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ANTARCTIC MICROBIAL BIODIVERSITY :

THE IMPORTANCE OF GEOGRAPHICAL AND ECOLOGICAL FACTORS

"AMBIO"

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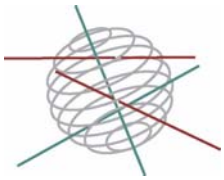

Biodiversity

FINAL REPORT PHASE 1

ANTARCTIC MICROBIAL BIODIVERSITY: THE IMPORTANCE OF GEOGRAPHICAL AND ECOLOGICAL FACTORS

"AMBIO"

SD/BA/01A

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Appendixes are available at the website of the Belgian Science Policy

http://www.belspo.be/ssd/

Acronyms, abbreviations and units

ANTAR-IMPACT:	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".
CA:	Correspondence Analysis
CACHE-PEP:	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
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 multi-proxy 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)
PAE / P2:	UGent laboratory for Protistology and Aquatic Ecology
PCA:	Principal Component Analysis
REGAL:	NIPR project "Research on Ecology and Geohistory of Antarctic Lakes"
rep-PCR:	repetitive extragenic palindromic PCR
RNA:	Ribonucleic acid
rRNA/rDNA,	ribosomal RNA/DNA (ribonucleic acid/deoxyribonucleic acid)
SCAR:	Scientific Committee on Antarctic Research
TM:	Transantarctic Mountains
WO:	West Ongul (East Antarctica)

Summary

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, 1988; 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; Ellis-Evans, 1996; Gibson et al., 2006; Taton et al. 2006a; Stackebrandt et al. 2004; 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; Gibson et al, in press). 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 characterisations 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. They also have a considerable potential for the study of the geographical distribution of microorganisms (Amann, 1995; Loisel et al. 2006). 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.

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, 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; 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 (Lawley et al. 2004, Gibson et al. 2006). 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. 2006). 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).

In the present project we aim 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 use a polyphasic approach combining morphologic characterization by microscopy with molecular techniques in order to reveal the diversity of bacteria (with special emphasis on Proteobacteria, Bacteroidetes), 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 on environmental samples and isolated strains in culture allows us to unveil the diversity.

After plating the samples on different types of media, different growth characteristics were observed for the isolated bacterial strains. Halophilic organisms were isolated from samples TM2 and LA3, which is in agreement with the saline nature of the lakes of origin. Other organisms seem oligortophic or require media with specific nutrient composition in order to grow.

Grouping by rep-PBR showed more than 700 rep-clusters so far but very few contained isolates from the same sample. This points to the uniqueness of bacterial microflora in each site. Because rep-PCR is a very fine typing technique, it does not permit to define site-specific species. 16S rDNA sequencing may be an answer for such question. However, this technique is showing particularly diverse samples (PQ1 and LA3 with respectively 32% and 21% of the isolates having a unique rep-profile.

In several of the groups, part of the isolates showed low similarity values with neighbouring sequences in the EMBL-database were observed. This points to the presence of potentially new taxa, particularly in the *Bacteroidetes* phylum. Potentially new representatives were also found in the *Deinococci* phylum which has relatively few cultivated representatives.

The results of 16S rDNA sequences from pure cyanobacterial strains, DGGE analysis and clone libraries showed 23 OTUs, 5 of them are endemic to Antarctica and 3 constitute a potentially previously undiscovered diversity. Those three OTUs included sequences from the BB, TM and EA which suggests a flux of microorganisms between these regions. On the

other hand, Florlidas Pond (high salinity, strong evaporation) and Lundström Lake (low salinity), both located in the TM and separated by a chain of mountains, share only one cosmopolitan OTU (OTU44). This dissimilarity in the cyanobacterial composition may be due to the different chemical characteristics of the lakes but also maybe to obstacles to the dissemination.

A Detrended Correspondence Analysis (DCA) was run with data from clone libraries from 20 samples of EA, TM and Antarctic Peninsula revealed that the OTU composition is geographically structured as each region has a more or less unique flora. 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.

Finally, the present data from TM, WO and BB show an impoverished diversity in the number of OTUs compared to the coastal lakes from Larsemann Hills, Vestfold Hills and Lake Fryxell (DV) studied by Taton *et al.* (2003, 2006a).

The high diversity of the green algal isolates suggests that this group successfully colonized the Antarctic continent. Two taxa (II11, VI11), were detected in most regions, suggesting that they are widely dispersed over Antarctica. More detailed data (ITS sequences) is required to characterize these taxa on the species level. Most of the studied taxa (10 out of 14) are only retrieved from one ice-free region, suggesting that the antarctic microalgal diversity remains undiscovered.

The study of antarctic sequences points to a unique antarctic flora. This suggests high rates of endemism compared to the results of the morphological work on terrestrial algae (Broady *et al.* 1996).

