)	0	0	Mostly terrestrial samples. Analysis of these samples done in the framework of ANTAR-IMPACT
Lützow Holm	92	36	15	33	1	3	4	24	
Framnes Mtns.	24	0	0	0	0	0	0	0	
Prydz Bay	91	34	22	30	(5)*	0	(26)*	(2)	
Macquarie Isl.	+	0	0	12	0	0	4	0	
McMurdo	2	2	0	2	(1)*		0	(1)	
N. Victoria Land	30	0	0	0	0	0	0	0	

II.1.1. 6. Selection of 83 samples for the uncultivated diversity analysis

The samples were selected using a stratified random sampling approach and Principal Component Analysis, which ensured that we captured the main limnological gradients present in Antarctic water bodies (see Verleyen et al. in press). Based on the available supporting data, we made a selection of a subset of samples to be analysed. This selection was performed using both 'practical' criteria (a-c) and criteria based on the (available) supporting environmental data (d): a) availability of sufficient sample material for a detailed analysis, b) availability of supporting information on the history of these lakes (through analysis of sediment cores within a project), c) conserved in a standard way (both frozen samples and ethanol samples are available), d) using a stratified random sampling for the regions with a wide range of samples. This stratified random sampling was performed using the environmental data available for these regions; i.e. pH, salinity, depth, nutrients and pigment data for the Prydz Bay area (Princess Elizabeth Land) and multi-meter data (pH, salinity, temperature, O_2) for the Lützow Holm bay area ("Syowa Oasis", Dronning Maud Land). Visual data exploration using the centered and standardized environmental data in a Principal Component Analysis allowed us to select both a set of samples covering the same environmental gradients and the samples that had unique environmental conditions (and thus potentially different microbial community composition). Using histograms we verified whether we covered the same range of environmental variation for specific variables. During the project, we have acquired the nutrient and ion concentration data for the lakes in the Lützow Holm Bay region. This allowed us to better compare the chemical limnology in East Antarctic lakes (PCA; **Fig 2**).

Finally, 12 samples from Macquarie Island have been added to investigate cyanobacterial diversity *via* DGGE. These have been selected based on microscopic observations of the 20 samples obtained by the AAD. Due to their late arrival in 2009, they could not be used for the other microbial groups.

We further selected eleven samples originating from the area near the new Belgian Princess Elisabeth base, based on the description in the field by Dr. D. Ertz. Additional samples from this region are studied in the framework of the BELSPO "ANTAR-IMPACT" project. If possible, the results from the AMBIO selection will be compared to those from ANTAR-IMPACT.

EV PCA1=0.846
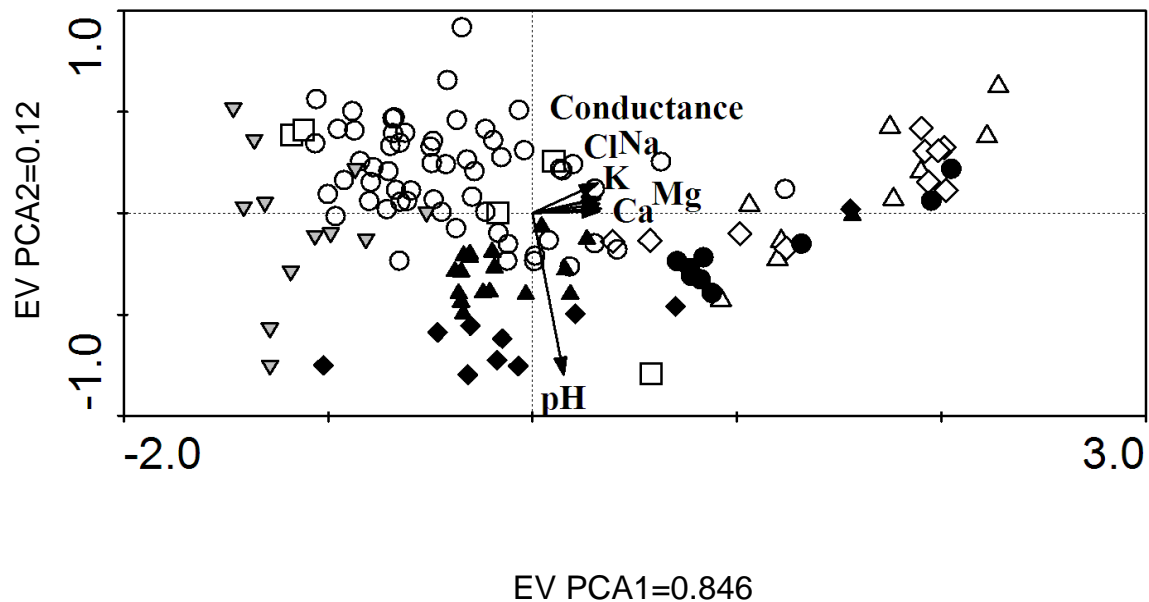
Fig. 2: PCA diagram of the standardized and centered environmental data for East Antarctica. The samples included in our analysis were selected based upon the criteria described above. Prydz Bay (open symbols): the Vestfold Hills (up-triangle), Rauer Islands (diamonds), Bølingen Islands (squares), Larsemann Hills (circles); Lützow Holm Bay (symbols filled with black): Ongul islands (up-triangles), Langhovde (circles), Skarvsnes (diamonds); and Schirmacher Oasis (down-triangle filled with grey).

II.1.2. CULTIVATED DIVERSITY

II.1.2.1. Bacterial strains

Isolation of bacteria

One gram of a sample (or up to 3 g if more was available) was weighed and a dilution series was made in physiological water. These were plated on Marine agar (MA), which is a saline medium, R2A and R2A/10, which are nutrient poor media and Peptone-Yeast-Glucose-Vitamin (PYGV) medium, which is a medium supplemented with glucose and vitamins. The use of both seawater and physiological water for the dilution series was tested. The plates were incubated at three relatively low temperatures (4, 15 and 20°C). Anaerobic incubation conditions were tested for the first three samples but did not result in additional taxa and therefore these conditions were not used further.

The plates were incubated for several weeks during which the number of colony forming units (CFU) was counted. When the number of CFU's had reached a maximum, isolation were started. Three colonies (or less in case of insufficient growth) of each morphological type were isolated and purified. All cultures were stored at -20°C and representatives were stored -80°C.

Genotypic and phenotypic characterization of bacteria
We used repetitive extragenic palindromic (rep)-PCR as a dereplication technique to screen the large number of isolates. Comparison of the whole-genome fingerprints it generated, allowed us to detect identical strains and group the isolates into rep-types (clusters of strains with a similar rep-fingerprint). Some of the isolates did not form part of a cluster and were also classified as separate rep-types. We have used a genetic technique rather than fatty acid analysis as originally planned because fatty acid analysis requires the use of a standard medium at a single temperature to obtain comparable results. This would have been impossible with our diverse conditions of growth.

The 16S rRNA genes of a representative of the each rep-type were partially sequenced (± 400 bp). The sequences were compared and grouped into phylotypes at 99.0% 16S rRNA gene sequence similarity [Acinas et al., 2004; Stach et al., 2003]. The classifier of the Ribosomal Database Project, containing the sequences of all described species, was for genus identification of the phylotypes [Wang et al., 2007]. Identifications with confidence estimates lower than 80% [Wang et al., 2007] were confirmed by phylogenetic analysis with all neighbouring taxa using Bionumerics software (Applied Maths). For samples BB50, BB115, TM2 and TM4, the 16S rRNA gene sequence for the representatives of the different phylotypes was completed (approx. 1500 bp) (Vancanneyt et al., 2004) to improve the identification to species level by further phylogenetic analysis. For the other samples this could not be done, because of time and budget considerations.

The 16S rRNA gene sequence of each phylotype was compared with sequences available in public databases (EMBL and NCBI) including cultured strains as well as environmental sequences (both from metagenomics and high throughput sequencing datasets). Based on the origin of sequences showing ≥ 99.0% sequence similarity, the phylotypes were classified as cosmopolitan, cold, bipolar or Antarctic. Phylotypes that showed no significant similarity with any other sequences were classified as Antarctic.

Flavobacterium isolates were studied in more detail by complete 16S rRNA gene sequence analysis. In addition the phylogeny of the more diverse *gyrB* gene was as determined. New primers were designed on the basis of available whole genome sequences and used to sequence the *gyrB* gene of all new *Flavobacterium* groups.

Phylogenetic analysis was performed with existing *Flavobacterium* species suing Bionumerics software (Applied Maths).

Groups representing new *Flavobacterium* species were further characterized by fatty acid analysis (Mergaert et al., 1993) and several morphological and phenotypical tests (Bernardet et al., 2002) were performed.

II.1.2.2. Cyanobacterial strains
Isolation of cyanobacteria
Isolation media spanning a range of salinities (with or without nitrogen) were used according to Taton *et al* (2006b) for four samples from the Transantarctic Mountains and Shackelton Range (TM1-4). The other isolations were performed on the BG11 medium containing cycloheximide (final concentration of 500 mg/L) to inhibit eukaryotic contaminants and incubated at 18°C with constant light.

Phenotypic and genotypic characterization of the cyanobacterial strains
The phenotypic characterization (morphology) was performed by microscopic analysis using the determination key of Komárek and Anagnostidis (2005). For the genotypic characterization, DNA extraction was performed with DNeasy Plant mini kit (Quiagen). PCR amplification of the 16S rDNA+ITS region was performed to amplify a fragment of ca.1800 bp (Taton *et al.* 2003).

II.1.2.3. Microalgal strains
Isolation and cultivation of microalgae
A total of 36 samples (9 from Trinity Peninsula and 27 from the Lützow Holm bay area) were enriched and screened for the presence of green algae and diatoms.

Diatom cultures were enriched in WC medium, while green algae were incubated on both solid and liquid DM, WC and Guillard-medium. Subsamples of the unialgal cultures were harvested and stored at -20ºC for DNA analysis and diatom samples were taken for oxidation and morphological characterization. Diatom strains were characterized using microscopical analysis of oxidized material.
Green algal isolates grown on solid media were screened using ARDRA and those growing in liquid media were microscopically screened for selecting the strains to be sequenced.

Phenotypic and genotypic characterization of the microalgal strains
The 45 green algal strains that were sequenced after microscopical and ARDRA screening were incorporated in an *ARB* sequence database and aligned together with closely related sequences from other regions studied within previous projects, or available in the Silva database (http://www.arb-silva.de).

Sequences were exported and aligned using Muscle 3.6. Phylogenetic analyses consisted of maximum likelihood (ML) and Bayesian inference (BI) tree searches under a general time-reversible model with a proportion of invariable sites and gamma distribution split into 4 categories (GTR+I+G), as determined by the Akaike Information Criterion in PAUP/Modeltest 3.6 (Posada & Crandall 1998, http://paup.csit.fsu.edu/about.html,). The phylogenetic tree was calibrated using absolute ages by setting the minimum and maximum age of the Chlorophyta–Streptophyta split at 700 and 1500Ma, based on the fossil record and molecular clock estimates (see De Wever et al. 2009 for a full description of the methods used).

For the diatoms, we focused on the globally occurring species complex *Pinnularia borealis*. From four different samples of Schirmacher Oasis (Dronning Maud Land), 19 strains of *P. borealis* were isolated and the *rbcL* and 28S regions were sequenced. Phylogenetic relationships of these strains were analyzed in a likelihood framework together with 32 additional strains of *P. borealis* isolated from European, Asian and South-American areas. The outgroup consists of representatives of the most closely related *Pinnularia* lineages as recovered by a five-marker molecular *Pinnularia* phylogeny (Souffreau et al. unpubl. data). Calibration of the molecular clock was based on the 95% HPD resulting from the preferred time-calibration of the *Pinnularia* phylogeny which was constrained by the fossil record.

II.1.3. Non-cultivated diversity

II.1.3.1. Non-cultivated cyanobacterial diversity

DNA extraction

The DNA was extracted as in Fernandez-Carazo *et al.* (2011). Briefly, nucleic acids were extracted from the cells with the SNT solution (500 mM Tris-HCl pH8, 100 mM NaCl, 25% saccharose), fresh lysozyme and SII solution (TRIS Base 500 mM, EDTA 500 mM, SDS 1%, phenol 6%) and by grinding the sample with glass beads (0.17 to 0.18 mm diameter; Braun Biotech) and a micropestle (Eppendorf, Hamburg, Germany). Then the DNA was separated in successive steps by addition of phenol, phenol-chloroform-isoamyl alcohol (25:24:1), chloroform-isoamyl alcohol (24:1). Finally, a standard Na acetate-ethanol precipitation was performed and the DNA was purified using the kit Wizard® DNA Clean-up System (Promega).

Clone libraries

Clone libraries were constructed as described by Taton et al. (2003) for 4 samples of the Transantarctic Mountains and Shackelton Range, sample WO4 from West Ongul, and a microbial mat sample from Triangular Lake at Livingston Island (Maritime Antarctica). The latter had an upper red layer and a lower greenish layer, that were separated using a sterile scalpel and forceps and a clone library was constructed for each layer.

The obtained sequences were screened for putative chimera using the Bellorephon and PINTAIL softwares. However, the visual analysis of unique sequences with BIOEDIT was often the only way to detect them.

The sequences were aligned with ClustalW using the software Geneious (Drummond et al. 2010). Using the Seqmatch tool of the Ribosomal database Project (RDP II, http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp), the closest strain as well as the closest uncultured organism were downloaded for each sequence and integrated into the alignment. Then, the sequences were grouped into Operational Taxonomic Units (OTUs) using MOTHUR (Schloss, 2009) at different thresholds.

Based on the works of Taton et al (2003), the OTUs were previously defined at a cut-off level of 97.5% of similarity on 405 bp of the 16S rRNA gene. Other studies, such as Acinas et al (2004) and Stackebrandt & Ebers (2006), have shown that the diversity of a microbial community was underestimated using cut-off levels lower than 99%. Therefore, we tried different cut-off levels of similarity to define the OTUs and finally used 98.5%.

The geographical distribution of OTUs was examined by comparison with the sequences present in Genbank.

DGGE
Semi-nested PCRs and **DGGE** were performed (Taton et al. 2003 with slight modifications, Boutte et al. 2006) on 87 samples. Seventy-two samples are from Continental Antarctica, 3 from Maritime Antarctica and 12 from Macquarie Island (Sub-Antarctic). The band patterns were analyzed using Bionumerics 5.0 (Applied Maths). Moreover, as many bands as possible were excised from the gels for further molecular analyses. Around 500 bands were excised from all 87 samples. The re-amplifications and sequencings focused first on neat and well separated bands.

All 87 samples were screened by DGGEa and b. The number of excised bands per sample varied from 1 to 20. Then, the DNA eluted from the bands was reamplified with both primers 359F and 781R, purified using the GeneJet™ PCR purification kit (Fermentas) and sequenced at the Genotranscriptomics Platform, GIGA, University of Liège (http://www.giga.ulg.ac.be/). The contigs construction and sequence analysis was performed with the software Geneious Pro 5.8.2. (Drummond et al. 2010). As the sequence quality was sometimes low, we have deleted those with a quality score less than 50%. If they were obviously similar to other sequences of better quality (except for ambiguous positions), the geographical information was added to the information on the distribution of the OTUs. The sequence analysis was performed as for the clone libraries.

II.1.3.2.Non-cultivated diversity of bacteria and microalgae

DGGE

Our DNA-extraction protocols were optimized by removing extracellular DNA (Corinaldesi *et al.* 2005) prior to bead-beating extraction of DNA from environmental sediment samples. This method was compared to the standard method used at our laboratory (Muyzer and Smalla 1998). In addition, we also compared the performance of these methods to the bead-beating extraction, preceded by the procedure for the removal of extracellular DNA. This method provided the best results (Fig. 3), revealing a higher number of bands and more intense bands. This method was selected for further analysis.

Fig. 3 (right): G: Corinaldesi method (2005) coupled to the Vetriani method (2003) with a few modifications as suggested by D. Gillan (ULB), C: combined method using extracellular DNA removal and bead-beating, M: Muyzer bead-beating method (1998).
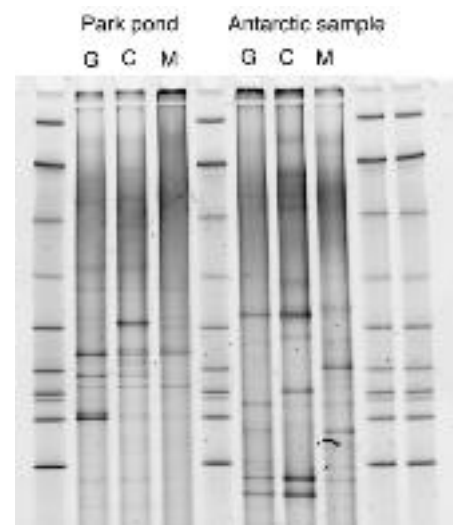
For the analysis of non-cultivated bacteria, we used the protocol developed during previous projects (Van der Gucht *et al.* 2007).

For the microalgae, we initially tested the primers Euk528f and CHLO02r (Zhu *et al.* 2005), as this revealed multiple bands for cultures, we tried a nested PCR approach using Euk1A-CHLO02r and Euk1A-Euk516r-GC (Díez *et al.* 2001), which gave better results.

Pyrosequencing

The initial plan was to use clone library techniques for bacteria in order to study a number of interesting samples and/or taxa in detail. Based on the DGGE analysis, we are unable to make a small (± 4 samples) selection of samples, as the DGGE results for Bacteria are very hard to interpret. First, DGGE banding patterns proved to be highly variable, with very little overlap between any of the samples and second, sequencing of the major bands revealed that for a high number of bandclasses, multiple sequences/taxa were observed at the same position. As we removed these bands, we lost a considerable amount of potential information. We therefore decided to run a parallel tag pyrosequencing analysis, which offers the advantage that we could retrieve a high number of sequences from a wider selection of samples compared with clone libraries.   In comparison to DGGE, which typically only targets the most abundant bacterial representatives, we were able to detect a higher number of taxa per sample and to study the rare biosphere (Sogin et al. 2006).

Secondly, we have completely avoided problems with 'mixed bandclasses', as the sequence data will allow us to delineate clear, unambiguous, operational taxonomic units (OTUs).

As the length of the amplicons during pyrosequencing is ±400bp when using a Titanium microtiter plate, we needed to select a region of the 16S rRNA gene that is variable enough and gives an optimal discrimination between the taxa of interest. Ten variable regions are distinguished in the 16S rRNA gene. We opted for primers which include the V1 up to the V3 region, as these regions give an optimal differentiation between taxa. Particularly the V3 region is frequently used in metagenomics literature and therefore available in the public databases.

We chose to perform amplicon sequencing on a genome Sequencer FLX Instrument (Roche/454) using a Titanium picotiter plate, which can yield around half a million reads. We aimed for around 10.000 reads per sample, so we could analyse 50 samples. We used the nucleotide barcodes proposed by (Parameswaran et al. 2007) in combination with the standard division of the picotiter plate in two regions to distinguish the different samples. We used one duplicate in order to check the physical separation of the plate in two regions. We selected 49 samples on the basis of the availability of environmental data (see above),

For the PCR amplicon library, we followed the 'Amplicon Library Preparation Method manual' for GS FLX Titanium Series by Roche/454 (October 2009). Fusion primers were constructed of the 454-sequencing adaptor, the barcode and a specific primer for the V1 up to the V3 region. The specific prokaryotic primers used are the pA (according to Edwards et al. 1989) and BKL1 (5'- GTATTACCGCGGCTGCTGGCA-3'). The PCR products were purified with a High pure PCR Product Purification kit (Roche). The quality of the amplicon libraries was controlled with a BioAnalyser DNA 1000 LabChip and the quantity was determined by fluorometry using the Quant-iTTM PicoGreen dsDNA Assay Kit (Invitrogen). The samples were subsequently pooled into two sets (picotiter plate is divided in two regions with a gasket) by combining equimolar concentrations of 25 samples with different barcodes. Pyrosequencing was performed by NXTGNT (Ghent University). The sequences were automatically corrected for pyrosequencing errors using PyroNoise (Quince et al. 2009). The sequences were subsequently mapped on the database of the Ribosomal Database Project (RDP) with BWA-SW (Li and Durbin 2010).