As for our phylogenetic studies, and using a range of 700 to 1200 million years, we speculate that the Antarctic taxa have been isolated between 5 and 566 million years ago.

As the full analysis of the DGGE results is not finished yet, the following conclusions should be regarded as preliminary. For the bacteria, the results seem to point to a minor importance of geographical variables, this may be due to the limited resolution of the technique in order to reveal patterns in the bacterial community composition. For cyanobacteria and green algae, different distribution patterns are observed, but more data is needed to refine the analysis.

I. Introduction

Microbial organisms dominate most Antarctic ecosystems and play a crucial role in their functioning and durability. Compared with temperate and tropical microbial diversity 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 lacked stability because of the plasticity of the morphology. The molecular era especially the studies based on the SSU rRNA gene have shown a quite 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. They also have a considerable potential for the study of the geographical distribution of microorganisms.

The present project builds on our combined expertise developed within the MICROMAT project and aims to extend the baseline information of microbial diversity through an integrated and standardized analysis of the microbial diversity of aquatic habitats in terrestrial environments of East, Maritime and Sub-Antarctic regions. We will use a combination of approaches, based on isolation and characterisation of strains, sequencing of molecular taxonomic markers and application of probes in bacteria (with special emphasis on Proteobacteria, Bacteroidetes), cyanobacteria and protists (with special emphasis on green algae and diatoms), which have been identified as interesting focal taxa during our earlier studies. Using a community fingerprinting approach based on general and group-specific primers we will construct a dataset covering different geographical regions, and spanning broad ecological gradients. This will enable us, for the first time, to assess the relative importance of ecological versus historical factors in explaining the geographical distribution of microbial communities in Antarctica and sub-Antarctica.

The objectives and outcomes of this project can be articulated as follows:

1. expand the existing database of rRNA gene sequences of bacteria, cyanobacteria and microalgae with new samples from Maritime and Continental Antarctica, as well as from Sub-Antarctica based upon isolates, clone libraries and DGGE of environmental DNA,

2. enlarge the existing collections of Antarctic bacteria (particularly Proteobacteria and Bacteroidetes), cyanobacteria, green algae and diatoms, with new documented isolates,

3. study the microbial diversity in wet terrestrial habitats in Sub-, Maritime and East Antarctica,

4. study the community turnover between different habitats (e.g., lakes, cryoconites and seepages) and among comparable habitats along ecological and geographical gradients to analyze the congruence and disparity in patterns of diversity and turnover observed for different taxa,

5. select in each of the groups particular taxa (genus to infraspecific level, phylotype) that display striking distribution patterns (e.g. potentially endemic or cosmopolitan with or without apparent environmental specializations), for further detailed study of these patterns. The presence of the selected taxa will be monitored with more specific and more sensitive genotypic methods in an enlarged set of samples so as to allow a more precise analysis and facilitate interpretation in relation to other ecological and historic factors,

6. identify regions of unique microbial diversity that deserve to be protected,

7. disseminate new information *via* peer-reviewed publications, presentations to the public and a project website.

AMBIO targets the terrestrial Antarctic diversity that is one of the priority themes of the present call. It will particularly contribute to one of the international initiatives which served as a basis for the present call, namely the SCAR program EBA (Evolution and Biodiversity in the Antarctic). The program has also been accepted as an IPY project (ID137), of which partners 1 and 2 are members.

AMBIO is also fully integrated in and will contribute to the MERGE proposal (ID55) of the International Polar Year which aims to study microbiological and ecological responses to global environmental changes in Polar Regions. The program's activities will thus strongly benefit from and contribute to the international interactions that will happen and will aid to fulfil Belgium's responsibilities during the IPY. Therefore, it will ensure that the Belgian expertise in microbial diversity will be maintained and further developed.

II. Material and methods

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-projects HOLANT and AMBIO).

Currently we have more than 267 samples (excluding duplicates and different conservation methods) available from 13 different ice-free regions in Antarctica (**Fig. 1**).

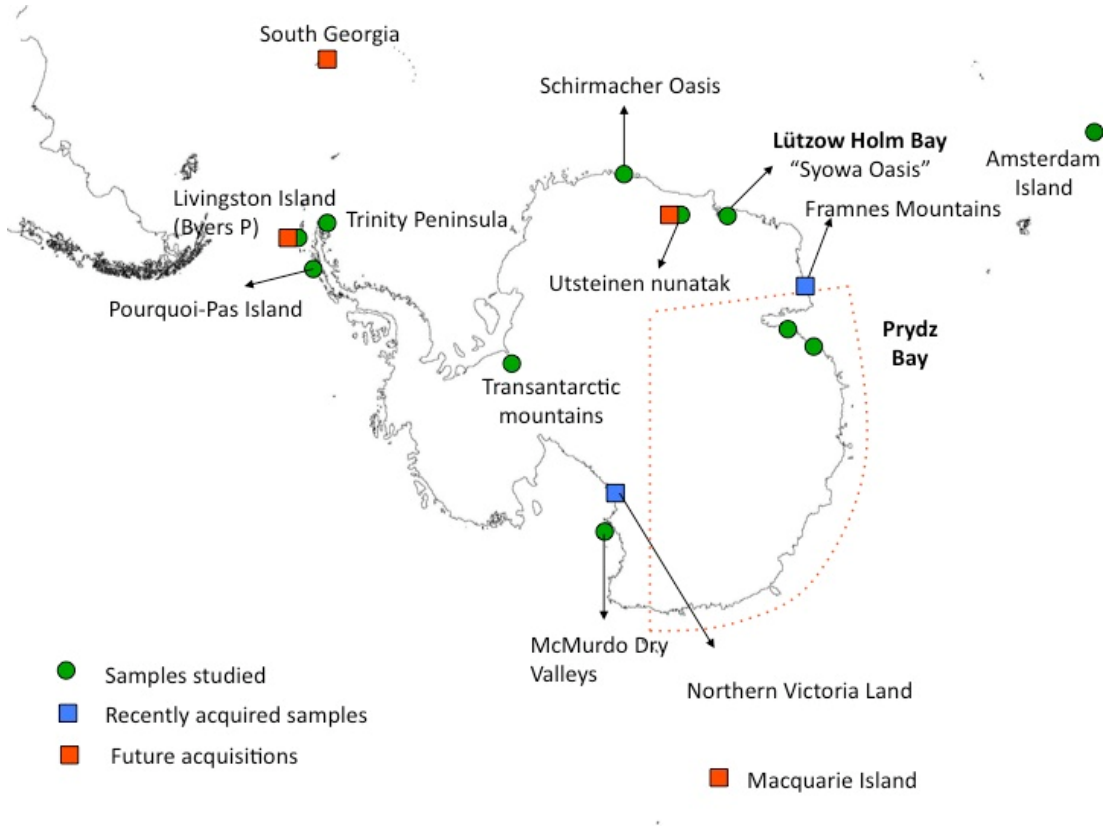

Fig.1: Map showing the different sampling locations: dots indicate samples from regions which were visited in the framework of previous and current projects of the AMBIO partners and are currently being studied. Squares are obtained through collaboration with international colleagues and during future field campaigns. The sector along the coastline indicated with a dashed line will be covered within a new research proposal ICEMATE (see text below).

i. 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), Livingstone Island were obtained during sampling campaigns/projects in which one of the project partners was involved.

Samples from the Transantarctic Mountains (Davis Valley and Shackleton range), Pourquoi-Pas Island, Alexander 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).

Framnes Mountains (Chapman ridge and Stillwell hills) samples were obtained through collaboration with John Gibson (University of Tasmania, Australia).

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

Samples from Amsterdam Island were collected by Bart Van De Vyver (University of Antwerp, Belgium) in December 2007. Only fresh samples for the isolation of microalgae were obtained, and the biotopes seemed too acid for the growth of microbial mats.

New samples for future analyses were obtained during the BELDIVA sampling campaign (Utsteinen). Others will be provided from the collaborations with A. Quesada (Byers Peninsula), D. Bergstrom (ICEMATE; Macquarie Island).

ii. MERLIN sampling campaign

Benthic microbial mats were sampled in four regions near Lützow-Holm Bay ("Syowa Oasis"): Skarvsnes, Langhovde, East Ongul and West Ongul Island, see also MERLIN field report). In addition, some samples were obtained from the Schirmacher oasis. We obtained surface sediment samples from both the deepest part of over 40 lakes (using a UWITEC glew corer) and the littoral zone from a wide range of water bodies from these regions.

iii. Sample storage

The majority of the samples consist of frozen 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 (for overview see **App. 3**). The biological samples taken during the MERLIN campaign were frozen and stored at -20°C; a subsample was kept cool in the dark and transferred to Belgium for the isolation of microalgae. A selection of the samples was preserved in ethanol (96%) in order to compare the effect of conservation method on the non-cultivated diversity techniques (WP4). As some of the available samples from other locations are only available as ethanol samples rather than as frozen samples, this enabled us to evaluate whether we could analyze samples conserved using different methods without having to infer differences due to the conservation method used.

iv. 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 MERLIN sampling campaign, environmental parameters (including temperature, conductivity, pH, salinity, oxygen concentration) were measured using a YSI water quality meter. Water samples for the analysis of nutrients and photosynthetic pigments were collected in acid-washed Nalgene bottles and frozen until analysis. These

data are incorporated in our database, which will allow us to assess the importance of ecological versus geographical variables in structuring the microbial communities.

v. 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 wasn't always possible for practical reasons.