PCR test for new Flavobacterium species
Three potential new *Flavobacterium* species were selected for the development of a PCR test that would allow the detection of their presence in environmental DNA extracts. Species-specific primers were designed on the basis of full 16S rRNA gene sequences and comparison with in public databases using the programs Kodon and Bionumerics (Applied Maths). The PCR reaction was optimized and tested for specificity using DNA extracts from target as well as non-target strains.

It was finally tested on community DNA samples that were prepared as described above for DGGE. The optimized test was applied on the same set of samples as used for bacterial cultivation supplemented with an additional sample from the region where the potential new species were isolated and several samples from the Sør Rondane Mountains.

Diatom analysis
For the diatom analysis and countings, the organic matter was removed with H_2O_2 (30%) following Renberg (1990). Naphrax was used as a high refractive index mounting medium. Observations were made using a Zeiss axiophot light microscope at a magnification of 10x100x. SEM pictures were taken using a JEOL JSM5600LV (JEOL, Tokyo, Japan). At least 400 valves were counted in each sample. Taxonomic identification was based on Sabbe et al. (2003) and Sterken et al. (in prep.).

II.1.4. MULTIVARIATE ANALYSES

Ordinations were used to identify those variables that significantly explain the variation in community structure between the samples. All ordinations, except those of the 454 pyrosequencing analysis (see below), were performed with CANOCO 4.5 for Windows [ter Braak and Smilauer, 2002]. Three different matrices were created. First, a biotic matrix contained the distribution of rep-types (cultured bacteria), OTUs (cyanobacteria), band classes (cyanobacteria, green algae, bacteria), sequences (bacteria) and diatoms relative abundances. Second, an environmental matrix contained the limnological variables. Third, a geographic matrix contained the eigenvectors corresponding to the positive eigenvalues after principal coordinate analysis of a truncated matrix of the geographic distances among the sampling sites (Borcard and Legendre 2002), which approximates the connectivity between sites. If the length of gradient, calculated using a detrended correspondence analysis, exceeded 1.5, linear ordination techniques were used. Forward selection using unrestricted Monte Carlo permutations tests (499 permutations, $p < 0.05$) was used to select the minimal number of variables explaining the variation in the biotic data.

Variation partition analysis was subsequently used to assess the unique contribution of the environmental versus geographical variables in structuring the microbial communities (see Verleyen et al. 2010). The forward selection procedure using Monte Carlo Permutation tests (999 permutations) in CANOCO 4.5 was used to select only those variables (geographical and environmental variables selected separately) that significantly explain variation in the biotic data. The variation partitioning analyses results in 4 fractions if at least one variable is significant in each of the different factor classes, namely (1) the unique effect of geographical variables, (2) the unique effect of environmental variables, (3) the combined variation due to joint effects of (1) and (2), and (4) the unexplained variation in biotic data.

Monte Carlo permutation tests (999 permutations) were used to assess the significance of the ordination axes in each model.

II. 2. RESULTS

II.2.1. BACTERIAL DIVERSITY
II.2.1.1. Isolation of bacterial strains

Isolation of strains started after about 2 weeks (15°C and 20°C) or 3 weeks (4°C) incubation, for aerobic growth conditions. Anaerobic growth was slower and the general yield was extremely low or zero. This might be related to the procedures used during sampling (i.e. under aerobic conditions) which likely harmed sensitive anaerobic bacteria. We therefore abandoned anaerobic incubation after the first three samples. The use of seawater instead of physiological water in dilution series for MA medium did not deliver much extra isolates and we therefore used it only to plate out saline samples (LA3 and WO10) on MA medium. The yield on the different media and at different temperatures is documented in **Table 2**.

Table 2. Results of the plate counts (10^5 cfu/g) for the different media and temperatures. The highest value per sample and per temperature is given in **bold**.

Medium	BB50	BB115	TM2	TM4	PQ1	LA3	SK5	WO10	SO6
4°C									
MA PFO	2.28	3.63	**22.12**	<0.01	<0.01	0.05	<0.01	**50.00**	0.13
MA SW	1.64					**5.47**	0	43.14	
R2A	2.68	6.20	2.61	**0.02**	**<0.01**	0	0.19	11.84	6.93
R2A/10	**3.16**	4.57	0.12	<0.01	<0.01	0	0.16	20.4	**5.76**
PYGV	1.18	**6.43**	0.59	0.01	<0.01	0	**0.21**	5.26	5.62
15°C									
MA PFO	12.10	10.10	**78.2**	0.05	<0.01	11.0	<0.01	**177.76**	0.40
MA SW						**17.8**	<0.01	55.71	
R2A	508.50	52.00	34.4	0.26	0.01	<0.01	0.86	57.63	**79.21**
R2A/10	**672.00**	**147.50**	12.4	**0.27**	**0.01**	<0.01	0.51	63.42	26.45
PYGV	118.00	126.33	26.5	0.24	0.01	<0.01	**1.38**	34.74	25.71
20°C									
MA PFO	5.75	7.00	**85.29**	0.04	<0.01	14.10	<0.01	**244.74**	0.61
MA SW	5.71					16.13	<0.01	48.00	
R2A	**432.00**	86.67	16.38	0.14	0.02	<0.01	1.89	114.21	19.91
R2A/10	231.00	141.67	18.85	0.14	**0.03**	**17.67**	0.90	30.00	24.34
PYGV	84.00	**157.17**	21.32	**0.15**	0.02	<0.01	**2.10**	37.89	**26.83**

We have, since mid-February 2007, processed 9 samples and purified about 3800 isolates. All have been stored on glycerol at -20°C or in Cryobank vials at -80°C. The number of isolates per sample is given below, in Fig. 4.

Best growth was generally obtained on the oligotrophic media, although samples TM2, LA3 and WO10 showed the highest number of CFU/g on the marine media. This is in line with expectations as these samples were known to be more saline. Incubation at 4°C yielded less colony-forming units than incubation at 15°C or 20°C.
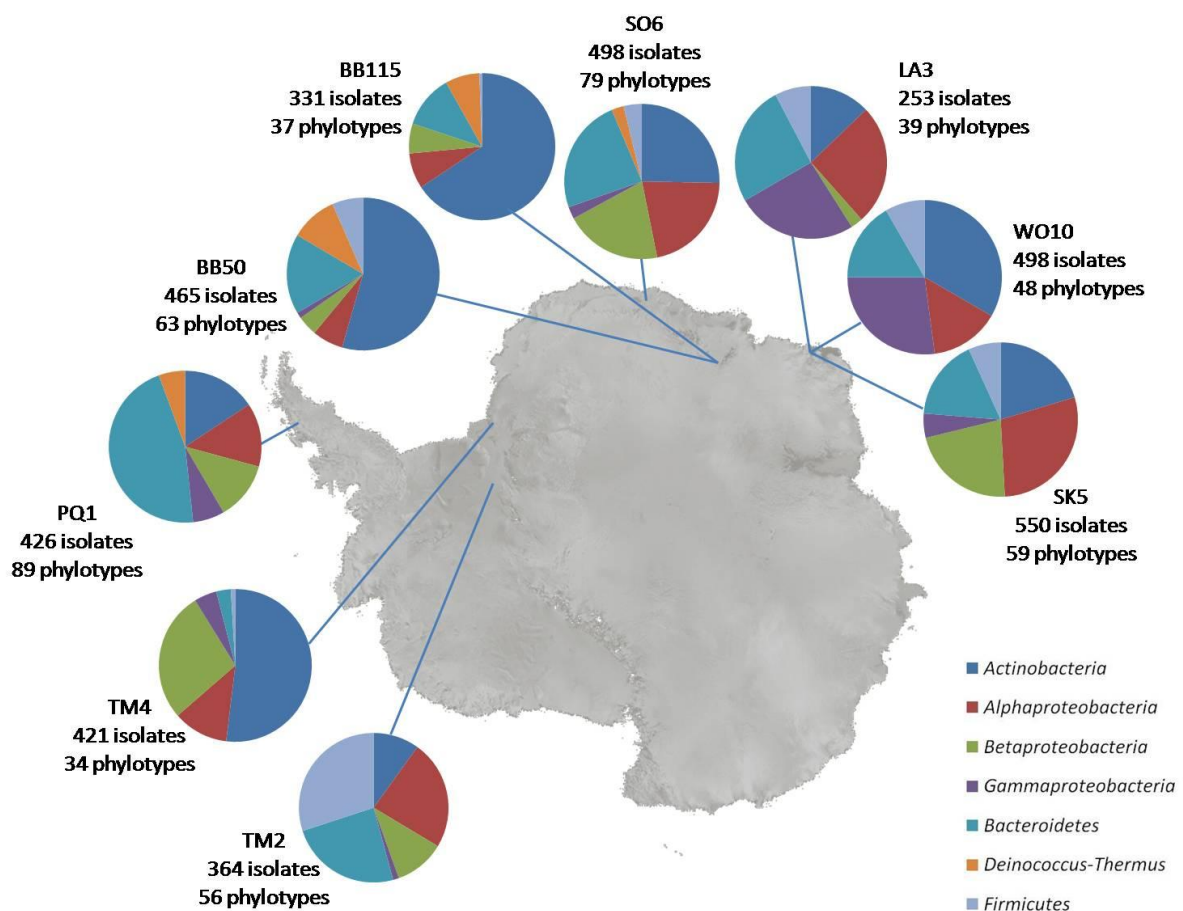
Fig. 4 Distribution of phylotypes over the different bacterial phyla or classes for the different samples. The number of isolates and phylotypes for each sample is listed.

II.2.1.2. Characterization of bacterial strains

After incubation, between 253 and 550 isolates were purified from each of the nine samples (Fig. 4). DNA from all isolates was subjected to a rep-PCR followed by electrophoresis to obtain a genomic fingerprint for each strain. Cluster analysis of fingerprint patterns using Bionumerics software revealed 1379 rep-types (cut-off level 80%).

Comparison of samples revealed that there were no universally present rep-clusters. Few rep-clusters were found in two (45/1379) or three (2/1379) different samples, illustrating the large diversity observed.

Partial 16S rDNA sequencing was performed on 1 to 5 representatives per rep-type. In total, about 1800 strains were sequenced and compared to the RDP database for generic identification. The results show a large diversity (Fig. 4), distributed over the major phylogenetic groups *Proteobacteria*, *Bacteroidetes*, *Actinobacteria* and *Firmicutes* that have been reported frequently from Antarctica (Aislabie et al. 2006b; Aislabie et al. 2008; Babalola et al. 2009; Bowman et al. 2000b; Bowman and McCuaig 2003; Cary et al. 2010; Selbmann et al. 2010) Isolates belonging to the phylum *Deinococcus-Thermus* were only recovered from samples BB50, BB115, PQ1 and SO6. The genus *Deinococcus* has been found previously in Antarctic soils and especially in the McMurdo Dry Valleys (Aislabie et al. 2006a; Aislabie et al. 2008; Cary et al. 2010; Niederberger et al. 2008) although several other studies focusing on Antarctic soils (Gesheva 2009; Shivaji et al. 2004) as well as on marine environments (Bowman et al. 2003; Bowman et al. 2000a) and microbial mats in Antarctic lakes (Brambilla et al. 2001; Van Trappen et al. 2002) did not report their presence. At genus level, variation between the samples is larger. None of the genera was recovered from all nine samples. The genera *Arthrobacter*, *Brevundimonas* and *Hymenobacter* were found in eight samples whereas the genera *Cryobacterium*, *Rhodococcus*, *Sphingomonas*, *Flavobacterium* and *Bacillus* were found in seven of the nine samples. Furthermore, some 37% (31/83) of the genera were recovered from only one sample (e.g. *Frigoribacterium*, *Saxeibacter*, *Aurantimonas*, *Caulobacter*, *Lysobacter*, *Maribacter*, *Brevibacillus*). Several halotolerant genera were present in the more saline samples TM2, LA3 and WO10 (e.g. *Gillisia* (Aislabie, et al. 2008) *Sporosarcina* (Yoon et al. 2001) and *Psychrobacter* (Zdanowski et al. 2004).

At finer taxonomic level, about 75% of the phylotypes – which can be regarded as proxies for bacterial species – were recovered from only one location, the other 25% were found in multiple samples. However, none of the phylotypes was found in all nine locations. Sample SK5 shared the highest percentage of phylotypes with other samples, especially with samples PQ1, LA3 and SO6. Also samples TM2 and WO10, TM4 and SO6 and PQ1 and SO6 shared an important percentage of phylotypes.

For samples BB50, BB115, TM2 and TM4, representatives of the phylotypes were completely sequenced. Forty-three phylotypes that showed ≥ 99.0% 16S sequence similarity with type strains of named species, were classified as belonging to this species. An important number of the phylotypes (120) could only be identified at genus level and were classified as potential new species within these genera.

These potential new species were distributed over all phyla recovered. Eight phylotypes were found equally related to multiple neighboring genera and were therefore tentatively classified as potential new genera in the *Actinobacteria*, *Alphaproteobacteria* and *Betaproteobacteria*. For the remaining five samples studied, only partial 16S rRNA gene sequences were obtained and phylotypes were therefore only identified at genus level. Nevertheless, an important number of phylotypes (26) was found to represent potential novel genera, mostly in the phyla *Alphaproteobacteria* and *Betaproteobacteria* and in samples LA3 and SO6. Further polyphasic studies are necessary to confirm the status of all these potential new taxa and to elucidate their classification.

For the new isolates of the genus *Flavobacterium,* the process of further characterization with a view to describing and naming new groups has been started. *Flavobacterium* isolates were present in almost all samples studied and several were only distantly related to existing species. They constitute 33 *Flavobacterium* phylotypes for which we performed a complete 16S rRNA gene sequence analysis including all existing species. In addition, to overcome the sometimes limited resolving power of 16S rDNA at species level (Probst et al. 1998), the more variable *gyrB* gene was also studied. This gene has been successfully used to study the phylogeny of other groups (Yamamoto et al. 1996; Kasai et al. 2000; Suzuki et al. 1999). We determined the full sequences for 33 new isolates of *Flavobacterium* representatives and for the 22 most closely related species. Phylogenetic analysis of both the complete 16S rRNA gene sequences and the *gyrB* gene sequences (Fig. 5) revealed that our *Flavobacterium* isolates formed 15 groups. One of these grouped with the species *F. micromati* that was previously described from Antarctica (Van Trappen et al. 2004b). The other groups formed new lineages in the genus.
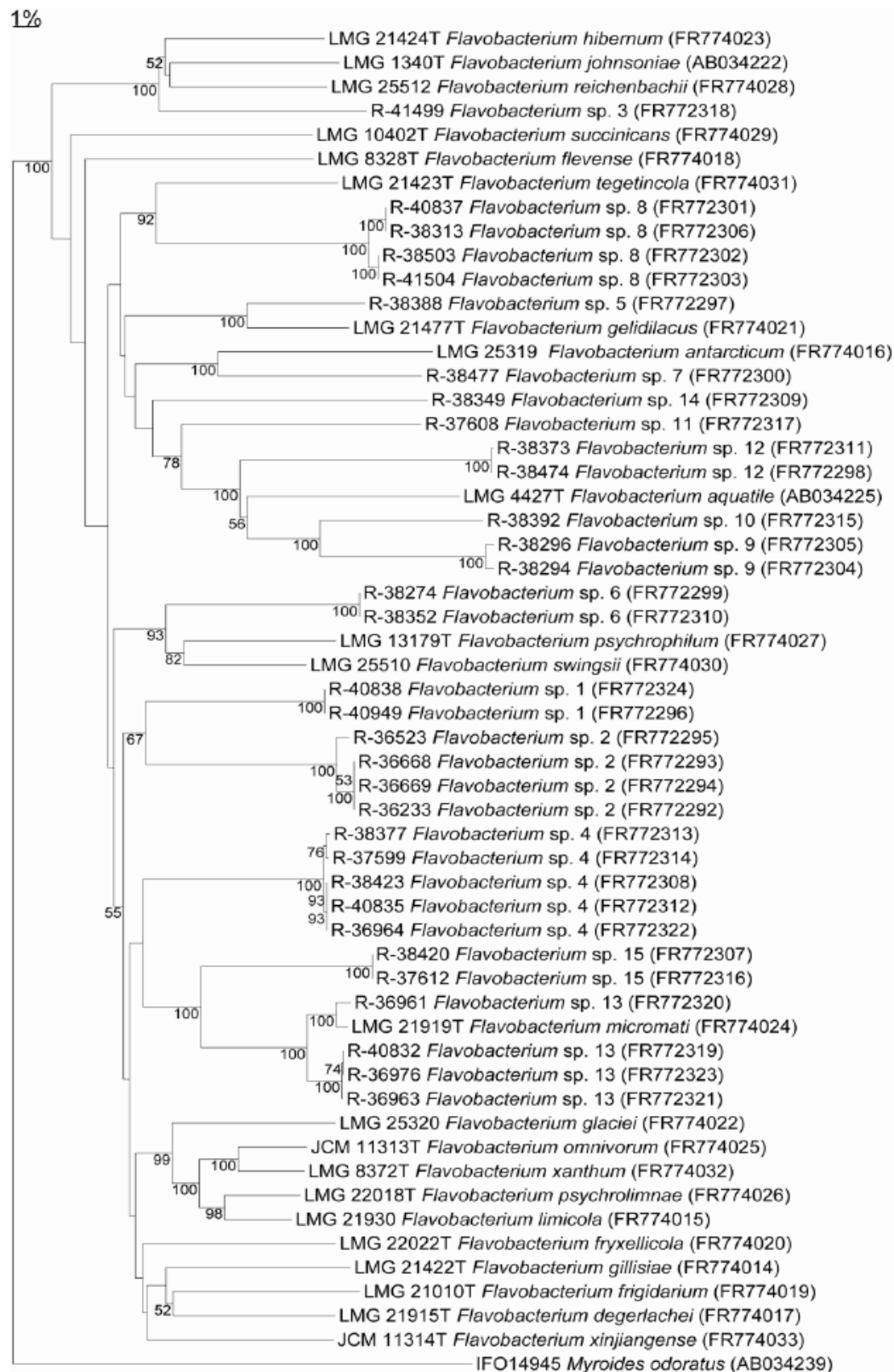
Fig. 5 Phylogenetic tree based on Neighbor joining analysis of the *gyrB* gene sequences (1006 bp long) of *Flavobacterium* isolates and closely related species. The numbers at branch nodes are bootstrap values shown as percentages of 500 bootstrap replicates (only values > 50% are shown).

Thirteen of these *Flavobacterium* groups, including twelve potential new species and the group close to *Flavobacterium micromati*, were characterized biochemically and chemotaxonomically to confirm their status in comparison to existing *Flavobacterium* species. Several closely related type strains were included in these tests for comparative purposes. The predominant fatty acids, together with some other characteristics, such as the rod shape of the cells, the gram-negativity, yellow or orange pigmentation of the colonies, and the observations that cellulose is not decomposed and indole is not produced, confirmed the affiliation of the selected Antarctic isolates with the genus *Flavobacterium* [Bernardet et al., 1996]. Although all the tests needed (Bernardet et al., 2002) have not yet been completed, it looks likely that twelve potential new *Flavobacterium* species can be differentiated from the other species and from the type strains based on a combination of several phenotypic characteristics confirming that they belong to new species. Phenotypic characteristics confirm that one of the *Flavobacterium* groups indeed belongs to *F. micromati* as already indicated in the 16S rRNA gene and *gyrB* gene phylogeny (Peeters et al.2011).

II.2.1.3. Non-cultivated bacterial diversity
DGGE analysis
DGGE analysis revealed 63 bandclasses (BCs). In total, 146 strong bands were sequenced, and they belonged to 45 OTU's. For a high number of bandclasses, multiple sequences/taxa were observed at the same position which biases the dataset. Using this sequence data, 6 BCs were identified as Cyanobacteria, 23 as other bacterial groups, 16 BCs could not be identified as belonging to a specific group due to the occurrence of different genotypes at the same band position. The most prominent groups identified during band sequencing are Cyanobacteria, Bacteroidetes, Alphaproteobacteria and Gammaproteobacteria.