The uncultivated 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 below). Samples for other analyses are generally a subset of the samples for this uncultivated diversity analysis. Samples from the BPB that became available during the project were also analyzed at the ULg (add. DGGE cyano in **Table 1**).

For bacteria cultures, a subset of 7 samples was selected based on the ordination analysis of both environmental data as well as DGGE data for bacteria.

For the cultures of cyanobacteria, the work has started on samples from West Ongul Island (Lützow Holm Bay, clone library and DGGE data were already available for WO4 sample). Further cultivation will target the same subset of 7 samples.

For the cultivation of microalgae we relied on the availability of fresh, cooled samples. All available samples were included.

Table 1: Overview of the samples studied showing the number of samples studied for the different tasks. +: future set: new samples will be analyzed or obtained during the second term of the project, ...: in progress, (): studied during earlier/other project(s), but will be included in AMBIO results. *: see Taton *et al.* 2003, 2006*a* and 2006*b*.

Region	Available samples	DGGE selection (all)	add. DGGE cyano	Clone libs. cyano	Bact isol.	Cyan. isol.	Microalg. isol.	Remarks
S. Georgia	5+	0		0	0	0	0	Currently no frozen samples, new samples expected
Livingstone Isl.	1+	0		(1)	0	0	0	Meltwater sample
Pourquoi-Pas Isl.	9	3		0	1	0	0	
Trinity pen.	65	0		0	0	0	9	Currently only fresh and ethanol samples available
Trans Ant. Mtns.	18	4		4	2	3	0	
Schirmacher Oasis	11	3		0	1	0	3	
Utsteinen	52+	(24)	5	0	(2)	0	0	Mostly terrestrial samples. Analysis of these samples will be done in the framework of ANTARIMPACT
Lützow Holm	92	36		1	3	...	24	
Framnes Mtns.	24	+?		0	0	0	0	
Prydz Bay	91	35		(1)*	(x)	(26)*	(2)	
Macquarie Isl.	+	+?		0	+	+	+	
McMurdo	2	2		(1)*		0	(1)	
N. Victoria land	30	+?		0	0	0	0	

vi. Selection of samples for uncultivated diversity analysis

The samples, which were analyzed during the first phase of the project, were selected using a stratified random sampling approach and Principal Component Analysis, which ensured we captured the main limnological gradients present in Antarctic water bodies. Based on the available supporting data, we made a selection of a subset of samples to be analysed during the first term of the project. 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", Enderby Land). Visual data exploration using the centered and standardized environmental data in a Principal Component Analysis (PCA; **Fig 2**) 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. Recently, we acquired additional nutrient data for a large number of samples obtained by BAS and the sampling campaign in the Lützow-Holm Bay region, which will allow us to further refine and check our sample selection. 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 will be 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.

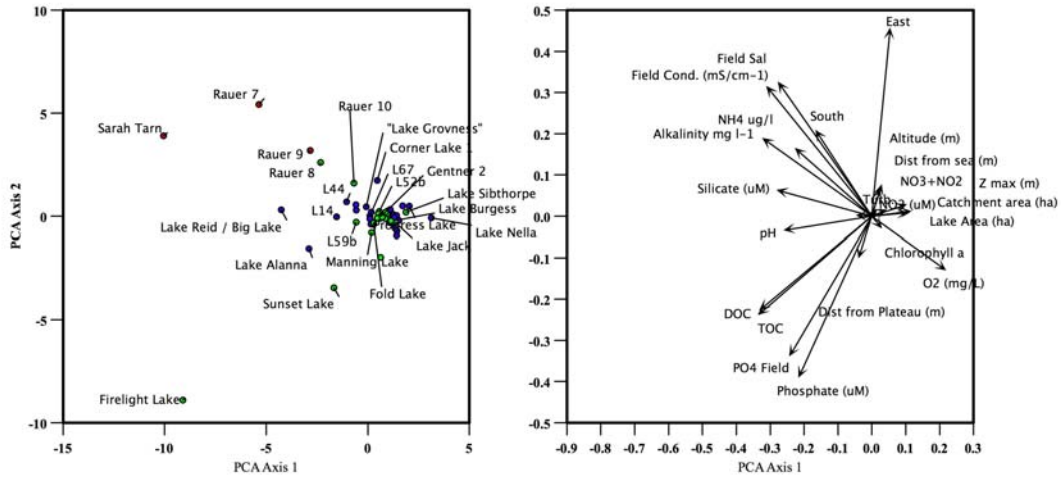

Fig. 2: PCA diagram of the environmental data for the Prydz bay area (right: sample data, right: explanatory variables). The samples that were selected for analysis are named in the left graph.

2. Cultivated diversity

i. Isolation of bacteria

One gram of a sample (or up to 3 g if more is 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 PYGV (Peptone-Yeast-Glucose-Vitamin)-medium, which is a rich medium. The use of both seawater and physiological water for the dilution series was tested.

The plates were incubated under different conditions. Three relatively low temperatures were used in view of the cold local conditions: 20°C, which would permit growth of psychrotolerant, but not psychrophilic micro-organisms and 15°C and 4°C, which would permit growth of psychrophilic micro-organisms. The plates were also incubated at different atmospheric conditions. Aerobic conditions were compared with anaerobic conditions where the oxygen level is reduced and the carbon dioxide level is elevated. 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.

Different isolates, selected based on the morphology of the colonies, were then purified. After pure cultures were obtained by a streak culture, a massive culture was made which was used for storage at -80°C and for preparation of DNA.

ii. Characterization of bacteria

We used repetitive extragenic palindromic (rep)-PCR fingerprinting as a dereplication technique to screen the large number of isolates. This allowed us to detect identical strains and group the isolates in a smaller number of rep-clusters of similar strains. Some of the isolates did not form part of a cluster and are called "separate isolates". We have used a genetic fingerprinting 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.

For a rapid preliminary identification of the rep-clusters, from every cluster, one to five isolates were selected for partial 16S rRNA gene sequencing. The resulting sequence (approximately 300 to 500 bp) was compared to the EMBL database to obtain a first approximate identification.

iii. Isolation of cyanobacteria

Isolation media spanning a range of salinity (with or without Nitrogen) were used according to Taton *et al* (2006b) for samples from the TM. The other isolations were performed on the BG11 medium containing cycloheximide (final concentration of 500g/mL) to inhibit eukaryotic contaminants and incubated at 18°C with constant light.

iv. 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).

v. Isolation, cultivation and characterization 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 characterisation. Diatom strains were characterised 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,. Recently, 12 new samples from Amsterdam Island were incubated and are being analyzed.

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 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, Swofford 1999).

3. Non-cultivated diversity

vi. Non-cultivated cyanobacterial diversity

The DNA was extracted as in Fernandez-Carazo *et al.* (in prep.) and purified using the Promega kit Wizard® DNA Clean-up System. Four samples (TM1, TM2, TM3 and TM4) were studied from TM, as well as eight from Utsteinen (Belgian Polar Base, 33, 34, 164, 183, 195 and 196), 12 samples from several lakes of East Antarctica and 18 samples from West Ongul Lakes (Table 1, App. 1).

Semi-nested PCRs and DGGE were performed (Taton *et al.* 2003 with slight modifications, Boutte *et al.* 2006). DGGE bands were excised reamplified and sequenced. The DGGE was performed on the TM, BPB samples, and eighteen others from West Ongul and Skarvsnes so far.

For the four samples of the TM and sample WO4 from West Ongul, clone libraries were constructed as described by Taton *et al.* (2006a).

vii. 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 *et al.* 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.

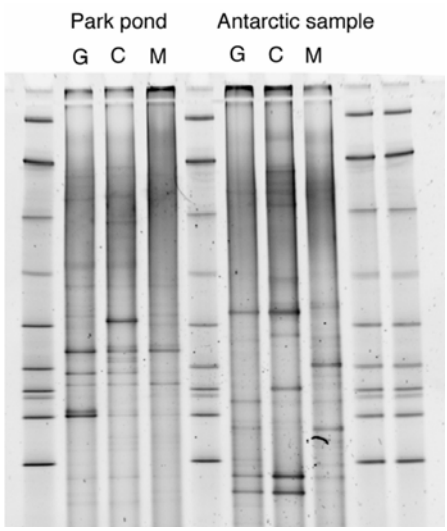

Fig. 3 (right): G: Corinaldesi method (2005) coupled to the Vetriani method (1999) with a few modifications as suggested by D. Gillan (ULB), C: combined method using extracellular DNA removal and bead-beating, M: Muyzer bead-beating method (1998).

For the analysis of non-cultivated bacteria, we used the protocol developed during previous projects (Van der Gucht *et al.* 2001).

For the microalgae, we focussed on green algae which are largely understudied in Antarctica. Initially we 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.

Clone library analysis

Currently, no clone library analysis has been performed for bacteria. This will be done during the second phase. The results obtained from these analyses will be compared to the cultivated bacterial diversity.