While indirect ordination analysis (PCA and CA) of the DGGE data of both all available data and the data on identified bands only did not reveal the occurrence of a divergent bacterial community composition in any of the regions studied, direct ordination techniques points to a significant contribution of spatial variables in explaining the variation in the species data. For bacteria (Fig. 6), about 7% of the data could be explained by the environmental variables conductivity, salinity and Si, and 3% could be explained by a spatial component.
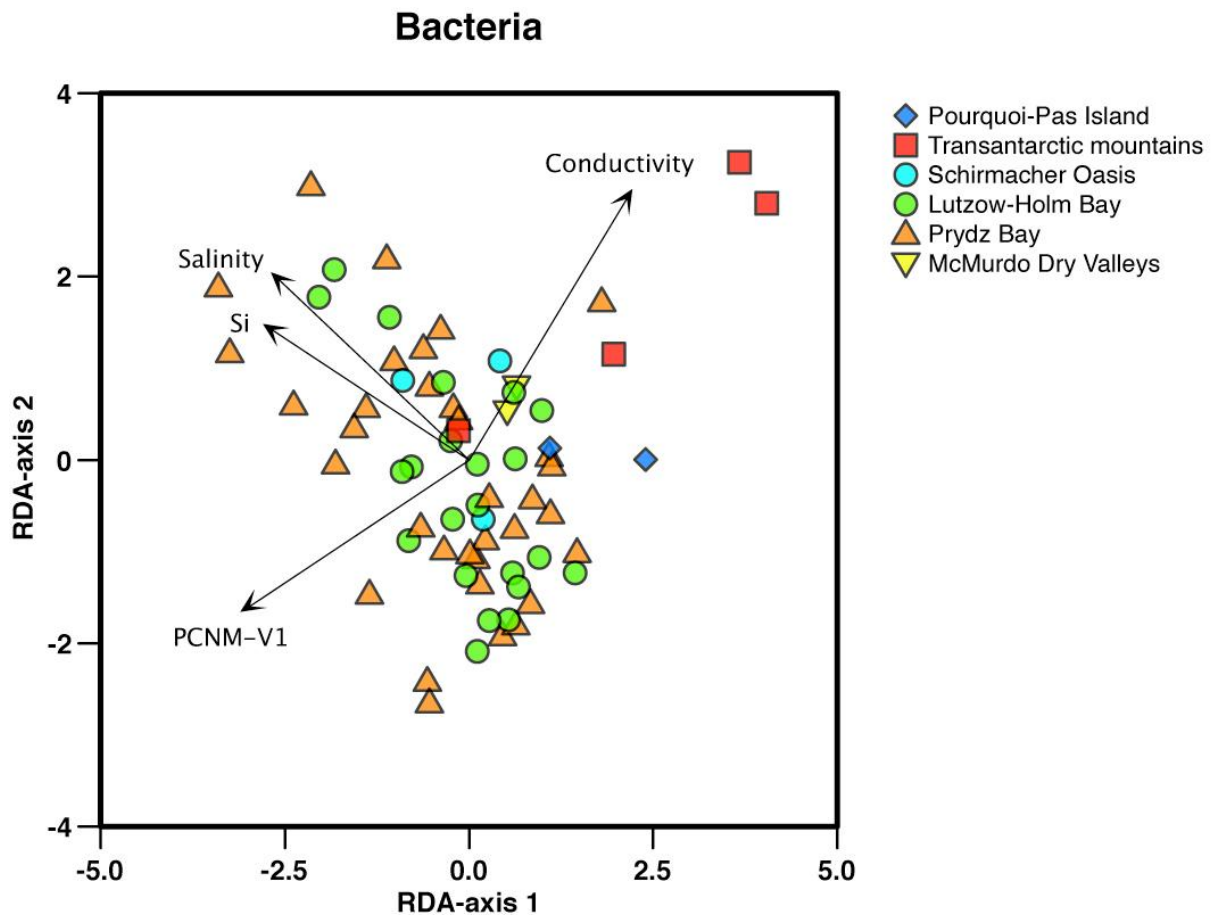
Fig. 6. Results of direct ordination analyses showing species samples and environmental (including spatial variables) scores for the bacterial DGGE data. Different regions are indicated as shown in the legend.

Pyrosequencing results
In total, 619.426 sequences were recovered in 49 samples. The groups of bacteria that were isolated, are also found in the pyrosequencing data. The dataset will be further analysed using multivariate statistics in the final five months of the project in order to (i) identify those variables that significantly explain the variation in community composition between the samples and (ii) calculate the amount of variation that can be explained by geographical versus local environmental samples.

PCR test for new Flavobacterium species
Three of the potential new *Flavobacterium* species (sp. 6, 14 and 9) were selected for development of a specific PCR test to investigate their presence in environmental samples without the need to cultivate.

Species-specific primers were designed and after optimisation of a nested PCR protocol, applied on extracts of community DNA from a first set of 32 Antarctic samples including 22 samples from the Sør Rondane Mountains (BELDIVA-campaign), nine samples previously used for bacterial isolations including sample PQ1 (Pourquoi-Pas Island) from which the new *Flavobacterium* species were originally isolated and sample CL2 from Horseshoe Island, located close to Pourquoi-Pas Island.

For one of our groups (sp. 9) it proved not possible to obtain specific PCR amplification and this group was therefore excluded. The following samples gave a positive result for the presence of both sp. 6 and sp. 14: PQ1, CL2, Beldiva66, Beldiva69, Beldiva75 and Beldiva87. Sample SO6 was positive for sp. 14 and sample Beldiva157 for sp. 6. The following samples were negative for both species: BB50, TM2, TM4, LA3, SK5, WO10, Beldiva27, Beldiva35, Beldiva51, Beldiva55, Beldiva61, Beldiva80, Beldiva83, Beldiva92, Beldiva98, Beldiva101, Beldiva112, Beldiva129, Beldiva131, Beldiva133 and Beldiva140. Samples BB115 and Beldiva145 could not be analysed because the DNA yield after extraction was too low. Absence of a PCR product indicates that the *Flavobacterium* species targeted was absent in the sample or present in a quantity below the detection limit of this PCR test. The positive result for sample PQ1 is in accordance with the cultivation results as both *Flavobacterium* species were isolated from this sample. Sample CL2 originates from an island close to Pourquoi-Pas Island, where sample PQ1 was taken. Its positive result may indicate that these *Flavobacterium* species are more generally distributed in this area. Interestingly, they were also PCR detected in some terrestrial samples from the Sør Rondane Mountains with more exposed, harsh conditions.

II.2.2. CYANOBACTERIAL DIVERSITY
II.2.2.1. Isolation of cyanobacterial strains

Five strains from 4 samples (TM1, TM2, TM3 and TM4) were isolated and their 16S rDNA gene and ITS sequenced. Four of them have been isolated and purified as unicyanobacterial cultures and were deposited into the BCCM/ULC collection of polar cyanobacteria at Ulg. Ten other samples (5 from Continental Antarctica and 5 from Macquarie Island) have been inoculated on BG11 and $BG11_0$ media. The isolation from 8 samples is undergoing: Manning Lake (AMB15, LH), WO10 (AMB74), SC6 (AMB60), LA7 (AMB55), MI06, MI13, MI18 and MI19 (Macquarie Island). Re-inoculations of single filaments or cells were performed twice on solid media. We will inoculate the strains in liquid media to obtain enough biomass for the cyanobacterial strains and characterize them using the methods described above.

II.2.2.2. Phenotypic and genotypic characterization of the cyanobacterial strains

The microscopical observations showed that the five strains belonged to the Oscillatoriales: *Leptolyngbya antarctica* (ULC73 from TM1), *Leptolyngbya cf foveolarum* (ULC129 from TM2 and TM3FOS129 from TM3), *Phormidium autumnale* (ULC130 from TM2) and *Phormidium pristleyi* (ULC131 from TM4). The 16S rRNA gene sequences of all strains were sequenced and aligned with DGGE and clone sequences from the same samples. This showed that there were 2 OTUs found only with cultivation, but not by DGGE nor clone library. They were probably minor components in the sample. This illustrates the need to use complementary methods to obtain a better picture of the diversity.

II.2.2.3. Non-cultivated cyanobacterial diversity
DGGE
Here, an analysis of 162 good-quality (Geneious) sequences (contigs of c.a. 431 bp length) of **56** samples is described. After downloading the closest sequences (1 isolate and 1 uncultured) from RDP II, all the sequences (270 in total) were aligned. A distance tree (Neighbor-Joining, Jukes & Cantor correction for multiple mutations, 500 bootstraps, Fig 7) was constructed using MEGA 5.05 (Tamura et al, 2011). *E. coli* K12 was used as an outgroup. The grouping into OTUs was performed as done for the clone sequences, and 33 OTUs were obtained.

The OTU richness varied between 1 and 4 OTUs per sample. The richest sample was AMB29 (Watts Lake, Vestfold Hills) with 4 OTUs. However, as written before, we could not recover all bands from the gels. Moreover, we often observed that one sequence corresponded to several bands in the same lane. This has already been observed by other authors and might be explained by the existence of several melting domains and a heterogeneous behaviour of the populations of PCR products (Boutte et al. 2006).

The OTUs cover well the major cyanobacterial orders; Chroococcales, Oscillatoriales and Nostocales (5 OTUs , all cosmopolitan).
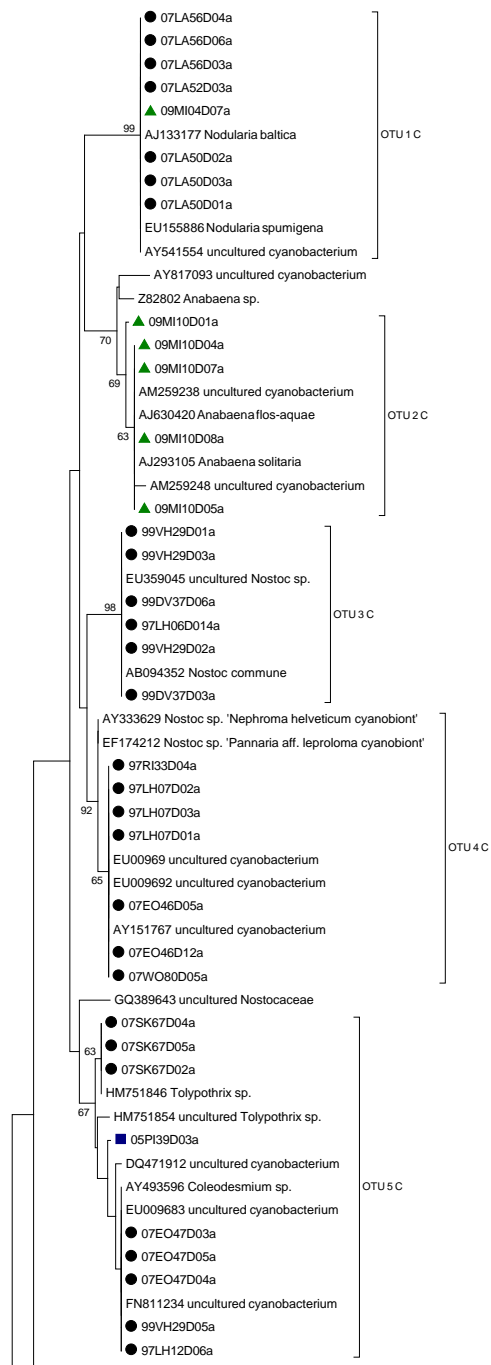
Fig. 7. Distance tree (Neighbor Joining) on 174 partial sequences (375 bp, *E. coli* positions 433 to 808) of the 16S rRNA gene. Bootstrap values are indicated when equal or greater than 70%. Sequences from this analysis are in **bold black** for Continental samples, **bold blue** for Maritime samples and **bold green** for sub-Antarctic samples. 80 closest relatives were downloaded from RDPII. OTUs were calculated at 0.015% of sequence similarity. Their distribution codes are as follows: C=cosmopolitan, CAP=cosmopolitan-antarctic-polar, CP=cosmopolitan-polar, E=endemic and EN=endemic-new.
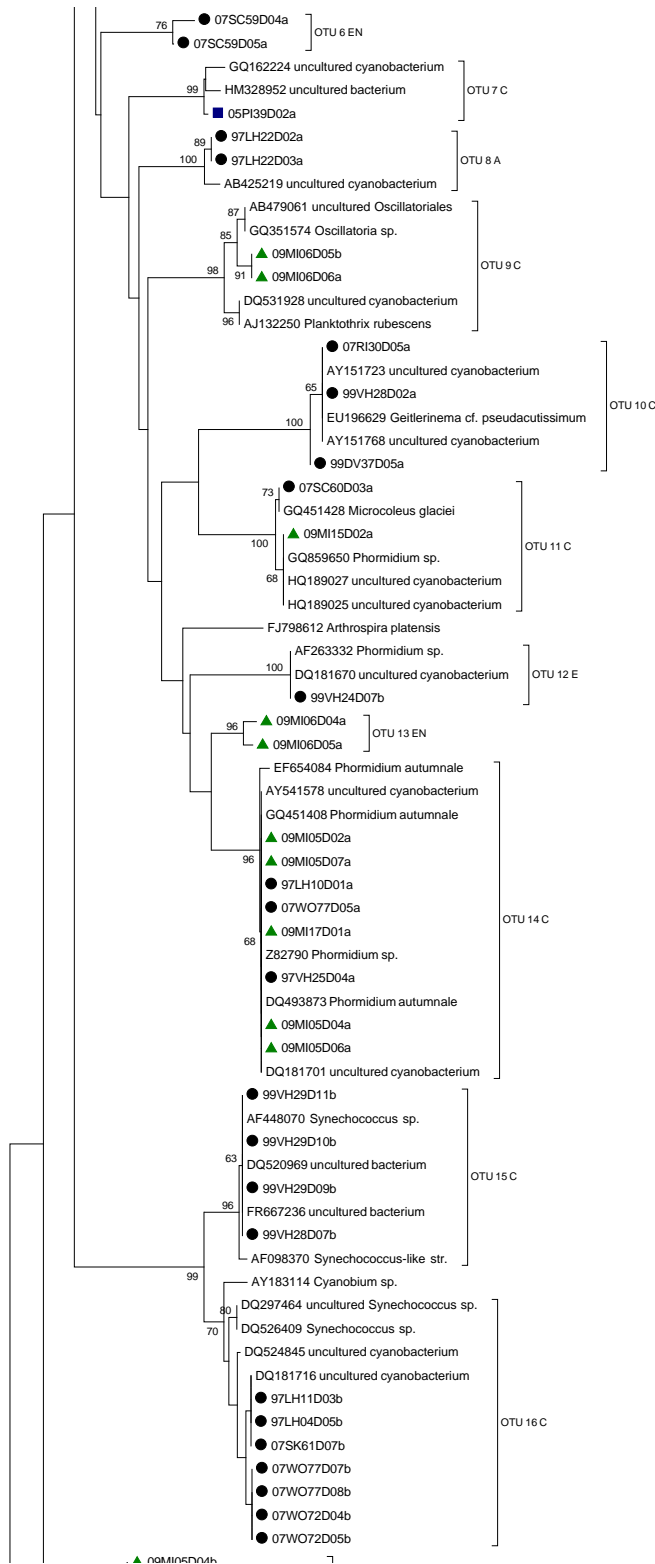
Fig.7 (continued)
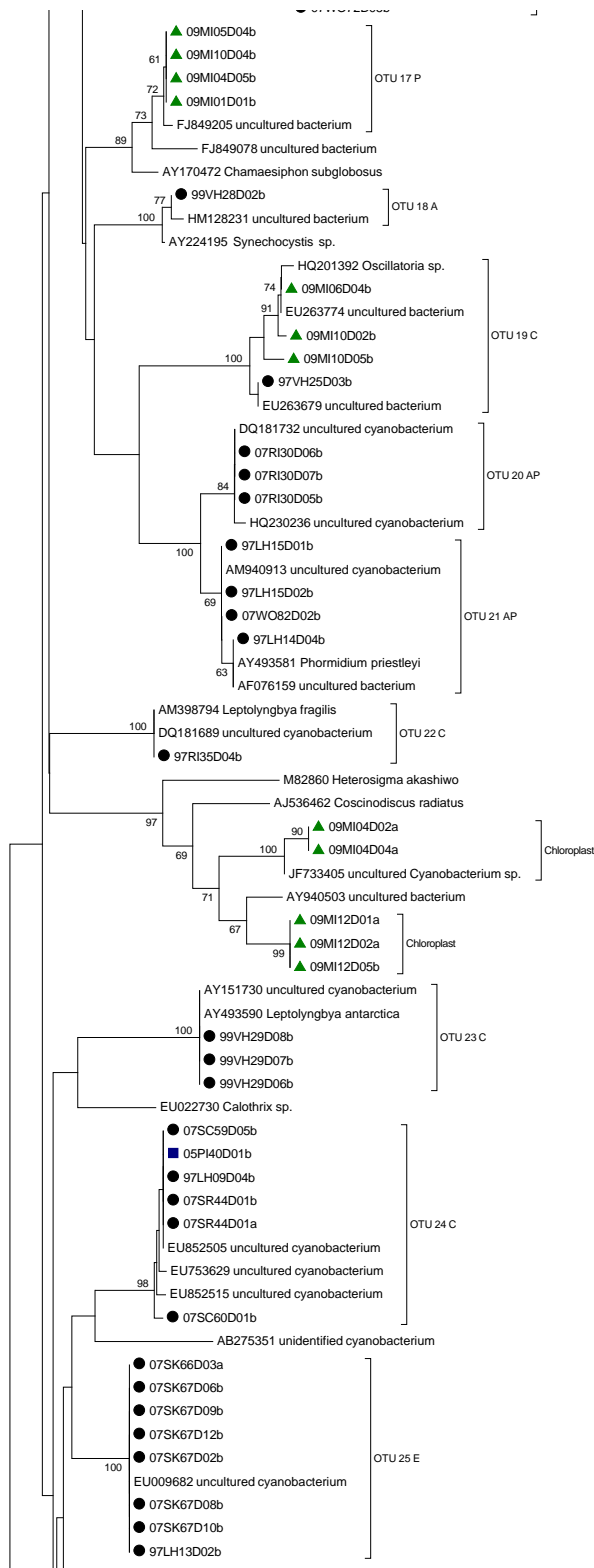
Fig. 7 (continued)
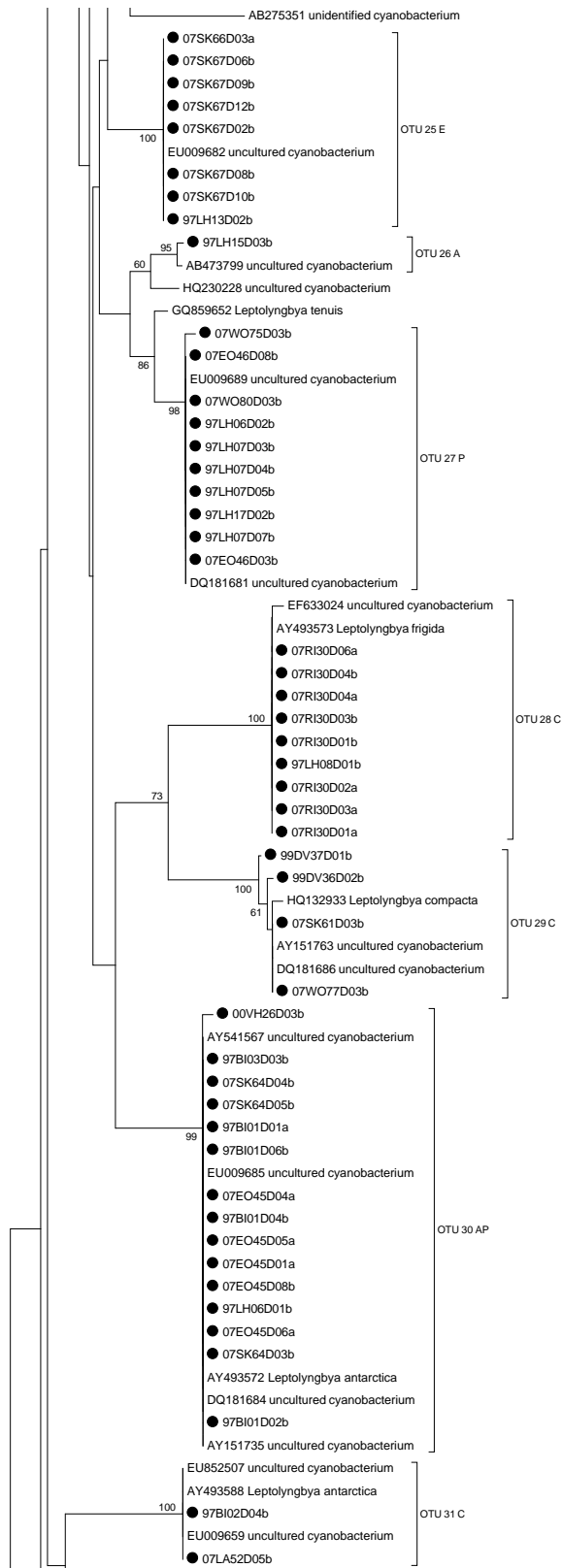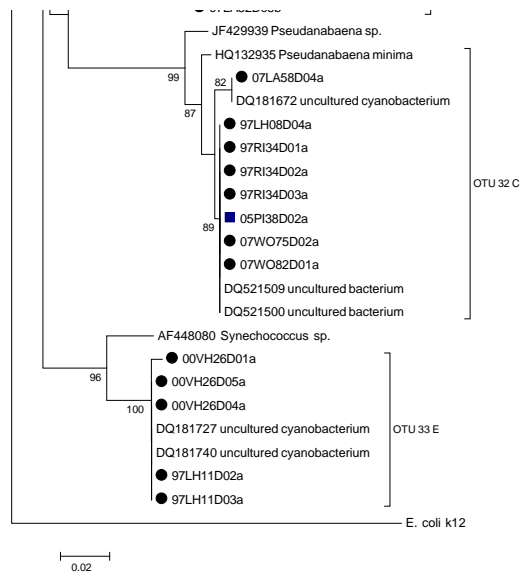
Fig. 7 (continued)

Fig. 7. (continued)

DGGE band classes analysis

The pictures of the gels were analyzed with the software Bionumerics and the bands were grouped into band classes (BC). As they come from two different PCRs that target different organisms (filamentous and unicellular cyanobacteria, respectively), the data of DGGEa and b were separated into two tables. A correspondence analysis was run on each of the datasets but showed no clear differences in the cyanobacterial communities of the 3 provinces. Nevertheless, in the BCb dataset (Fig. 8), 7 samples are separated from the rest of the dataset. The 4 WO (AMB73, 79, 82 and 83, surrounded by a red circle on the figure) all come from the littoral part of the lakes and seem to have similar cyanobacterial communities. AMB31 (Rauer Islands) and AMB48 (Langhovde) come from lakes with very high salinities, 24.9 and 49 respectively, which might explain their particular position on the plot whereas the salinity in the lake of AMB32 is 4.6. Also note that the samples from Macquarie Island are closely grouped whereas the continental ones are more spread.
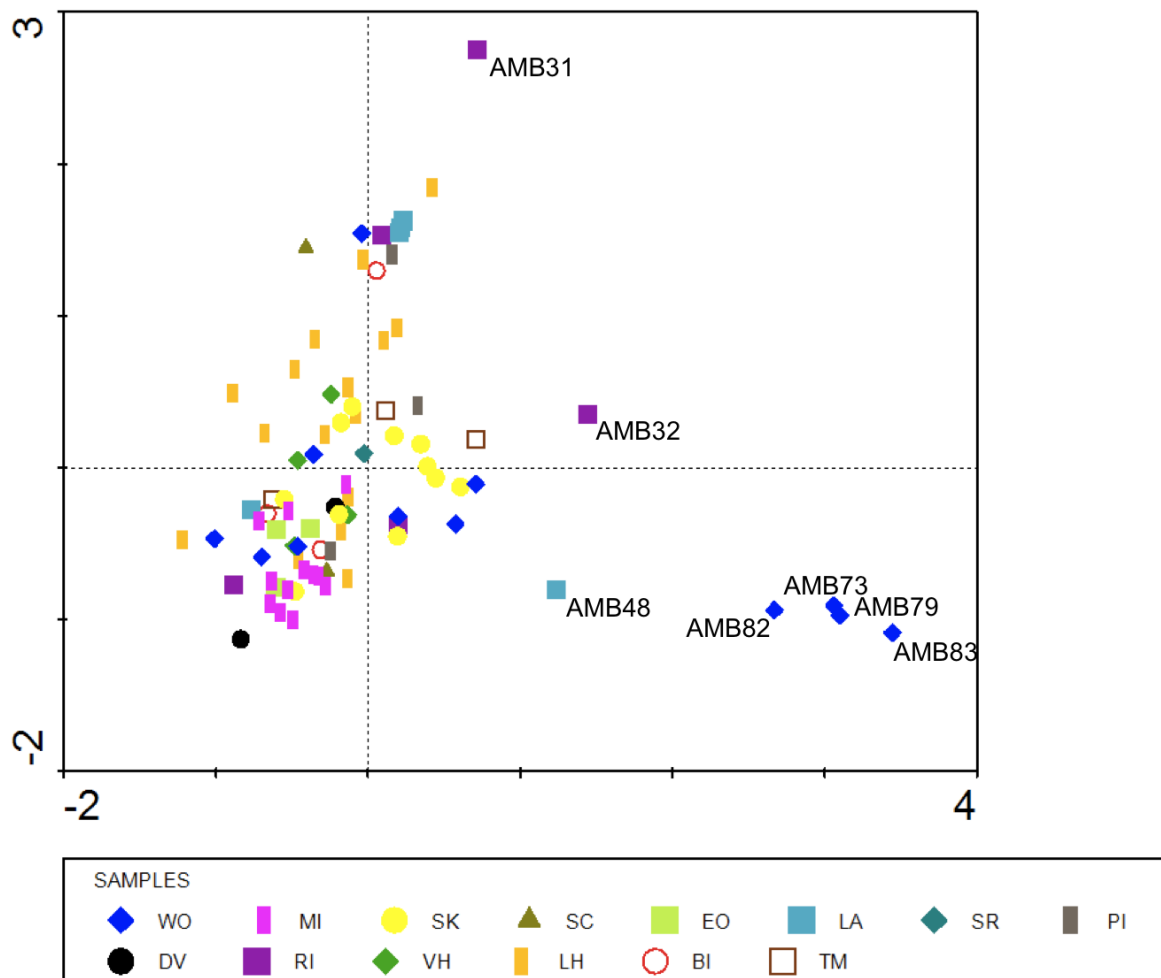
Figure 8. Correspondance Analysis of the band classes for DGGEb showing the sample scores.

Clone libraries for the 3 TM and the Shackelton Range samples

The samples come from 2 lakes located inland. In Forlidas Pond (Davis Valley), TM1, TM2 and TM3 come respectively from the deepest part of the lake, the littoral zone and from a terrestrial mat located near the lake. Sample TM4 comes from the littoral zone of the Lundström Lake (Shackelton Range).

A total of 264 clones with an insert of the correct size were obtained from the 4 clone libraries. To assign clones to taxonomic clusters, the 16S rDNA of 100 clones chosen randomly was partially sequenced (*E. coli* positions 364-1044) and the remaining 164 were studied by ARDRA. Chimerical sequences were absent from the TM1 sample, but accounted for 22% of the clones for TM2 sample, 10% for TM3 sample and 3% for TM4 sample. They were excluded from the analysis. One or two complete sequences were determined for each OTU, and 12 complete sequences (including the ITS) were obtained for 10 OTUs in total.

More details are given in Fernandez-Carazo et al.(2011).

Clone libraries, results for the W0 4 littoral sample

A total of 60 clones were obtained (20 analyzed by direct sequencing and 40 by ARDRA). Only two OTUs were found, in agreement with the DGGE pattern showing two main bands. After aligning the sequences, 2 were eliminated as they are expected to be chimera. We studied the distribution of the OTUs by comparing the clones to GenBank. The first OTU includes sequences from East Antarctica, McMurdo Dry Valleys, Nepalese and alpine (Switzerland) glaciers and Svalbard. The second OTU seems to be restricted to Polar Regions, as it includes only sequences from East Antarctica, McMurdo Dry Valleys and Svalbard.

Clone libraries for sample 41 of Triangular lake (Maritime Antarctica)

One hundred and fifty-eight sequences (clones) of sufficient length (above 500 bp) were obtained and they originated as follows: 91 from the upper layer and 67 from the lower one. The sequences were corrected and aligned. The screening for chimera showed that 37 sequences were chimeric and they were then removed from the analysis, since they were all represented through their parent-sequences. Thirty-four chimera originated from the upper layer and only 3 from the lower one. After alignment, 12 different Operational Taxonomic Units (OTUs) have been delineated at a level of 97 % 16S rRNA sequence similarity, of which the two layers shared 8. One OTU appear to include only Antarctic sequences (OTU12), and the others are cosmopolitan. Based on the BLAST results of the clones, the global distribution of the OTUs is described in Table 3.

Table 3. Distribution of the OTUs obtained from the sample 41 of Triangular lake (Maritime Antarctica)

OTU	Distribution
1	Antarctic regions, Swiss Alps, Tibetan glacier
2	Antarctic regions, Swiss Alps, Tibetan glacier
3	Antarctic regions, Andes (Peru), Tibetan snow, Airforce base New Mexico, USA
4	Antarctic regions, Tibetan and German glaciers, Finnish lake, Yellowstone Park and Bogota River (Columbia)
5	Antarctic regions and deglaciated soil (uncited origin)
6	Eastern Antarctica and Swiss Alps
7	Eastern Antarctica and Swiss Alps
8	Antarctica, Arctic, Swiss Alps, Tychonema strain from an Irish lake, East Java and other temperate zones
9	Strains of Phormidium murrayi and clones from Antarctica

10	Regions of Antarctica and Swiss Alps, temperate zones (Loa River in Chili, Lac Erie in Michigan)
11	Limnothrix morphotype from both Antarctic, alpine (Switzerland) and temperate samples (Polish lake, hospital shower USA...)
12	Lake Fryxell, East Antarctica (Pseudanabaena morphotype), McMurdo Dry Valleys

Clone libraries for 20 samples of 5 regions (Prydz Bay, McMurdo Dry Valleys, Transantarctic Mountains, Schackleton Range, Antarctic Peninsula)

Using the clone libraries' data from AMBIO and two past projects (LAQUAN and MICROMAT), we have performed a preliminary ordination analyses to check whether regional patterns in the cyanobacterial community composition may be masked by the environmental grouping of the samples. All environmental and species (OTUs) variables were log-transformed prior to multivariate analysis to reduce or remove skewness in the data. A Detrended Correspondence Analysis (DCA) was run with data from clone libraries from 20 samples of EA, TM and Antarctic Peninsula..

The DCA revealed that the OTU composition is geographically structured as each region has a more or less unique flora. The sample TMLU4re (Lundström lake, Shackleton Range) is deviant from the other samples. The observed geographic differences might be underlain by several reasons, such as differences in limnological properties between regions or rather the result from dispersal limitation among cyanobacteria. We can also observe that saline samples are grouped together (TMFO1, RIR8, RIR2 and VHAL) (Fig. 9).

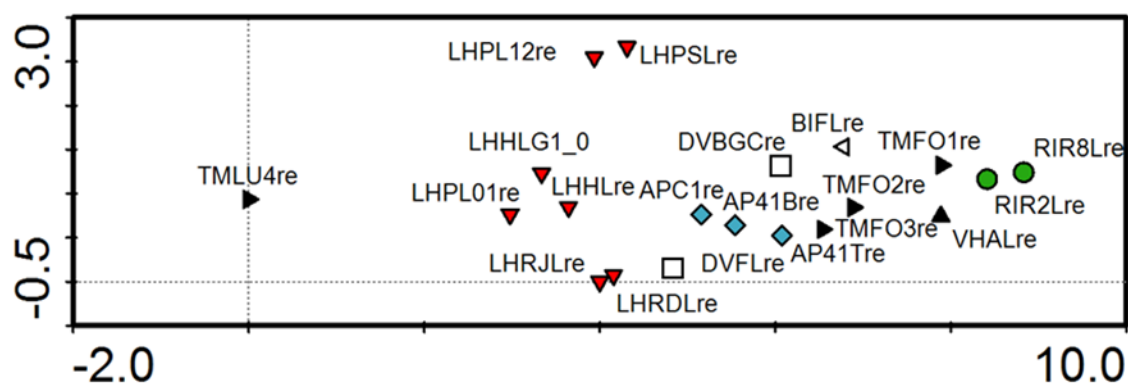**Fig. 9.** Detrended Correspondence Analysis on clone libraries' data for samples of TM, EA, DV and Antarctic Peninsula showing that each region is more or less unique in terms of OTUs composition. Samples close to each other are similar, each region has a different symbol. LH: Larsemann Hills, BI: Bolingen Islands, VH: Vestfold Hills, RI; Rauer Islands, DV: Dry Valleys, AP: Antarctic Peninsula and TM: Transantarctic Mountains

II.2.3. MICROALGAL DIVERSITY
II.2.3.1. Isolation of microalgae, with focus on green algae and diatoms
In total 56 diatom strains and 139 green algal strains were successfully isolated.

The green algal strains belong to 14 different clades and point to a high diversity of this group (Fig. 10). Ten of these taxa were detected in only one region, 2 in two regions and 2 were found at a majority of the sites. The unicellular green algal taxa detected during this study are widely spread among the Chlorophyceae and Trebouxiophycea classes. The strains belonging to the *Scenedesmus* clade were highly similar to an Antarctic strain from Victoria Lake, Gondwana Land (Moro *et al.* unpub). Three Antarctic strains belong (VI11, VI2, VPL1-3) to the Chlorellaceae. The 18S rDNA gene sequence of strain VI11 is identical to an Antarctic isolate (location not specified; Xu & Hu unpub) and closely related to the freshwater isolate *Chlorella* sp. MDL4-1 (Fawley *et al.* unpub). Strain SC2-2 from Schirmacher Oasis is the deepest branching taxon of our isolates and its position among the Trebouxiophyceae is not fully resolved. If we consider the distribution of the isolated taxa, especially representatives of the *Scenedesmus* and *Chlorella* were frequently observed. Even among the Antarctic sequences found on Genbank, these two clades seem well represented. The taxa showing high similarity to one of our isolates include *'Scotiellopsis terrestris'* and *Scenedesmus* sp. CCMP 1625 strains isolated from Mount Erebus (resp. Hanagata 1998, Lesser *et al.* 2002), *Chlorella vulgaris*, sp. FACHB31, YEL and NJ-18 (Xu & Hu unpub). and a *Chlorella* strain from Bratina Island (Morgan-Kiss 2008). The time-calibrated phylogeny (Fig. 10) provides absolute age estimates for evolutionary events. Estimated ages of the Antarctic lineages (determined as the node separating the Antarctic sequence from its non-Antarctic sister) range from 2.7–9.9 Ma for a number of chlamydomonad isolates (Chlorophyceae) to over 17–84 Ma for the majority of the sequences and 330–708 Ma for the trebouxiophycean isolate SC2-2.

In the diatoms, the different Antarctic strains of *Pinnularia borealis* formed a single, distinct cluster and were highly related (Fig. 11). The divergence time of the Antarctic clade from its sister clade (strains from Belgium and the French Alps) was estimated to lay between 2 and 15 million years ago with an average of 7.7 million years ago.

II.2.3.2. Non-cultivated diversity of microalgae
DGGE analysis performed with green algal primers revealed 51 bandclasses (BCs). In total 141 strong bands were sequenced, these belonged to 41 OTU's. Using this sequence information 11 BCs were identified as green algae, 25 as other eukaryotic groups, 5 BCs could not be identified as belonging to a specific group due to the occurrence of different genotypes at the same band position. The green algae belonged to the following groups, Chlorophyceae, Prasinophyceae, Ulvophyceae and