III. Results

1. Bacterial diversity

i. Isolation of bacteria

Bacteria were isolated from samples kept frozen since the time of collection. For the isolation, we processed one sample at a time because the large number of plates involved occupies all incubator space available. The samples used include 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; two samples from the Trans-Antarctic Mountains: **TM2** from a cyanobacterial mat at the bottom of Forlidas Pond littoral (Pensacola Mountains) and **TM4** from Lundström Lake littoral (Shackleton Mountains); a littoral sample **PQ1** from Pourquoi-Pas Lake (Pourquoi-Pas Island) and samples **LA3** (Langhovde Peninsula), **SK5** (Skarvsness Peninsula) and **WO10** (West Ongul Island) were taken from three lakes at Lützow-Holm Bay, Syowa and sample **SO6** was from the Schirmacher Oasis.

Isolation of strains could start after about 2 weeks (15°C and 20°C) or 3 weeks (4°C) incubation, for aerobic growth conditions. Anaerobic growth was slower and generally yield was extremely low or zero. We presume that because of the aerobic way in which samples were originally collected from the environment, sensitive anaerobes may not be present. 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 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 over 4700 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 Table 3.

Both samples from the Princess Elisabeth Station site gave the highest absolute number of heterotrophic colonies. Best growth was generally obtained on the oligotrophic media. Incubation at 4°C yielded less colony-forming units than incubation at 15°C or 20°C.

ii. Characterization of bacterial strains

DNA from all isolates was subjected to a rep-PCR followed by electrophoresis to obtain a genomic fingerprint for each strain. From samples PQ1, LK3, SK5, WO10 and SO4 some isolates still remain to be included in rep-PCR. Cluster analysis of fingerprint patterns using Bionumerics software so far revealed 719 clusters (cut-off level 80%) of similar

strains and a number of separate isolates. An overview of the results is given in **Table 3**. Comparison of samples revealed there were no universally present rep-clusters. Few rep-clusters were found in two (31/719) or three (3/719) different samples, illustrating the large diversity observed.

Table 3. Results of the bacterial isolations and rep-clustering.

	BB50	**BB115**	TM2	**TM4**	**PQ1**	LA3	**SK5**	**WO 10**	**SO4**
Total # of isolates	527	353	452	442	490	351	648	741	707
# of rep-clusters	103	57	89	61	101	51	93	83	81
# of separate isolates	76	51	75	47	155	74	89	89	114
Isolates yet to be included in rep-PCR		14	48	25	27	81	76	293	330

Partial 16S rDNA sequencing was performed on 1 to 5 representatives per rep-cluster and on the separate strains. In total 905 strains were sequenced so far and compared to the EMBL database for preliminary identification. The work for samples WO10 and SO4 is still ongoing. Sequenced strains were stored in Microbank tubes at -80°C. An overview of the preliminary identifications per sample is given in **Fig. 4**. It is summarized as the distribution of rep-clusters and separate isolates over different bacterial classes recovered. More than 97% sequence similarity indicates probable species identification. Values of 95% to 97% indicate fairly reliable genus identification, but species assignment within the genus is not possible (because no similar sequence is present in the database), such groups may represent new species. Less than 95% means no genus identification was possible because no similar sequence is present in the database. Such groups may represent new genera.

The results of the partial 16S rRNA gene sequencing show a large diversity, distributed over the major phylogenetic groups (*Proteobacteria*, *Bacteroidetes*, *Deinococci*, *Actinobacteria* and *Firmicutes*). For both **BB** samples, most isolates belong to the class of the *Actinobacteria* and second most abundant are *Bacteroidetes* and *Deinococci.* For the for **TM 4** sample *Actinobacteria* and *Betaproteobacteria* were the most abundant. For sample **TM 2**, most isolates belong to the *Firmicutes* followed by the *Alphaproteobacteria*. Sample **PQ 1** was very diverse with *Actinobactera, Bacteroidetes, Alpha-*, *Beta-* and *Gammaproteobacteria* as well as *Deinococci* being recovered in important amounts. In sample **LA3** the class of *Bacteroidetes* was best represented, but *Alpha-* and *Gammaproteobacteria* were also recovered in important amounts. For sample **SK5**, most isolates belong to the *Alphaproteobacteria* followed by *Betaproteobacteria* and *Actinobacteria.*