Trebouxiophyceae. Direct ordination (Fig. 12) revealed that 21% of the variation could be explained by the significant explanatory variables. Almost 16% could be explained by a spatial component and 11% by an environmental component. This suggests that at least part of the data is spatially structured and that regional factors may important for explaining the distribution of the An tarctic green algae
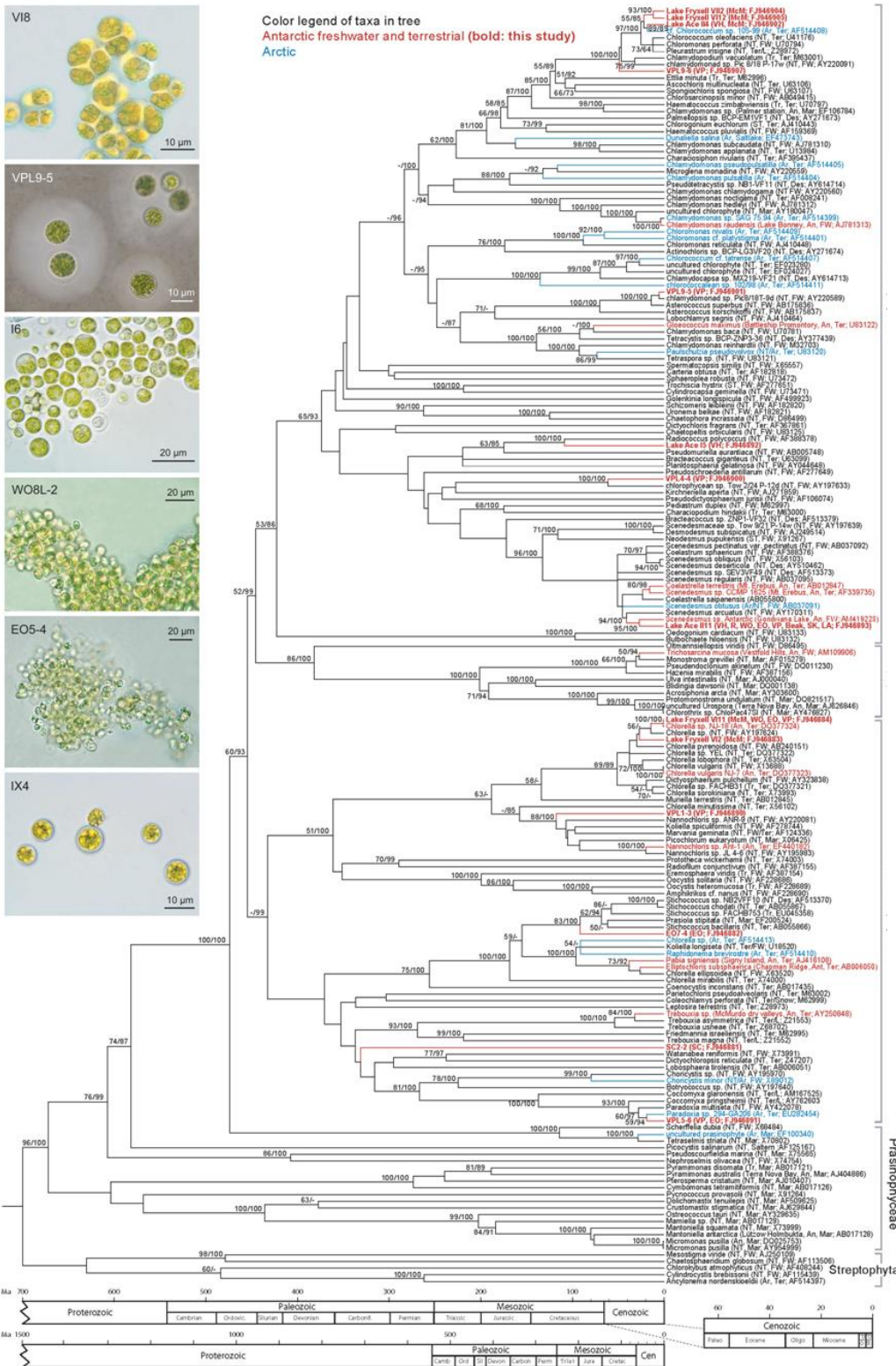
Figure 10: ML tree of the green algae inferred from 18S rDNA sequences with branch lengths fitted to a molecular clock using penalized likelihood, and absolute ages estimated by setting the split of Chlorophyta and Streptophyta at 700 and 1500 Ma, respectively. Numbers at nodes indicate statistical support: ML bootstrap proportions (more than 50) and BI posterior probabilities (more than 85). Phylogenetic positions of freshwater and terrestrial Antarctic sequences are indicated in red, Arctic sequences in blue. Strains obtained during this study are highlighted and indicated by strain name.
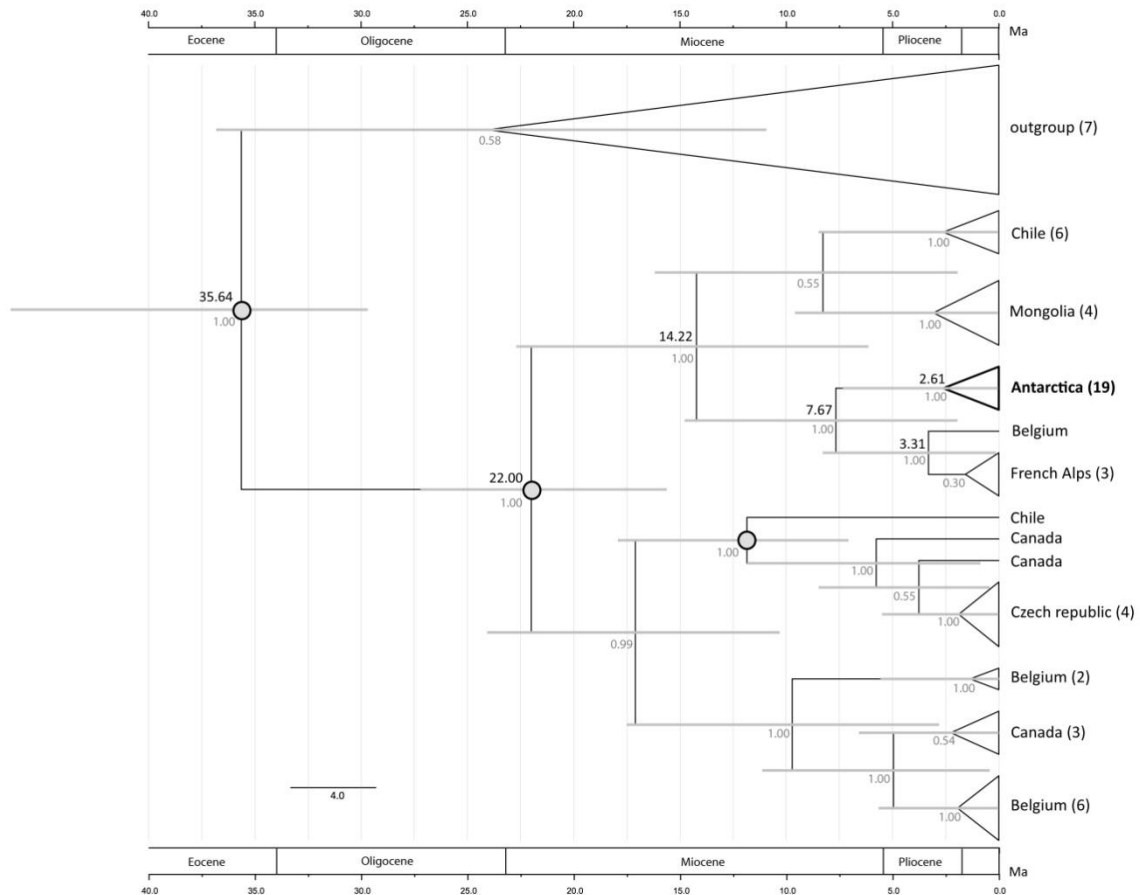
Fig.11: Time-calibrated molecular phylogeny of the diatom morphospecies complex *Pinnularia borealis* showing a divergence of the Continental Antarctic lineage from a western European lineage around 7.7 Ma (2-15Ma, 95% Highest Posterior Probability interval (HPD)) (indicated by an arrow), based on concatenated rbcL and LSU (D1-D2 region) sequence data. Triangles indicate different phylogenetic species as identified by the modified general mixed Yule-coalescent model of phylogenetic species delineation.
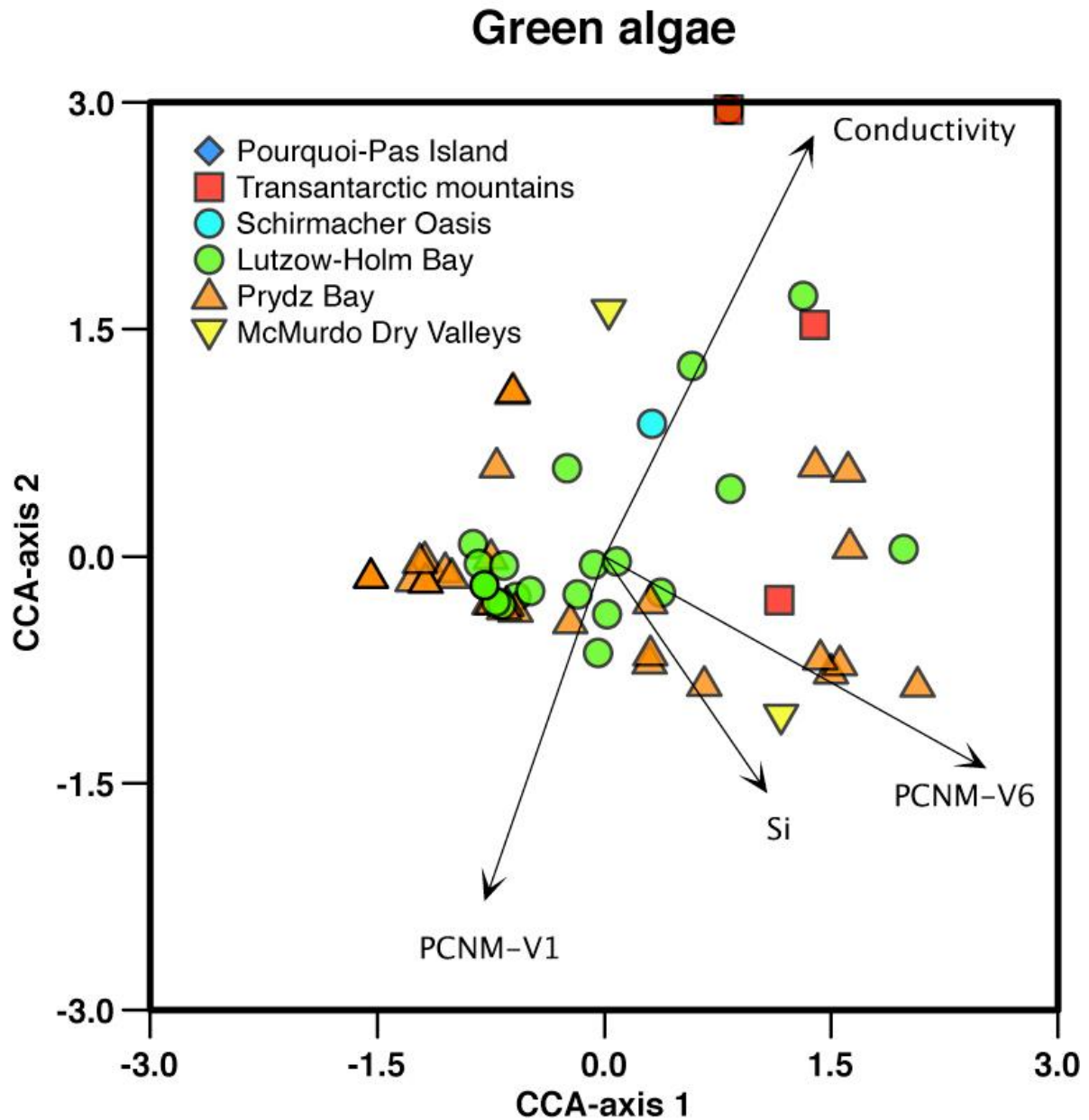
Figure 12: Results of direct ordination analyses showing sample scores and environmental (including spatial variables) scores for the green algae DGGE data. Different regions are indicated as shown in the legend.

II.2.3.3. Diatom counts.

In total, 64 diatom species were found in 74 samples. Environmental and spatial data were available for 60 samples. The most abundant species are *Amphora cf. venata*, *Diadesmis australis* and *Pinnularia microstauron s.l.* The lakes in the Rauer islands are generally more saline and characterized by the presence of *Navicula* cf. *shackletoni* and *Navicula phyllepta*. The freshwater lakes in the Larsemann Hills are dominated by *Stauroforma inermis* and *Psammothidum abundances*, while similar lakes in the Lützow Holm Bay region are characterized by the presence of *Diadesmis australis*. Hence, our data point to geographical differences in the diatom community structure, which is confirmed by a variation partitioning analysis (Fig. 13). Almost 23% of the variation could be explained by the environmental variables conductivity, pH, sampling depth, Na, K, Ca and ammonia, while 11% of the variation was explained by the spatial variables. The incidence of endemism is relatively high, with values ranging between 60% in Continental Antarctica and c. 30% in Maritime Antarctica. Interestingly, some species only occur in a few lakes in one region, such as a unidentified centric diatom which is restricted to the Larsemann Hills. Particularly genera that are dominated by terrestrial species, were species-rich and contained a high number of endemic species.
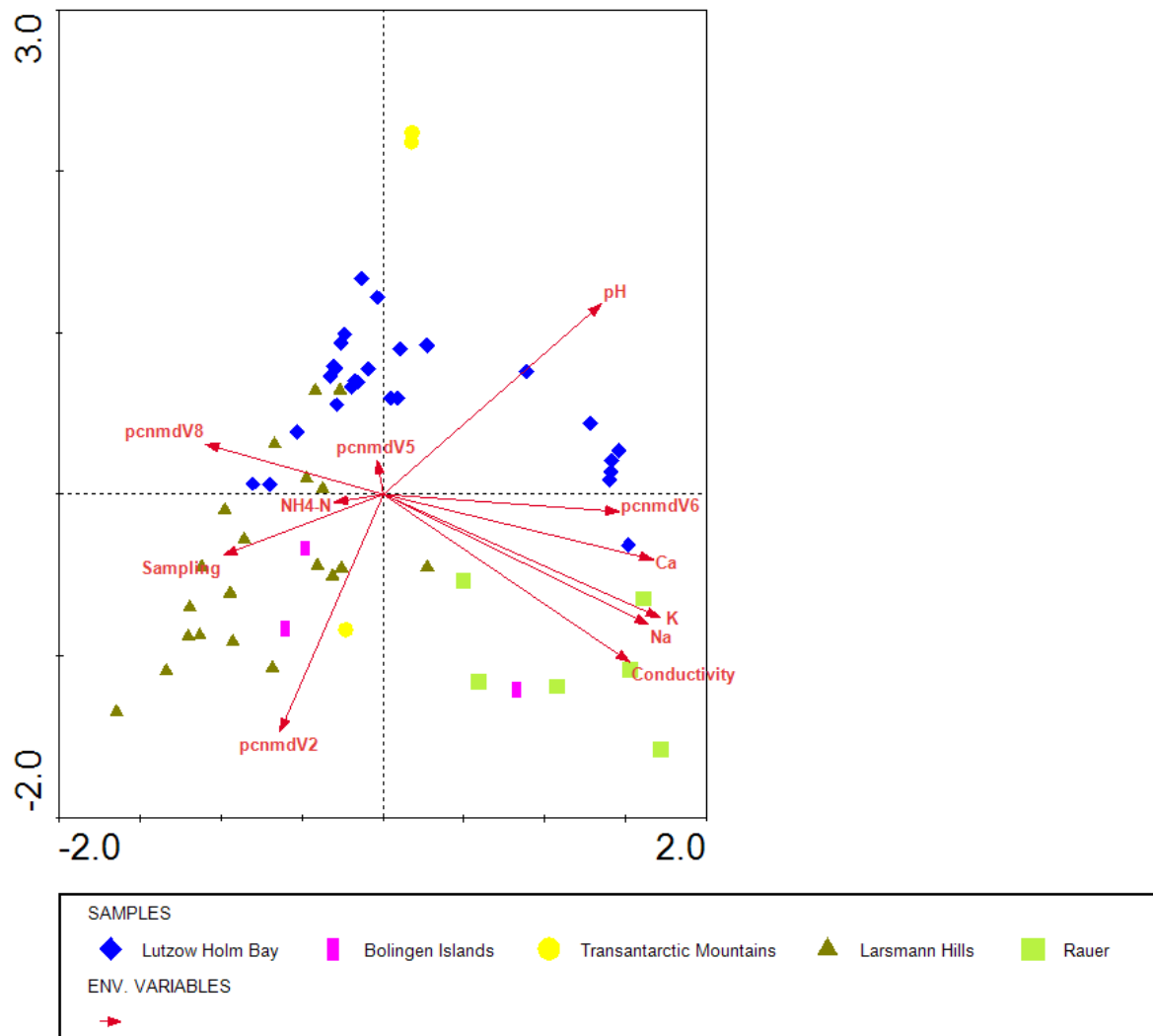
Figure 13: Results of direct ordination analyses showing sample scores and environmental scores (incl. the spatial variables) for the diatom data. Different regions are indicated as shown in the legend.

II.2.4. BIOGEOGRAPHICAL ANALYSES

II.2.4.1. Geographical distribution of cultured bacteria

For all nine samples a large number of the phylotypes (36.6-79.4%) showed a cosmopolitan distribution (Table 4) with the highest value for sample TM4 and the lowest value for sample PQ1. All samples also contained a large number of Antarctic phylotypes and many of these shared no significant similarity with any other sequence in public databases. In general, only small numbers of phylotypes were classified as cold or bipolar. For most phyla/classes phylotypes cultured from the nine samples were predominantly cosmopolitan with some variation between the samples (data not shown). Notable exceptions were the phyla *Bacteroidetes* and *Deinococcus-Thermus* where the majority of phylotypes were Antarctic, many of

them without significant sequence similarity with any other sequence. Again here, the relatively poor representation of much of the existing bacterial diversity in sequence databases should be taken into account.

Table 4. Distribution type of phylotypes recovered from the different samples. Distribution types were assigned to phylotypes by evaluating the geographic origin of highly similar sequences (≥ 99.0%) present in public databases and originating from cultured strains as well as environmental samples and clone-libraries.

Distribution	BB50	BB115	TM2	TM4	PQ1	LA3	SK5	WO10	SO6
% cosmopolitan	52,4	48,6	67,9	79,4	36,6	47,6	63,9	59,2	64,4
% cold	1,6	2,7	1,8	0,0	5,4	2,4	4,9	10,2	4,6
% bipolar	3,2	5,4	5,4	0,0	0,0	2,4	1,6	8,2	2,3
% Antarctic[a]	42,9	43,2	25,0	20,6	58,1	47,6	29,5	22,4	28,7
	(36,5)	(40,5)	(17,9)	(17,6)	(41,9)	(26,2)	(23,0)	(4,1)	(27,6)

[a] In brackets, the number of phylotypes that shared no significant similarity with any other sequence in the public databases.

II.2.4.2. Multivariate analysis relating cultured bacterial diversity to water chemistry and geographic location

Only the seven aquatic samples were included in the PCA as no environmental data were available for the terrestrial samples BB50 and BB115. The PCA of the samples showing the differences in bacterial diversity at genus level based on the number of rep-types is shown in Figure 14 (left). Samples PQ1, SK5 and SO6 were located quite close to each other, indicating that they have some genera in common, whereas the other samples were scattered over the plot. The RDA of the water chemistry data was performed for different combinations of samples and parameters because some values were not available. For all combinations, conductivity was found to be most correlated with the first axis of the scatter plot whereas the RDA of the distance matrix revealed that none of the geographical factors was correlated with the distribution of the samples.

A similar PCA was also done at phylotype level. Figure 14 (right) shows that the samples are dispersed over the scatter plot. RDA of the water chemistry data with similar combinations for the different samples and parameters (see above) showed no significant correlation with any of the environmental parameters whereas the RDA of the distance matrix showed a correlation with one of the geographical factors.
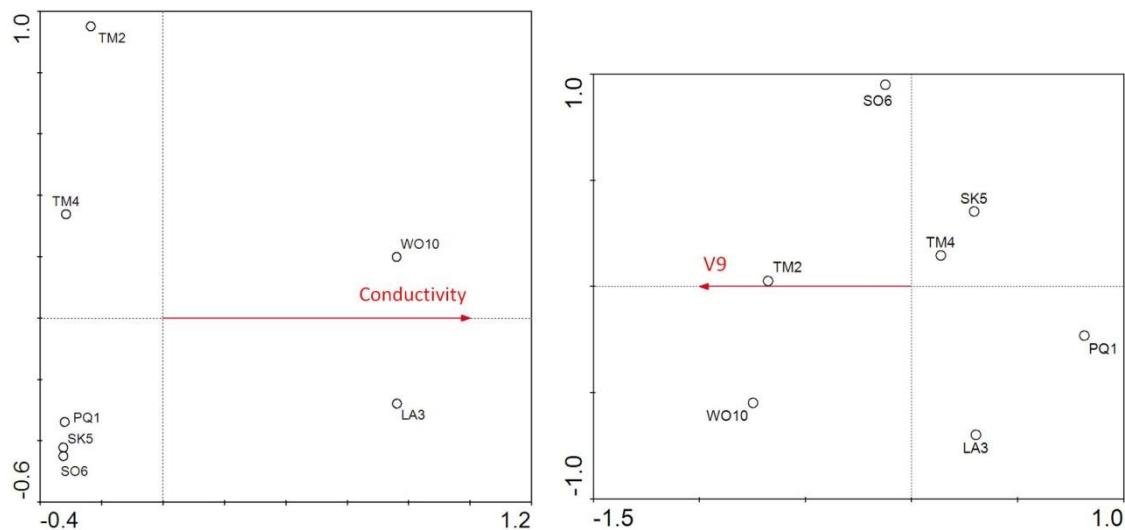
Fig. 14. PCA and RDA of the samples taking into account bacterial diversity at genus level (left) revealed a structuring role of conductivity. The PCA and RDA at phylotype level (right) showed the structuring role of one of the geographical factors (V9).

II.2.4.3. Geographical distribution of the cyanobacterial OTUs obtained with DGGE

Based on the alignment, the DGGE band sequences were grouped into 33 OTUs (0.015 cut-off, average neighbor method in MOTHUR). With the BLAST results, we examined the geographical distribution of these OTUs. The results are summarized in Fig. 15. According to their distribution, we separated these OTUs in the following groups: cosmopolitan (C), alpine-polar (AP), alpine (A), polar (P), endemic to the Antarctic or to the studied region (E), endemic and not described before (EN).

Twenty OTUs have a cosmopolitan (C) distribution. We define a cosmopolitan OTU as a group including at least one sequence from non-Antarctic origin. Those are found in various regions and habitats (monuments, deserts, oceans, lakes, hospitals, even on the human skin).

Remarkably, 13 OTUs (39.4%) are only found in polar and alpine regions (AP, P, A, E and EN), and they seem to be restricted to the cold habitats (other regions of Antarctica, Arctic regions, Andes, Alps, Pyreneans, Himalayan and Nepalese glaciers). This finding supports the study of Jungblut et al (2010) that have documented the distribution of cyanobacterial ecotypes in the "cold biosphere". OTUs 8, 18, and 26 are only found in Antarctica and alpine regions (Swiss Alps and Tibetan lake) but not in the Arctic (A), whereas OTUs 17 and 27 are only found in polar regions (P, Macquarie Island, a stream in Alaska, McMurdo Dry Valleys, East Antarctica, Svalbard, High Canadian Arctic).

Among the 13 "cold" OTUs, this study revealed the presence of 5 potential Antarctic endemic ones (OTU6, 12, 13, 25 and 33). 2 OTUs were novel and did not contain any sequence already deposited in Genbank (endemic-new, EN). OTUs 6 and 13 are found in Schirmacher Oasis (AMB59) and in Macquarie Island (MI06), respectively. The other three OTUs had already been found in Antarctica but only there. OTU12 contains sequences from the Vestfold Hills and Bratina Island (McMurdo Ice Shelf). OTU 25 seems to be endemic (E) to the East Antarctic region. It is found in the Larsemann Hills (AMB13) and in Skarvsness (AMB66 and 67) and its entire sequences match (more than 99.7% similarity) with a DGGE sequence (EU009682) from Lake Grovness (Larsemann Hills). The sequences composing OTU33 originate from Larsemann Hills (AMB11) and Vestfold Hills (AMB26). They are also related to sequences from the same two regions (lakes Heart, Reid, Rauer 7 and Pup and Fresh ponds) and the Transantarctic Mountains. Thus, the cyanobacterial taxa in this study show some biogeographical patterns: there are cosmopolitan OTUs, OTUs that seem to be restricted to cold, polar and alpine habitats, and others that are potentially endemic to the Antarctic continent or to a particular Antarctic region, as seen in Macquarie Island and Schirmacher Oasis.
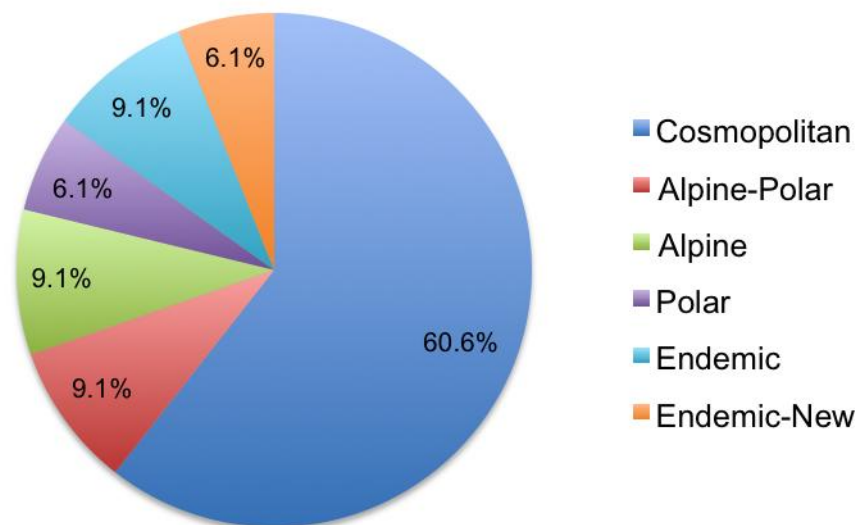
Figure 15. Distribution of the OTUs (98.5% 16S rRNA similarity).

It is also interesting to look deeper into the OTU 13 (EN) and 17 (P) that both include sequences from Macquarie Island.. OTU13 is new and contains only sequences from this sub-antarctic island. OTU17 includes also a sequence (FJ849205) that comes from a stream in Noatak National Preserve, Alaska.

Although the reserve is located just North of the Arctic Circle, it is known to be a transition zone for plants and animals between Arctic and sub-Arctic environments and the climate is milder (higher temperature and humidity) than at the poles (Binkley et al, 1994; Stottlemeyer et al, 2001). Thus, these 2 OTUs do not include any real polar cyanobacterial sequences and their distribution seems to be limited to the sub-polar regions. Considering the effects of climate change on the polar habitats, these organisms might become indicators of global warming if their distribution would extend towards the poles in the future.

As previously mentioned, OTU 25 appears to be potentially endemic to East Antarctica, corroborating previous studies (Taton et al, 2006a; Verleyen et al, 2010). Therefore, it would be interesting to monitor the evolution of the microbial communities in this region as it is experiencing marked changes in climate. These changes include increased wind speeds (Gillett and Thompson, 2003) that influence the salinity in lakes and changing patterns of snow and ice accumulation (Hodgson et al, 2006).

II.2.4.4. Polyphasic study of the cyanobacterial diversity in four samples of the Transantarctic Mountains and Shackelton Range (80-82°S)

These four samples were the only ones studied by a combination of strain isolation, DGGE and clone libraries. They come from two sites, Davis Valley (Transantarctic Mountains) and Shackelton Range (80-82ºS), that are amongst the most southerly locations where freshwater-related ecosystems are present. Cyanobacteria are the dominant phototrophs in these extreme environments. Results from the combination of methods showed a low cyanobacterial biodiversity, with only 3-7 Operational Taxonomic Units (OTUs) per sample. As shown in Table 5, each of the three approaches was needed to maximize the detection of the OTUs.

Table 5. Detection of OTUs by different methods. Presence/absence of OTUs from clone libraries, DGGE gels and isolated strains. Presences are marked by dark cases.

	16ST02	16ST07	16ST11	16ST14	16ST16	16ST44	16ST49	16ST53	16ST57	16ST63	16ST80	16ST92
TM1				D			D			C+S+D		
TM2				C		C+S+D	C+D			C+D	S	
TM3		D				C+D	C+D			D	S	
TM4	D		S		C	D		D	C			C+D

The origins of the sequences from an OTU in a sample are noted: "D" for DGGE, "C" for clone library and "S" for an isolated strain. The OTU numbering is the same as in the PhD thesis of A. Taton (2006).

Interestingly, 4 OTUs are common to Forlidas pond (TM1 + TM2) and the well developed terrestrial mats in the vicinity (Fig. 16). This shows the persistence of the same taxa in aquatic and terrestrial biotopes, as was already detected by Gordon et al. (2000).
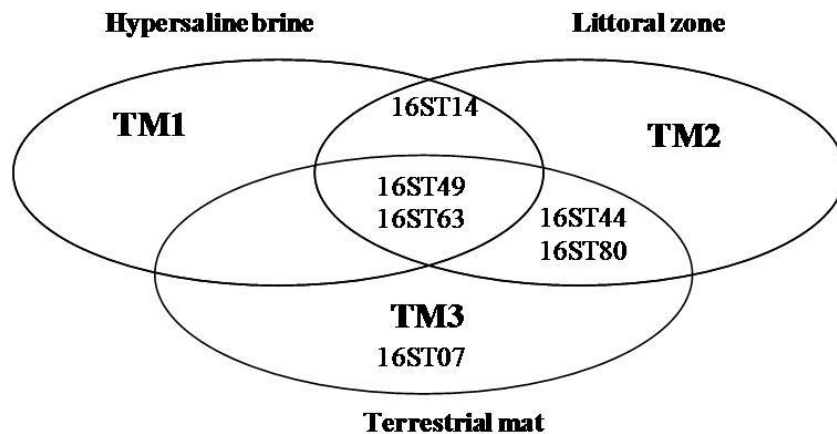
Figure 16. Spatial distribution of cyanobacterial OTUs in and around Forlidas Pond (Davis Valley).

Eleven of the 12 OTUs identified were cosmopolitan and therefore probably adapted to a wide range of habitats in both polar and temperate latitudes. The remaining OTU was potentially endemic to Antarctica and is present in several regions of the continent.

The ITS sequences were also determined and were shown to further discriminate different genotypes within some of the OTUs. A study of the geographic distribution based on ITS sequences seems promising as already shown by Taton et al. (2006a) but is still impeded by the lack of data in Genbank. However, when such comparison was possible, we have observed that the ITS sequences from Antarctic locations appear more closely related to each other than to a non Antarctic sequence (Romania).

This data is the first molecular study of cyanobacterial diversity in these most southern locations (Fernandez-Carazo et al. 2011).

II.2.4.5. Multivariate analysis relating uncultivated diversity to environmental variables and geographic location

DGGE analysis with specific primers for cyanobacteria, bacteria and green algae and pyrosequencing with prokaryotic specific primers was performed on 41 samples for which environmental and spatial data was available. This approach enabled us to compare the patterns found in the different taxonomic groups. Diatoms were present in 38 of these samples. RDA with forward selection and unrestricted Monte Carlo permutation tests of the cyanobacteria dataset revealed that silicate significantly explains 4.2% of the variation in DGGE bands.

RDA of the green algae data revealed that 9.4% of the variation in DGGE band patterns is explained by conductivity and Na. SO_4 explains 4.4% of the variation in DGGE bands in het prokaryotic data, while 33% of variation in the diatom dataset is explained by pH, DOC, sampling depth, Na, Cl and SO_4 . Spatial parameters explain 3.4%, 18% and 19.3% of the variation in DGGE bands in the cyanobacteria, green algae and diatom data respectively.

Variation partitioning analyses allowed us to statistically assess the unique contribution of environmental versus geographical variables in explaining the differences in community structure (Fig. 17). The environmental variables explained 3.4%, 6.8%, and 24.3% of the total variance, independent of the geographical variables in the cyanobacteria, green algae and diatom data respectively (all ordination axes were significant at p≤0.05). The geographical variables were less important in the cyanobacteria data and explained only 2.6% of the total variance independent of the environmental variables. In contrast, the geographical variables were more important in the diatom and green algae data, with 10.6% and 15.4% respectively of the total variance being explained independent of the environmental variables. None of the geographical factors significantly explained variation in the DGGE bands obtained using general prokaryotic primers.
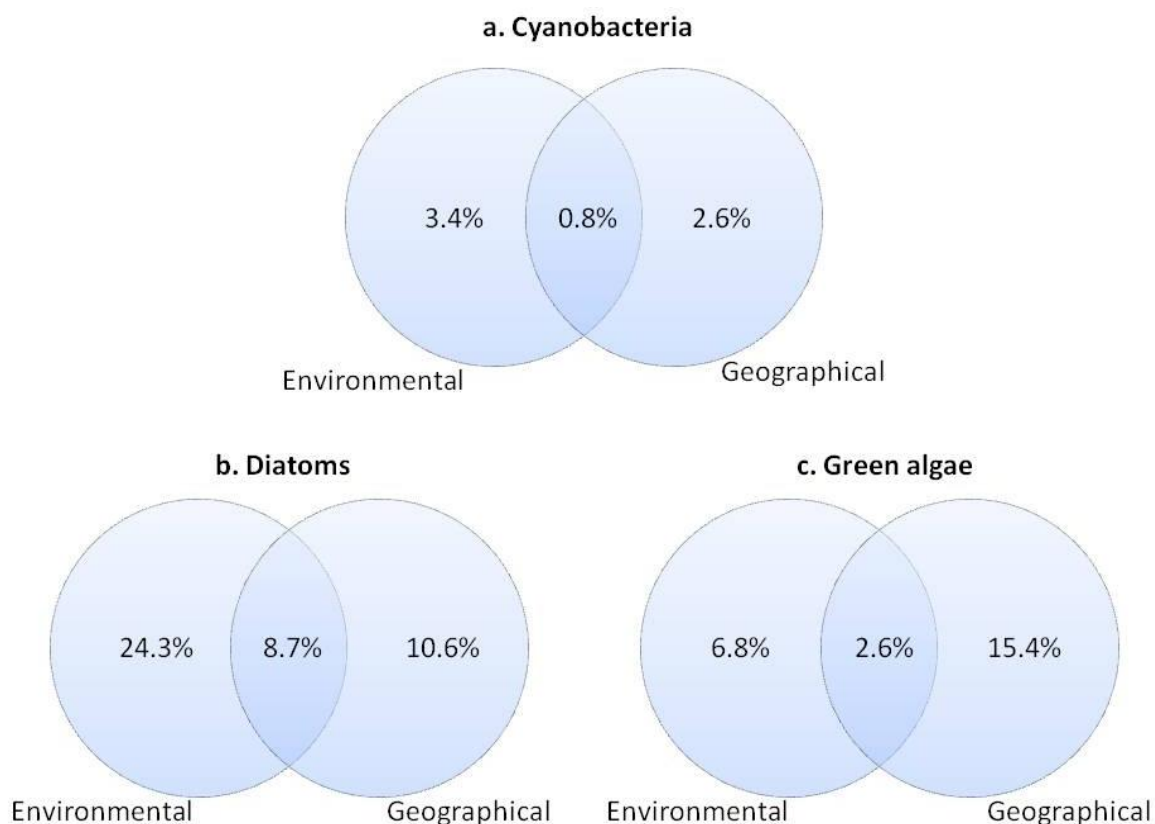
Figure 17: The amount of variation in community composition of the Cyanobacteria, Diatoms and Green algae uniquely explained by the environmental and geographical factors and the overlap between the different fractions as assessed using variation partitioning analysis.

II.3. DISCUSSION AND CONCLUSIONS

II.3.1. DIVERSITY OF CYANOBACTERIA

II.3.1.1. Strain isolation

Strains belonging to four OTUs have already been obtained. Isolation of cyanobacteria requires more time than for bacterial isolates as their growth rates are very low. However, this is worthwhile to perform, as illustrated by the fact that strains from the Transantarctic Mountains and Shackelton Range have allowed to find new OTUs not observed by cloning or DGGE techniques.

II.3.1.2. Clone libraries

Twenty-six OTUs have been observed thanks to clone libraries in four regions (Livingston Is., Syowa Oasis, Transantarctic Mountains, Shackelton Range).

In **Syowa Oasis** and the two remote continental regions (**TM, SR**), the number of OTUs was below the average of 8.5 OTUs per lake that was found in the mats of coastal lakes in the Larsemann Hills, Vestfold Hills and lake Fryxell in the Mc Murdo Dry Valleys (Taton *et al.*, 2003, 2006a).

The two lakes of the **Transantarctic Mountains** and **Shackelton Range** share only one OTU (OTU44) that is cosmopolitan and was found also outside Antarctica. Though the lakes share a similar size and are at a distance of ca 400 kms, they are separated by a chain of mountains and have different chemical characteristics. The high salinity of Forlidas Pond suggests a long history of evaporation in this lake. In contrast, Lundström lake shows a quite low salinity, with only a slight increase at the base of the water column. Because of their different histories, we can infer that they have also been subjected to different ecological processes and that this is the reason why the cyanobacterial diversity found in each lake is so dissimilar. This illustrates that geographic obstacles and different chemical compositions are important factors in the cyanobacterial colonization of new habitats.

In the **Triangular Lake** (sample 41, Byers Peninsula), the results indicate a large molecular diversity of cyanobacteria in this sample from the Antarctic Peninsula (12 OTUs). This can be explained by the milder conditions that prevail in this region, compared to what could be found in other Antarctic regions. After removing the chimeric sequences from the analysis, the lower layer appeared to be slightly more diverse that the upper one (11 OTUs versus 9). The abnormal large proportion of chimera in the upper layer (30%) could be due to a degradation of the DNA, most likely due to UV radiation. Eight OTUs out of 12 were common to the two layers. The others were distributed as follows: 2 OTUs were only present in the upper layer and 2 OTU only in the lower layer.

The presence of OTUs including sequences from both layers could be partially due to the fact that the mechanical separation of the two layers was not perfect and some bits of the green lower layer stayed trapped with the red upper one. It seems that the greatest difference between the two layers is not so much the diversity but rather the likely bad state of the organisms in the red upper layer. However, this hypothesis is based on the high % of chimera during PCR but was not visible by microscopic observation. The upper layer could shield the lower layer from high UV and light intensities thanks to a higher content of protective pigments, and thus help to keep the mat functional as a whole.

II. 3.1.3. DGGE

Here, we used two methods to study the diversity of benthic mat-forming cyanobacteria from Antarctic lakes based on PCR-DGGE. This approach inevitably brings biases such as not detecting the rare taxa (abundance lower than 1%, Casamayor et al, 2000).

The first method was to analyse the band patterns using Bionumerics by grouping the bands that have migrated to the same position into band classes. A matrix of the presence/absence of the BC in the samples was analysed using a correspondence analysis which did not provide conclusive results. More in particular, no particular community structure was observed, except for the isolation of the 7 samples discussed earlier (see II.2.2.3). Moreover, the variation partitioning analysis showed that both environmental and geographical variables had a very small influence (3.4% and 2.6% respectively with 0.8% overlap) on the distribution of the cyanobacterial BC. However, the analysis may have been impacted by two technical difficulties with which we have struggled (i) standardisation of the DGGE gels even with a unified protocol, and probably most important,(ii) the mixed band classes.

The second method was to sequence the bands and perform a phylogenetic analysis and distribution analysis. We have observed 33 OTUs that encompassed a large range of diversity, corresponding to the classical orders Chroococcales, Oscillatoriales and Nostocales. Although not all the bands were sequenced or provided good quality sequences, we were able to retrieve a clear biogeographical pattern from our data, in contrast with the band patterns results. Indeed, we found a majority (60.4%) of cosmopolitan OTUs, which is in agreement with the ubiquity and wide distribution of these microorganisms. More important are the OTUs (39.6%) that were only found in the "cold biosphere" (including 5 OTUs endemic to the Antarctic) in agreement with the recent survey of Jungblut et al (2010). Some OTUs found in regions having sub-polar climates (Macquarie Island and Noatak National Preserve of Alaska), might be unable to colonize polar habitats in the present climatic conditions.

Moreover, we observed 5 OTUs that seem to be endemic to some regions of Antarctica (Larsemann Hills, Vestfold Hills, Schirmacher Oasis and Macquarie Island). This supports the existence of geographical restrictions of the cyanobacterial taxa. Following our study, organisms that were thought to have no limits of dispersal seem to be subject to clear biogeographical patterns. Therefore, in reference to Baas Becking's saying (1934), all cyanobacteria are not everywhere.

However, the OTU composition suggests that there is a flux of microorganisms on a large scale. Indeed, we have OTUs including sequences from DV (McMurdo) and East Antarctic (EA) regions (OTUs 3 and 12), MI and EA (OTUs 1 and 11), PI and EA (OTU5), and between SR, EA and PI (OTU24) (Fig.7).

Of course, these results are influenced by the available sequences in the public database, which will certainly grow with time. Thus, OTUs that are now – potentially – endemic might not be in the future. Since our first reports of potentially endemic cyanobacteria restricted to Antarctica (Taton et al. 2003, 2006a,b; Zakhia et al. 2007), many diversity studies have focused on the polar biotopes during the International Polar Year, and the emerging high throughput methodologies will enable a more precise distribution analysis on a global scale.

Another remark is that the average rate of 16S rRNA evolution in bacteria is estimated at 1% per 50 million years (Ochman & Wilson, 1987), and thus, differentiation of the cyanobacterial OTUs would have happened before the opening of the Drake Passage.

II.3.2. DIVERSITY OF MICROALGAL ISOLATES AND MOLECULAR CLOCK ANALYSES
From five well-distributed regions in both maritime and continental Antarctica, 14 distinct microchlorophyte sequences were recovered. Although there is no absolute threshold of 18S sequence divergence for defining green algal taxa, most Antarctic sequences are divergent enough to be considered distinct species, genera or even higher order taxa (Lewis & Flechtner 2002; Lewis & Lewis 2005). Our results thus indicate a **wide phylogenetic diversity of apparently endemic Antarctic lineages** at different taxonomic levels. In our diatom model, the species complex *Pinnularia borealis*, we found a well-defined **Antarctic clade**. Combined, these findings have at least two important implications.

First, our results clearly indicate that **the true diversity and the incidence of endemism in coccal green algae and diatoms is likely to be an underestimation** when species identification is based on the morphological characteristics. Moreover, our molecular phylogenies and even the morphospecies based assessments of the diatom diversity suggest a relatively high amount of **endemism** in both the diatom and green algal floras, which support the notion that Antarctica has developed a

distinct regional flora. These findings contradict previous studies which suggested that Antarctic green algal and diatom communities are dominated by cosmopolitan species (Broady 1996, Van de Vijver & Beyens 1999).