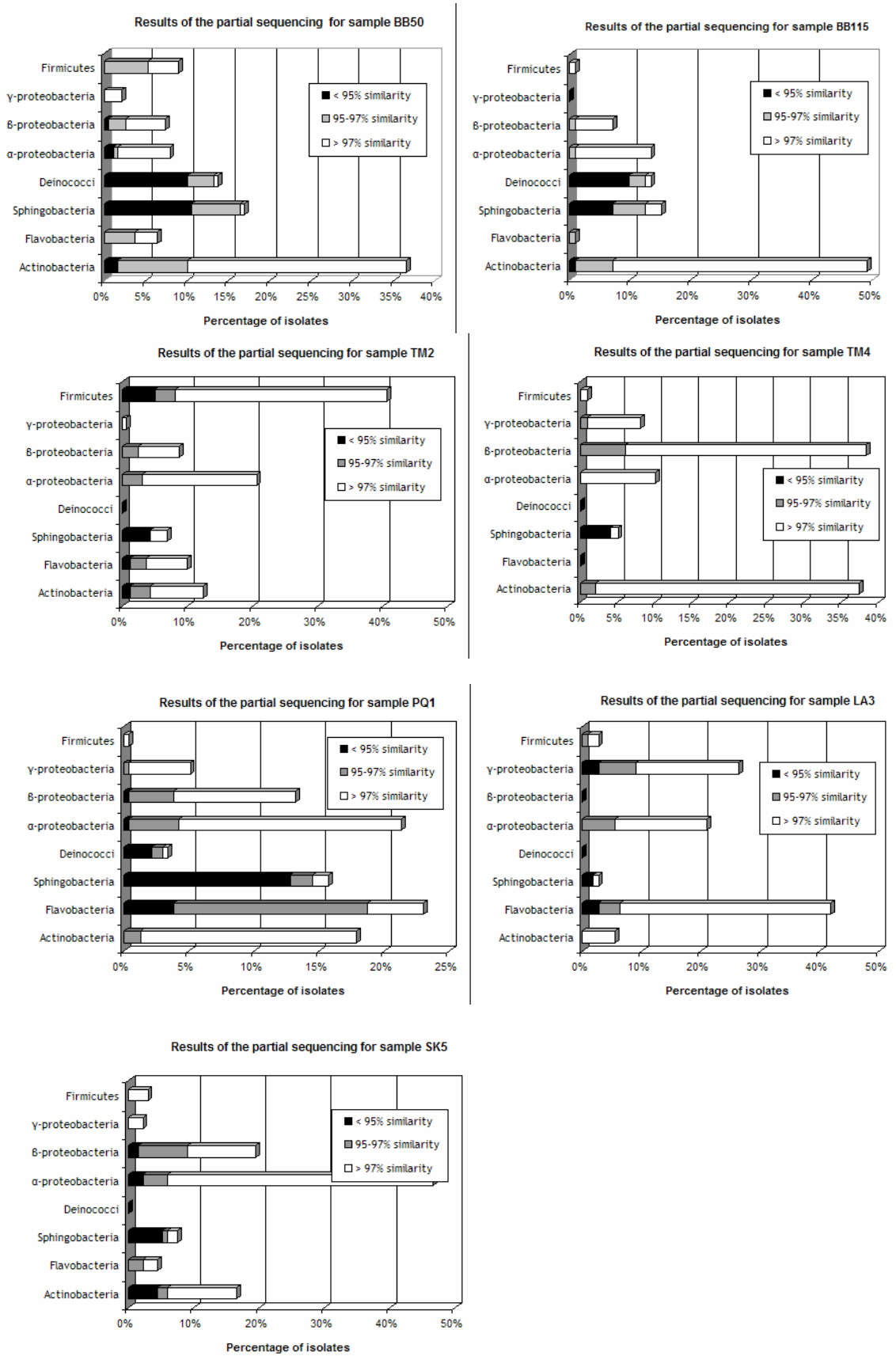

Fig. 4. Bar dendrograms showing the group identifications. The first seven samples are shown. Representatives of the Bacteroidetes are divided in the classes Flavobacteria and Sphingobacteria.

iii. Non-cultivated bacterial diversity

DGGE analysis revealed 63 bandclasses (BCs). In total 146 strong bands were sequenced, these belonged to 45 OTU's. 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. 5**), 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.