Second, our results point to several **independent but rare colonisation events over a long time frame in green algae and long-term survival in glacial refugia** as evidenced by the different branch lengths of the Antarctic the diatom and green algal lineages. The time-calibrated phylogenies allowed us to obtain rough estimates for the divergence times of the different Antarctic microchlorophyte and diatom strains. The majority of the chlorophyte lineages (16 out of 26) have estimated ages between 17 and 84 Ma and, based on available sequence data, likely diverged from their closest relatives around the time of the opening of Drake Passage (30 to 45 Ma) during the Eocene, which initiated the first transient glaciations on the continent. The lineages with longer branch lengths, including SC2-2 (330 to 708 Ma), have estimated ages that precede the break-up of Gondwana (65 to 100 Ma). The time-calibrated molecular phylogeny of *Pinnularia borealis* showed a divergence of the Continental Antarctic lineages from a western European lineage around 7.67 Ma (14.77-1.95 Ma, 95% Highest Posterior Probability interval (HPD). These findings therefore support the hypothesis of the existence of refugia during successive glacial cycles (Convey & Stevens 2007; Convey et al. 2008) and contradicts the 'recolonisation hypothesis' which proposes that high colonisation rates have resulted in the dominance of cosmopolitan species in Antarctica. Hence, our findings highlight the importance of past climate and geological changes in shaping the current diversity among eukaryotic microalgae, which is in agreement with recent evidence in multicellular organisms. For example, survival in glacial refugia throughout glacial-interglacial cycles since the formation of Drake Passage c. 35 Ma ago likely led to the high endemicity observed in the terrestrial arthropod fauna and the lichen flora [see Convey et al. (2008, 2009) and Peat et al. (2007) for a review].

II.3.3. DIVERSITY OF BACTERIAL ISOLATES

Yield on different media. **The use of different media and growth conditions was useful because it allowed us to target different organism.** Marine media were used for the more halophilic organisms in saline samples; oligotrophic bacteria preferred the poorer media R2A and R2A/10 while organisms with more specific nutrient requirements grew best on PYGV medium that contains added vitamins and glucose.

Grouping by rep-PCR clustering. Among the 1379 rep-types detected, we found very few (3.4%) that contained isolates from multiple samples. This points to the **uniqueness of the bacterial microflora in the different sites**. Because rep-PCR is a very fine typing technique, a single species may contain several rep-clusters.

Thus, this observation of sample-specific rep-clusters does not preclude that the same species may be present at different sites. This can be established by, for example, 16S rDNA sequencing.

Identification by 16S rRNA sequencing. **The diversity recovered is large and belonged to four major phyla, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria* and *Firmicutes*. From some samples also the phylum *Deinococcus–Thermus* was recovered.**

The majority of the genera obtained were previously observed in Antarctica, however, **our study is the first report from Antarctica for 23 other genera**. Most of these belonged to the *Alphaproteobacteria* (6 new reported genera), Firmicutes (5) and Bacteroidetes (4). The genera *Rothia* and *Aeromicrobium,* which were first reported in Antarctica from the Utsteinen region (Peeters et al. submitted), were also found in some of the other samples, indicating they are more generally distributed in Antarctica.

An important number of phylotypes was found to represent potential novel taxa. Our collection of isolates thus represents a valuable resource for future research of potentially new substances and functionalities. Furthermore, we obtained new strains related to species that are presently represented in culture collections worldwide only a one or a very limited number of cultures (e.g. *Chryseobacterium jeonii* and *Knoellia aerolata*) or to genera that currently consist of a single species (e.g. *Rhodoglobus*, *Saxeibacter*, *Enhydrobacter* and *Marisedimicola*). **Our new phylotypes may give more insight in the diversity of these species and genera that are currently underrepresented in culture collections.**

Detailed study of *Flavobacterium.* Our results demonstrate that the *gyrB* gene has a higher discriminatory power than the 16S rRNA gene sequence. Both gene phylogenies including existing *Flavobacterium* species, indicate that **the new Antarctic *Flavobacterium* isolates belong to 14 potential new species and *F. micromati.*** Twelve of these potential new species were confirmed to be distinct from existing species by phenotypic testing.

PCR tests that were developed to test for the presence of two of the new groups in environmental samples demonstrated that these groups are not restricted to the samples they were originally cultivated from. In the future, these tests can be used for the detection of these *Flavobacterium* species without the need for cultivation.

II.3.4. NON-CULTIVATED BACTERIAL DIVERSITY

Comparison cultured vs. uncultured approach for bacteria. The cultivation approach for the exploration of diversity is known to have limitations because only a small part of the diversity present is culturable [Amann et al. 1995] and cultivation imposes a selection in function of the conditions used. The cultivated bacteria may belong to abundant or more rare taxa. Novel large-scale pyrosequencing approaches on DNA extracts of the whole community in a sample can provide a detailed inventory without the bias of cultivation. A comparison with preliminary pyrosequencing results for the same samples (Obbels et al., personal communication) showed that our bacterial isolated indeed comprise both abundant and rare species. Nevertheless, pyrosequencing has its own limitations [Kunin et al. 2010; Quince et al. 2009]. Some of the cultured genera were not represented in the pyrosequencing results (e.g. *Arthrobacter* in sample TM4 and *Psychrobacter* in sample WO10). They may belong to the "rare biosphere" and may become more dominant when environmental factors change [Sogin et al. 2006]. These taxa may serve as a reservoir of genomic innovation and will permit the community to adapt to new conditions. Our observations confirm that **cultivation and culture-independent approaches are complementary in exploring the diversity of a particular habitat** (Amann et al. 1995; Dolci et al. 2009; Sun et al. 2010).

II.3.5. BIOGEOGRAPHICAL PATTERNS

II.3.5.1. Geographical distribution of cultured bacteria

By comparison with public sequence databases, phylotypes were classified as cosmopolitan when they showed high similarity with at least one non-cold/bipolar/Antarctic sequence, although this does not necessarily mean they are present everywhere as defined by Baas-Becking (Baas-Becking 1934). It should also be noted that labels describing geographic distribution reflect current and therefore limited knowledge of bacterial diversity and ecology (Curtis & Sloan 2004).

In most samples the majority of species obtained in culture were cosmopolitan. However, also 20 to 58% was found to be restricted to Antarctica, according to current knowledge. **All samples thus contain biodiversity unique to Antarctica.**

For the samples from Utsteinen, an important part of the cultured diversity is restricted to Antarctica. This may be explained partly by the terrestrial, more exposed nature of these samples that originated from the pristine environment of the new Princes Elisabeth Station. These samples were taken inland, whereas most microbial studies on terrestrial samples in Antarctica have focused on regions closer to the coast (Aislabie et al. 2006b; Chong et al. 2009; Shivaji et al. 2004).

The other samples in our study originate from locations closer to the ocean and research stations and may have experienced inflow of non-Antarctic species, which may have contributed to the lower percentage of phylotypes with an Antarctic distribution. A notable exception was sample **PQ1 from the Antarctic Peninsula, closer to the ocean and to research stations, where an important percentage of Antarctic phylotypes was recovered.**

The majority of the phylotypes belonging to the *Actinobacteria*, *Proteobacteria* and *Firmicutes* have a more general distribution whereas most *Bacteroidetes* and *Deinococcus-Thermus* phylotypes are restricted to the Antarctic continent. This high number of Antarctic phylotypes within the *Bacteroidetes* with several potential new taxa is in agreement with the important number of new species described from Antarctica within this phylum (Bowman et al. 1997; Bowman et al. 1998; Bowman and Nichols 2002; Hirsch et al. 1998; McCammon et al. 1998; Shivaji et al. 1992; Van Trappen et al. 2003; Van Trappen et al. 2004a,b; Yi et al. 2005; Yi and Chun 2006). Our observations seem to indicate that **cosmopolitan as well as Antarctic organisms with a more restricted dispersal and which likely evolved in isolation are present.** Papke et al. (2003) already suggested that ubiquitous dispersal is not a universal trait as some studies found a cosmopolitan distribution for some groups (Hewson & Fuhrman 2004; Kuske et al. 2002) while others were found to be endemic. Furthermore, Bissett et al. (2010) showed that geographical distance had no influence on the community structure of organisms known to have survival stages, but the converse was true for organisms thought to be less resistent.

II.3.5.2. Cultured bacterial diversity in relation to environmental (water chemistry) and geographical factors

To study the relative importance of environmental and geographical factors on the diversity recovered from the samples, PCA was applied at different taxonomic levels. Although only a limited number of samples and variables were included, some interesting observations can be made. **At genus level, our analyses showed that the variation between the samples could best be explained by the conductivity.** LA3 and WO10, located together and separately from the other samples on the scatter plot, also had the highest growth on the marine medium. It has been previously reported that salinity, which is correlated with conductivity, may be the major environmental determinant of microbial community composition rather than extremes of temperatures, pH, or other physical and chemical factors (Tamames et al. 2010). *Betaproteobacteria* have been reported to be less salt tolerant (Philippot et al. 2010), which may explain their very limited recovery from saline samples LA3 and WO10. Our analyses also revealed that **at genus level, there was no correlation between the diversity recovered and the geographic distance between the samples**.

Thus, at the genus level, the distribution of bacteria is correlated with environmental factors and not with geographic distance, in line with the Baas-Becking theory (Martiny et al. 2006).

In contrast, similar analyses at species (phylotype) level showed a correlation with one distance factor and no correlation with environmental data, indicating that **the distribution of species may be related with geographical factors**. This is in conflict with the Baas-Becking theory (1934). A plausible explanation is that colonisation of lakes by microorganisms from adjacent lakes is more probable than from more remote lakes (Reche et al. 2005). The existence of biogeographical patterns and the role of spatial factors have been found before for several bacterial taxa (Green & Bohannan 2006; Horner-Devine et al. 2004; Papke et al. 2003; Reche et al. 2005; Whitaker et al. 2003).

Contrasting results for PCA at different taxonomic levels (Fig. 2) have been observed before by Cho & Tiedje (2000). They found evidence for the correlation of geographical factors with the genetic distance of the isolates using BOX-PCR but not with ARDRA and ITS-RFLP. This indicates that geographical factors might be important at fine taxonomic levels and the use of very high-resolution genetic markers may be necessary (Bisset et al. 2010; Green & Bohannan 2006; Papke et al. 2003).

Some of our observations, however, challenge the importance of geographical factors. Samples BB50 and BB115 originated from the same general area and yielded a comparable bacterial diversity at phylum level but not at genus level. Furthermore, there were clear differences between the three samples from the Syowa Oasis, especially between SK5 and the other two samples (LA3 and WO10), while SK5 did share an important number of phylotypes and rep-types with the remote sample SO6. Most probably, these differences may be the result of the limited number of isolates and samples studied and the cultivation approach that cannot cover the complete diversity and/or the limnological conditions of the lakes.

To study biogeography more profoundly, a larger dataset is necessary, containing a number of samples originating both from the same region, comparable regions and different regions with complete environmental datasets. This may elucidate whether remote environments with comparable chemistry have a comparable diversity and chemically distinct environments in the same geographical region have differences in diversity which would be in line with Baas-Becking (Green & Bohannan, 2006; Papke et al., 2003).

II.3.5.3. Uncultured diversity in relation to environmental and geographical factors

Conductivity, or variables related to salinity (e.g. concentration of ions), are the most important environmental variables in all groups studied (see also Verleyen et al. 2010). This corroborates previous findings in particular taxonomic groups, such as diatoms studied at the morphospecies level in east and maritime Antarctic lakes (e.g., Jones et al. 1993, Verleyen et al. 2003, Sabbe et al. 2004, Gibson et al. 2006a) and cyanobacteria genotypes in supraglacial meltwater ponds on the McMurdo Ice Shelf (Jungblut et al. 2005) whose community structure exhibited a close relationship with environmental factors. HPLC analysis of the photosynthetic pigment composition in east Antarctic microbial mats similarly revealed that the major groups of autotrophic organisms are constrained by these groups of climate-related environmental factors (Hodgson et al. 2004).

The **importance of environmental versus geographical factors** in explaining the uncultured diversity of bacteria, cyanobacteria, green algae and diatoms was assessed using a subset of 41 samples for which limnological variables were available. In both **diatoms and green algae, geographical factors explained a significant part of the variation in community structure between the lakes.** These results thus refute the ubiquity hypothesis and stress the importance of dispersal and migration in structuring diatom and green algal communities. Instead, our results are consistent with predictions from the theory of island biogeography and metacommunity concepts. Hence, dispersal limitation, in combination with survival in glacial refugia, thus likely underlie the strong provinciality and endemism observed in the relatively isolated diatom and green algal floras in Antarctica. Our findings are also in agreement with a recent, global-wide analysis, which revealed that factors shaping the communities of multicellular organisms also operate in diatoms (Vyverman et al. 2007, Verleyen et al. 2009).

In contrast, **in prokaryotes in general the geographical factors failed to explain part of the variation in community structure, while their effect is small on cyanobacterial communities**. These findings are in agreement with the ubiquity hypothesis (Baas-Becking 1934) and a recent study in planktonic bacteria which showed a strong impact of local environmental factors, and a marginal impact of spatial distance (Van der Gucht et al. 2007). However, we are aware that the lack of correlation between environmental and geographical factors in the DGGE data obtained using the universal prokaryote and cyanobacterial primers might be related to the relatively high amount of mixed bands, which prevents an in-depth analysis and biases the multivariate analysis. The results of the 454 pyrosequencing analyses will enable us to confirm or reject these findings.

If the importance of geographical factors is indeed different between the different taxonomic groups, and not related to bias, then differences in life cycles characteristics likely underlie these patterns. For example, diatoms have a sexual

phase in their life cycle. It therefore takes a male and female strain from the same species to successfully establish a community. In contrast, bacteria and cyanobacteria reproduce asexually and the successful colonization of a new habitat can potentially result in a population if the environmental conditions are appropriate. We hypothesize that **findings from one particular microbial group cannot be generalized to microbes as a whole**.

II.3.6. *FUTURE PERSPECTIVES*
a) Extension of the geographical range of the biogeography studies
Because of delays in obtaining samples from the Sub-Antarctic Islands, we could not fully study these samples. As the Sub-Antarctic Islands experience a climate that is 'intermediate' between the Antarctic and neighbouring continents, and they could serve as 'stepping stones' for disseminating microorganisms, extension of the geographic extent of the present study would significantly help to complete our database and perform comparative analyses.

b) Improvement of the molecular methodology thanks to next-generation sequencing.
When the AMBIO project was written in 2006, the 454 methodology was still in infancy and not commonly available. The **DGGE** was the only tool at our disposal to obtain quite quickly community fingerprints based on partial 16S rRNA. However, we have encountered two main problems:

- For several groups, the cyanobacteria and bacteria, it became obvious that an analysis based solely on the band patterns (band classes) was not possible because there was a high amount of **mixed band classes**. For cyanobacteria, it was also possible to find almost identical sequences at different positions in the same lane.

- This problem could not be solved by the extraction of bands from the gels, reamplification and sequencing because the bands were too numerous for bacteria, and they were often of bad quality for cyanobacteria. Cloning these bands would have slowed the study further..The DGGE is based on partial 16S rRNA sequences. In 2006, it was our experience that this marker was able to distinguish potential endemics and cosmopolitan cyanobacterial OTUs (Taton et al., 2006a). The OTUs were based on a threshold of 2.5 % sequence similarity as proposed by Stackebrandt and Goebel (1994). Since that time, the database has grown considerably and most of the potential endemic OTUs in 2006 now include non-Antarctic sequences and have become cosmopolitan (sometimes restricted to polar and alpine biotopes). Moreover, bacterial taxonomists now recognize that the 3 to 2.5% threshold is too large for species definition, and should be closer to 1.5 % (Stackebrandt & Ebers, 2006). However, it is difficult to get high quality sequences from DGGE bands.

> Therefore, a better tool than the DGGE, but still less time-consuming than clone libraries, would be recommendable. The 454 pyrosequencing seems to be the most promising tool for this aim. It would allow us to use different loci as molecular markers, like spacers and house-keeping genes.

During the last year of AMBIO, we have tested the **pyrosequencing** method to study the bacterial diversity in 49 samples and this seemed really promising. In addition, this 454 run was used to select a model taxon for a recently started PhD project aimed at identifying salinity and stress stress-related genes. In addition to biodiversity surveys using primers that target molecular taxonomic markers (16S rRNA but also other loci like the ITS), a metagenomics approach could yield functional information on the microbial communities.

c) A focus on the evolutionary history of relatively recent organisms

When inferring rather recent evolutionary events (like the ones concerning the Antarctic continent), the evolutionary signal from organisms that evolved more recently, like the protists, was shown to be informative. For protists, targeted studies using isolated strains to build time-calibrated multigene phylogenies and to quantify population genetic structure, should be able to achieve a better understanding of their evolutionary history and to address the following questions:

- Do they have a long association with the continent and did they really survive in glacial refugia, comparable with multicellular biota? In other words, what is the importance of allopatric speciation in shaping the contemporary diversity ?

 - Can the imbalanced flora in some groups (e.g. speciose terrestrial genera) be explained by adaptive radiation?

 - What was the effect of past geological and climatic events, such as the opening of Drake Passage (35Ma ago) and the Mid Miocene cooling event (14Ma ago) and subsequent formation of the East Antarctic Ice Sheet on the evolution of Antarctic microbial eukaryotes?

 - Are Quaternary glacial-interglacial cycles reflected in their population genetic structure? In other words: what are the source populations for regions which became ice-free after the Last Glacial Maximum?

 - Are the biogeographical patterns found in multicellular organisms also present in microbial organisms?

 In order to answer these questions, we need to extend our datasets towards Maritime Antarctica and the three Sub-Antarctic biogeographical provinces observed in multicellular organisms, and develop time-constrained phylogenies of genera, which are relatively speciose in Antarctica.

d) Elaboration and extension of strain collections of all microbial groups

The AMBIO project has shown the usefulness of strain collections to directly study the evolutionary history of particular microorganisms. The presently available collections in the world contain generally only few Antarctic microorganisms, and they are not representative. Therefore, it would be worthwhile to obtain more Antarctic cultures of more microbial groups.

III. SUPPORT TO POLICY

The findings of many bacterial, diatom, green algal (and to some extent, cyanobacterial) species unique to Antarctica and new to science demonstrate the large value of Antarctica as a relatively unexplored territory that represents an immense resource for biotechnological, biomedical and environmental and applications. It is important to protect this continent and its inhabitants from human impact as much as possible. The discovery that Antarctic lakes are dominated by endemic microbial organisms has important implications for the conservation of these ecosystems. More in particular, the identification of Antarctic Special Protected Areas (ASPAs) was traditionally based on the diversity/presence of multicellular organisms. Because microorganisms, together with a few mosses, lichens, two flowering plants and small invertebrates, are the only permanent inhabitants, they should be an additional criterion for the delineation of ASPAs. For example, an endemic, as yet unidentified diatom species occurs in a handful of lakes in the Larsemann Hills. The presence of this taxon is likely related to the fact that some of the lakes acted as glacial refugia during past glacial maxima (Hodgson et al. 2001). The protection of this region should thus be a priority. Unfortunately, it was only categorized as ASMA (Antarctic Specially Managed Area) and a new Indian station is being built in close vicinity to one of these lakes. We have submitted an abstract for the IPY meeting in Montreal in 2012 concerning the use of microbial diversity for ASPA definition.

Less potential endemicity could be shown for cyanobacteria (based on the 16S rRNA sequences) than for bacteria and protists, but it can be used to demonstrate the biological value of Bauer Bay in the Macquarie Island and lake SC2 in Schirmacher Oasis. Moreover, the use of more variable molecular taxonomic markers and High Throughput methods (like the 545 pyrosequencing) could improve the resolution of the studies and better detect endemicity.

It is also apparent that we have only just caught a glimpse ('the tip of the iceberg') of the biodiversity that inhabits Antarctica. Further studies are needed and will undoubtedly yield even more novel organisms and insights. In view of the anticipated increased effects of global warming (such as rising temperature, increased desiccation, changes in UV radiation and snow/ice cover), it seems urgent to further assess particularly the impact of global change on Antarctic biota. Indeed, in addition to endemics, the total microbial biota is important for the ecosystems and might be impacted by the climate change effects. Moreover, we want to stress that particular care should be taken to avoid the import of alien species from outside Antarctica, and also between different regions of the continent and the sub-Antarctic islands. This is one of the priorities at the agenda of the Committee for Environmental Protection (CEP).

However, the IPY ALIEN project was studying mostly propagules of plants, fungi, etc with limited research on microbes. Therefore, AMBIO has produced valuable data on a rather understudied topics and has identified avenues for further research.

To facilitate international access to our data, it has been or will be submitted to the database ANTABIF, or directly to GBIF

(e.g. http://data.gbif.org/datasets/resource/11295/ for the diatoms data)

IV. DISSEMINATION AND VALORISATION

IV. 1. WEBSITE
The AMBIO website (http://www.ambio.ulg.ac.be/) includes information about the project, the activities, publications and news, and links to other relevant websites. Hyperlinks allow to find documents to be downloaded.

IV. 2. BIOLOGICAL RESSOURCES
All the bacterial, diatom and green algal isolates that have been obtained during the project have been preserved. They have been identified to genus level and some also to species level. For the bacteria, many were shown to potentially represent new species and genera that will be described and named in future. Representative strains from named species of bacteria will be transferred to the BCCM/LMG culture collection. The diatom and green algal strains will be integrated in the BCCM culture collection hosted by PAE. The cyanobacteria are currently deposited into the BCCM/ULC collection hosted by CIP at Ulg. They will be available for future research for fundamental research and applied purposes (e.g. on useful properties or compounds that these organisms may harbor).

IV. 3. COLLECTION OF SAMPLES
We have gathered a representative collection of samples that also could be used for later projects (see future outcomes) and made available to the scientific community.

IV. 4. OUTREACH AND EDUCATION

IV.4.1. ARTICLES IN NEWSPAPERS, MEDIA ACTIVITIES
Becquevort, S., A. Borges, D. Cardinal, P. Claeys, M. De Batist, C. De Broyer, F. Dehairs, B. Delille, G. Feller, T. Fichefet, H. Goosse, P. Huybrechts, C. Lancelot, R. Lorrain, F. Pattyn, K. Sabbe, V. Schoemann, J.-L. Tison, A. Vanreusel, F. Volckaert, A. Willems, and A. Wilmotte. 2007. Opinie & Analyse: Omdat Antarctica belangrijk is. De Standaard, 1-2 September 2007.

Becquevort, S., A. Borges, D. Cardinal, P. Claeys, M. De Batist, C. De Broyer, F. Dehairs, B. Delille, G. Feller, T. Fichefet, H. Goosse, P. Huybrechts, C. Lancelot, R. Lorrain, F. Pattyn, K. Sabbe, V. Schoemann, J.-L. Tison, A. Vanreusel, F. Volckaert, A. Willems, and A. Wilmotte. 2007. Forum – Carte blanche: Station belge dans l'Antarctique: "emission zero" pour un gain maximal? Le Soir, 4 September 2007.

Participation to the CANVAS TV-documentary 'De Prinses Elisabethbasis: een missie op Antarctica' of Anne Willems, Annick Wilmotte, Karolien Peeters, Elie Verleyen

Interview of E. Verleyen for the television program 'De zevende dag' in the framework of the inauguration of the Belgian Princes Elisabeth Base – 15/02/2009

Interview of A. Willems for Radio France International at the occasion of the inauguration of the Belgian Princes Elisabeth Station – 17/02/2009

Newspapers article on A. Wilmotte 'De l'Antarctique à l'espace' 16/08/2010 La Libre Belgique, page 40.

IV.4.2. VALORISATION FOR A WIDE AUDIENCE

1. Wilmotte A. 'Le printemps des sciences' 19.03.07 - 23.03.07 (Liège, Belgium) : « Comment les cyanobactéries exploitent au mieux la lumière ». A workshop for secondary school students and presentation of the International Polar Year and some related projects: MERGE and AMBIO.
2. Wilmotte A. 'Semence de curieux' 25.03.07 - 01.04.07 (Liège, Belgium). 2 Radio Broadcasts of RTB: Interview on the cyanobacteria in Polar regions with Dr Annick Wilmotte
3. Verleyen E. 05/06/2007. Organisation of workshop for primary school teachers about global warming and its effect on Antarctic microbial biodiversity, Dendermonde, Belgium
4. Wilmotte A. Conference, "L'Antarctique est un continent microbien" 23.08.07, (Gembloux, Belgium). In the Congress of the 'Science Professor Society', Faculté Agronomiques de Gembloux, by Dr Wilmotte
5. Wilmotte A. Conference, 'L'Antarctique, un continent microbien' 09.09.07 (Tour et Taxis, Brussels, Belgium). at the occasion of the inauguration of the Belgian polar base
6. Verleyen E. Conference, "Reconstructie van vroegere klimaatsveranderingen met behulp van de Antarctische kustmeren als natuurlijke archieven", 09.09.07, in dutch. Wide audience conference presented at the occasion of the inauguration of the Belgian polar base. The use of "natural archives" from Antarctica in order to reconstruct the climate changes is presented. Tour et Taxis, Brussels (Belgium)
7. Wilmotte A. Conference, "Genetic diversity and distribution of cyanobacteria in Antarctica" 20.09.07 (University of Amsterdam, The Netherlands). Faculteit der Natuurwetenschappen, Wiskunde en Informatica, Instituut voor Biodiversiteit en Ecosysteem Dynamica (IBED),
8. Zakhia F. European 'Researcher Night' 28.09.07, Liège (Belgium). Wide audience activities in the frame of the european "Researcher Night", at the Institut de zoologie, Quai Van Beneden, Dr Zakhia showed polar cyanobacteria at the visitors.
9. Verleyen E. 12/04/2008. Global change and biodiversity. Conference for primary school children. Cultureel centrum Lokeren, Belgium
10. Verleyen E. 23/05/2008. Conference for a wide audience regarding climate change in Antarctica and its effect on ice sheet dynamics and biodiversity. BSGO 't Vlasbloempje, Grembergen, Belgium
11. Verleyen E. 04/06/2008. Conference for primary school children regarding Antarctica. BSGO 'De bijenkorf', Sint-Gillis bij Dendermonde, Belgium
12. Verleyen E. 19/02/2009. Conference for high school students of the Katolieke Hogeschool Kortrijk regarding climate change in Antarctica, Ghent University

13. Peeters K. 28/09/2009. Presentation for KAV Baasrode. "Eerste groep wetenschappers naar het nieuwe Belgische onderzoeksstation op Antarctica."
14. Verleyen E. 23/11/2009. Conference for a wide audience (Kiwanis Brugge) regarding microbial diversity and climate change in Antarctica.
15. Peeters K. 07/12/2009. Presentation for Markant Berlare. "Eerste groep wetenschappers naar het nieuwe Belgische onderzoeksstation op Antarctica."
16. Verleyen E. 08/12/2009. Conference regarding Antarctica and climate change. Volkssterrenwacht Urania, Hove.
17. Peeters K. 10/02/2010. Presentation for Okra Lebbeke. "Eerste groep wetenschappers naar het nieuwe Belgische onderzoeksstation op Antarctica."
18. Fernandez-Carazo R. & Annick Wilmotte. 13/08/10. Article on Reflexions (outreach website of the University of Liège): http://reflexions.ulg.ac.be/cms/c_27953/des-cyanobacteries-decouvertes-pres-du-pole-sud
19. Verleyen E. 26/10/2010. Conference for secondary school children regarding Antarctica, climate change and scientists at work. International Polar Foundation, Brussels, Belgium
20. Wilmotte A. 20/11/2009 : Conférence sur invitation « Darwin en Antarctique : diversité et phylogénie », Colloque annuel 'Evolution de la vie et de la matière' Société Royale des Sciences de Liège, Liège, Belgique
21. Verleyen E. 11/01/2011. Conference for secondary school children regarding Antarctica, climate change and scientists at work. International Polar Foundation, Brussels, Belgium

IV.4.3. TRAINING AND EDUCATION

The Ulg team hosted an Erasmus fellow (Prof. J Elster, U. South Bohemia, CZ) for 10 days and two graduate students (S. Chfalmi and B. Gillard) for a technical training of 12 weeks in the frame of AMBIO. They were trained in molecular diversity of Antarctic cyanobacteria. A bilateral cooperation project for training and cooperation concerning the polar cyanobacteria taxonomy was obtained and supported by CGRI/Czech Republic

The LMG team hosted one Erasmus student (Poland, female) who did a laboratory training of 4 months in 2008 and two master students (both Belgian, female, 4th year Biology) who did 4 weeks of laboratory training. Two master students (Biology and Biotechnology) did their final master thesis research in the framework of AMBIO in the laboratory of P3 during 2009 and 2010.

The PAE team hosted two bachelor students for a 1-month study on (i) the morphology and phylogeny of Antarctic green algal strains and (ii) resistance to freezing and desiccation in coccal green algae.

IV.X. FINAL WORKSHOP

A final workshop was organized at the University of Liège, on 31 May 2011. The title was 'Antarctic Biodiversity: Status and trends'. It was organised with EBA and APECS (with a special session dedicated to young scientists in the afternoon). Two lectures were given by videoconference. Japanese (at NIPR) and Uruguayan colleagues attended the meeting by videoconference. (http://www.cip.ulg.ac.be/newsite/pages/workshop_antarctic_2011.php).

V. Publications

V.1. ARTICLES IN PEER-REVIEWED INTERNATIONAL JOURNALS

De Wever A., Leliaert F., Verleyen E., Vanormelingen P., Van der Gucht K., Hodgson D.A., Sabbe K., Vyverman W. (2009) Hidden levels of phylodiversity in Antarctic green algae: further evidence for the existence of glacial refugia. Royal Society B - Biological Sciences 276: 3591-3599.

Hodgson D., P. Convey, E. Verleyen, W. Vyverman, S. McInnes, C. Sands, R. Fernández-Carazo, A. Wilmotte, A. De Wever, K. Peeters, I. Tavernier, A. Willems. (2010) The limnology and biology of the Dufek Massif, Transantarctic Mountains 82° South. Polar Science 4:103-113. (DOI: 10.1016/j.polar.2010.04.003).

Vyverman W., E. Verleyen, A. Wilmotte, D. Hodgson, A. Willems, K. Peeters, B. Van de Vijver, A. De Wever, F. Leliaert, K. Sabbe. (2010) Evidence for widespread endemism among Antarctic micro-organisms. Polar Science 4:197-214. (DOI: 10.1016/j.polar.2010.03.006).

Verleyen E., Sabbe K., Hodgson D.A., Grubisic S., Taton A., Cousin S., Wilmotte A., De Wever A., Van der Gucht K. & Vyverman W. (2010) Structuring effects of climate-related environmental factors on Antarctic microbial mat communities. Aquatic Microbial Ecology 59: 11–24.

Verleyen E., Hodgson D.A., Gibson J., Imura S., Kaup E., Hoshino T., Kanda H., Kudoh S., De Wever A., Hoshino T, McMinn A., Obbels D., Roberts D., Roberts S.J., Sabbe K., Souffreau C., Tavernier I., Van Nieuwenhuyze W. Van Ranst E., Vindevogel N. & Vyverman W. Chemical limnology in coastal East Antarctic lakes: monitoring future climate change in centres of endemism and biodiversity. (In press). Antarctic Science.

Peeters K., D. Ertz, A. Willems. (2011) Culturable bacterial diversity at the Princess Elisabeth Station (Utsteinen, Sør Rondane Mountains, East Antarctica) harbours many new taxa. Syst. Appl. Microbiol., 34:360-367

Peeters K., Verleyen E., Hodgson D.A., Convey P., Ertz D., Vyverman W., Willems A. (2011) Heterotrophic bacterial diversity in terrestrial and aquatic microbial mat communities from Antarctica.Polar Biology doi: 10.1007/s00300-011-1100-4.

Peeters K., S. Verscheure and A. Willems (2011). The *gyrB* gene is a useful phylogenetic marker for exploring the diversity of *Flavobacterium* strains isolated from terrestrial and aquatic habitats in Antarctica. FEMS Microbiol. Lett., 321:130-140.

Peeters K., D. A. Hodgson, P. Convey and A. Willems. (2011) Culturable diversity of heterotrophic bacteria in Forlidas Pond (Pensacola Mountains) and Lundström Lake (Shackleton Range), Antarctica. Microb. Ecol. 62:399-413. (doi:10.1007/s00248-011-9842-7)

Rafael Fernandez-Carazo, Dominic A. Hodgson, Peter Convey & Annick Wilmotte.(2011). Low cyanobacterial diversity in biotopes of the Transantarctic Mountains and Shackleton Range (80-82ºS). .FEMS Microbial Ecology, 77, 503-517.

Rafael Fernández-Carazo, Zorigto Namsaraev, Marie-Jose Mano, Damien Ertz, Annick Wilmotte. Cyanobacterial diversity for an anthropogenic impact assessment in the Sør Rondane Mountains area (Antarctica). (in press) Antarctic Science.

V.2. CHAPTERS IN BOOKS

Zakhia, F., Jungblut A.-D., Taton, A., Vincent, W.F. and Wilmotte A. (2007) Cyanobacteria in cold Ecosystems (chapter 8) In 'Psychrophiles: from Biodiversity to Biotechnology'. Eds: Margesin, R., Schinner, F., Marx, J.-C., Gerday, C., Springer, pages 121-135.

V.3. ARTICLES SUBMITTED TO PEER-REVIEWED INTERNATIONAL JOURNALS

V.4. ARTICLES IN PREPARATION FOR REFEREED INTERNATIONAL JOURNALS

Rafael Fernandez-Carazo, John A.E. Gibson, Annick Wilmotte. A synthesis of molecular studies (16S rRNA) of Antarctic lacustrine cyanobacteria, including new data from the Mawson coast area. Manuscript in preparation.

Verleyen E., Van de Vijver B., Souffreau C., Sabbe K., Hodgson D.A., Nedbalová L., Vanormelingen P., Antoniades D., Convey P., Vyverman W. Poles apart: Interhemispheric contrast in diatom diversity driven by climate and tectonics.

V.5. ARTICLES IN NON-REFEREED JOURNALS

Wilmotte, A. 2007. L'API, Année Polaire Internationale : Focus sur les poles. Bulletin de l'ASBL Sciences et Culture, Liège, Belgium n° 406, p 52-58. mars-avril 2007

A. Wilmotte, W. Vyverman, A. Willems. 2009. AMBIO: Antarctic Microbial Biodiversity: The Importance of Geographical and Ecological Factors. SCAR-EBA Newsletter 3:4.

V.6. ORAL PRESENTATIONS AT SCIENTIFIC MEETINGS

Verleyen E., Hodgson D.A., Sabbe K., Wilmotte A., De Wever A., Vyverman W. Biodiversity assessments of lacustrine microbial communities in Antarctic lakes. Astrobiology workshop, Royal Academy of Sciences, Brussels, 12/06/2007.

De Wever A., Verleyen E., Sabbe K., Taton A., Wilmotte A., Cousin S., Van der Gucht K., Hodgson D.A. and Vyverman W. Patterns in diversity and distribution of Antarctic microbial mat communities determined by environmental and geographical factors. 10th Symposium in Aquatic Microbial Ecology, Faro (Portugal), 2-7/09/2007.

Verleyen E., Wilmotte A., Hodgson D.A., De Wever A., Zakhia F., Fernandez-Carazo R., Waleron K., Sterken M., Sabbe K., D'Hondt S., Dasseville R., Van der Gucht K., & Vyverman W. Spatiotemporal patterns in Antarctic microbial community structure in response to climate related environmental changes. SCAR/IASAC IPY Open Science Conference, Saint Petersburg, Russia, 4-11/07/2008.

De Carvalho Maalouf P., Zakhia F., Chfalmi S., Namsaraev Z., Mano M-J. and Wilmotte A. Diversité moléculaire des cyanobactéries antarctiques. Ecologie des Communautés Végétales (EcoVeg V), Gembloux, Belgium, 8-10/04/2009.

Verleyen E., Van der Gucht K., Martens K, De Meester L and Vyverman W. Patterns in microbial diversity and community structure along multiple spatial scales. 2nd Diversitas Open Science Conference, Cape Town, South Africa, 13-16/10/2009.

Verleyen E., Van de Vijver B., Hodgson D.A., Souffreau C., Tavernier I., Sabbe K. and Vyverman W. Historical processes underlie endemism and biogeographical regionalism in Antarctica diatom communities. XXXI SCAR OSC, Buenos Aires, Argentina, 3-6/08/2010.

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Fernandez-Carazo R., Namsaraev Z., Hodgson D., Convey P. & Wilmotte, A. 2010. Cyanobacterial diversity close to the South Pole (Antarctica). Annual workshop FNRS "Astrobiology: from stars and planets to extreme life' contact group, Brussels, Belgium, 8/12/2010.

De Carvalho Maalouf P., Lambion A., Gillard B., De Wever A., Verleyen E., Wyverman W. and Wilmotte A. Cyanobacterial diversity and distribution in microbial mats from Antarctic and Sub-Antarctic aquatic environments. Youg Botanists' Forum, Meise, Belgium, 19/11/2010.

V.7. POSTER PRESENTATIONS AT SCIENTIFIC MEETINGS

Verleyen E., Hodgson D.A., Sabbe K., Wilmotte A., De Wever A., Vyverman W. High latitude lacustrine microbial biodiversity in response to future climate change. Belgian Biodiversity Platform conference: Biodiversity and Climate Change, 21-22 May 2007, Brussels, Belgium. Poster

Fernandez-Carazo, R., Zakhia, F., Taton, A., Wilmotte, A. Antarctic cyanobacterial communities: baseline data on their diversity and distribution. Belgian Biodiversity Platform conference: Biodiversity and Climate Change, 21-22 May 2007, Brussels, Belgium.

Sabbe K., Verleyen E., Taton A., Wilmotte A., Cousin S., Van der Gucht K., De Wever A., Hodgson D.A. and Vyverman W. influence of environmental and geographic factors on the diversity and distribution of cyanobacteria and protists in microbial mats of Antarctic lakes. 4th European Phycological Congress, Oviedo, Spain, 23-27/07/2007. Poster.

De Wever A., Verleyen E., Van der Gucht K., Zakhia F., Fernandez-Carazo R., Sabbe K., Wilmotte A., Vyverman W. Biogeographical patterns in Antarctic microbial communities. Third International Conference on Polar and Alpine Microbiology, Banff, Alberta, Canada, May 11 - 15, 2008. Poster

Peeters K., E. Verleyen, D. Ertz, D. Hodgson and A. Willems. 2008. Bacterial diversity of Antarctic samples through cultivation. Third International Conference on Polar and Alpine Microbiology, Banff, Alberta, Canada, May 11 - 15, 2008.

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Verleyen E., De Wever A., Leliaert F., Hodgson D.A., Sabbe K., Boitsios S., Convey P., Vanormelingen P., Van der Gucht K. & Vyverman W. Hidden levels of phylodiversity in Antarctic green algae: further compelling evidence for the existence of glacial refugia. 10th SCAR Biology Symposium, Sapporo, Japan, 26-31/07/2009.

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De Smet, W., C. D'Haese, J. Elster, D. Ertz, R. Fernandez-Carazo, A. Mangold, M. J. Mano, Z. Namsaraev, D. Obbels, K. Peeters, S. J. Roberts, I. Tavernier, W. Van Nieuwenhuyze, B. Van de Vijver, J. Van Wichelen, E. Verleyen, W. Vyverman, A. Willems, A. Wilmotte. 2010. Exploration of the microbial diversity and assessment of the environmental impact in the region of the Antarctic research station "Princess Elisabeth". Belgian National Committee on Antarctic Research – International Polar Year Symposium. Brussels, 26 May 2010.

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Verleyen E., De Carvalho Maalouf P., De Wever A., Obbels D., Peeters K., Vyverman W., Willems A., Wilmotte A. The importance of historical and ecological factors in shaping Antarctic microbial biogeography. XXXI SCAR OSC, Buenos Aires, Argentina, 3-6/08/2010. Poster. **Awarded as best poster presentation**.

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VI. ACKNOWLEDGEMENTS

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Annex 1 & 2 : see additionnal online files