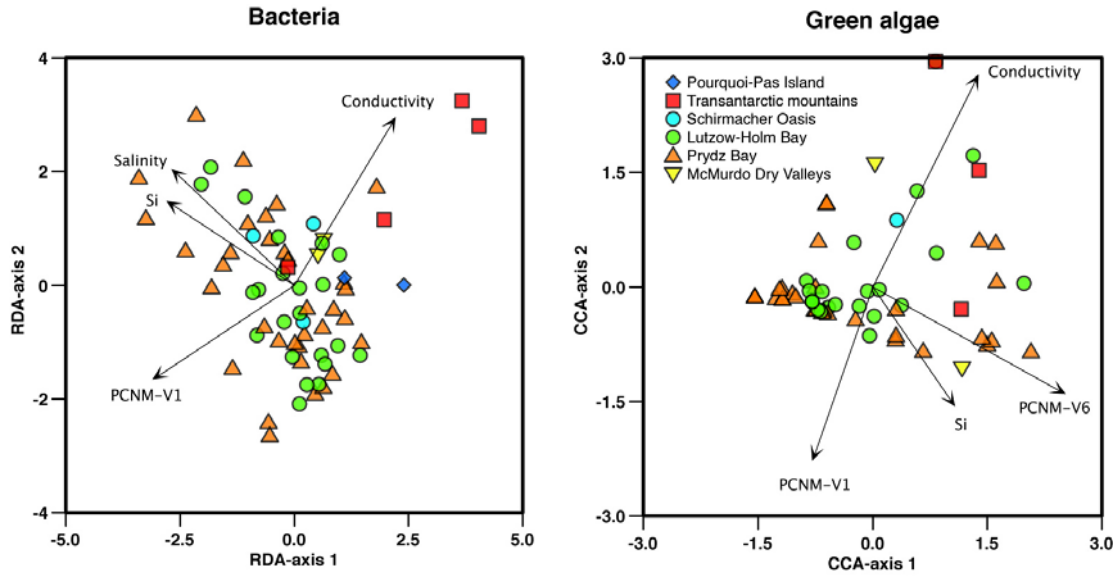

Fig. 5. Results of direct ordination analyses showing species scores and environmental scores for the bacterial and green algal DGGE data. Different regions are indicated as shown in the legend of the second panel.

2. Cyanobacterial diversity

i. Isolation of cyanobacteria

Four strains from 3 TM samples (TM1, TM2 and TM3) have been isolated and conserved as pure cultures. Their 16S rDNA gene as well as the ITS were sequenced. The isolation of 14 samples has started (Transantarctic Mountains (TM), West Ongul and South Antarctica). The isolation from other Antarctic samples (WO, SK, SO, LA, PQ) are still in progress.

ii. Phenotypic and genotypic characterization of the cyanobacterial strains

The microscopical observation showed that the four strains belong to the Oscillatoriales: *Leptolyngbya antarctica, Phormidium sp., Leptolyngbya cf pauciramosa* and *Phormidium pristleyi.* The 16S rRNA gene of all strains was sequenced, aligned with DGGE and clone sequences and a distance tree was constructed (**Fig. 1, App. 1**). The isolation allowed finding a new OTU, not found by other techniques, but probably quite rare in the samples.

iii. Non-cultivated cyanobacterial diversity

DNA extraction

The DNA of 5 samples of the BELARE expedition (Belgian Polar Base), 4 of the TM, 14 from Stillwell Hills and Chapman Ridge, and 23 from the MERLIN expedition (West Ongul and Skarvsnes lakes) has been extracted. 16S-ITS PCRs showed a band of adequate length (ca 1800 pb) in 36 cases (4 for BELARE samples, 4 for TM, 10 for SH and 18 for WO samples).

DGGE

All samples were screened by DGGEa and b (**App. 2**), except 12 samples for which only DGGEb was performed.

The number of obtained (intense) bands varied but was low: 1 to 2 bands maximum per sample for WO samples, 1 to 3 for BB samples, 1 to 4 for samples of SH and TM.

The bands were excised, reamplified and sequenced. The sequences were then cured and aligned using ARB and a distance tree was constructed (Van de Peer, 1997). Sequences were compared with the closest public sequences (Genbank) and then grouped into OTUs (Table 3, App. 1) and a distance tree was constructed (**Fig. 1, App. 1**). OTUs are groups of sequences sharing more than 97.5% of 16S rRNA gene sequence similarity. A global analysis is given below.

Clone libraries, results for the 4 TM samples:

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 were obtained for 10 OTUs. Interestingly, 3 OTUs are found in Forlidas pond and the terrestrial mats in the vicinity. This supports the mobility between aquatic and terrestrial environments that was already detected by Gordon et al. (2000).

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 (16 and 19) were found, in agreement with the DGGE pattern showing two main bands.

iv. Global results of isolation, DGGE and cloning for cyanobacteria

Combining the results of 16S rDNA sequences from 4 pure strains, DGGE analysis and clone libraries, we delineated a total of 23 OTUs (**App.2** and **Fig. 1, App.1**). The OTUs are distributed as follows: 15 cosmopolitan, grouping diverse sequences from different regions of the world (**App.2**), 5 were found so far only in Antarctica and 3 constitute potential new diversity. The TM samples were distributed in 10 OTUs: 8 cosmopolitan, one Antarctic and one new. The new OTU80 comprised 16S sequences of 2 strains obtained by isolation. The polar Belgian base sequences were distributed in 7 OTUs encompassing also sequences from the Transantarctic Mountains. They encompassed 3 cosmopolitan, 2 new and 2 Antarctic OTUs. The West Ongul samples were distributed in 8 OTUs, 6 of which were cosmopolitan and 2 Antarctic. Sequences from Stillwell Hills were distributed in 3 cosmopolitan OTUs and one Antarctic.

Preliminary ordination analyses were performed by P2 to check whether regional patterns in the bacterial 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 obtained in MICROMAT, LAQUAN and the start of AMBIO.

The DCA revealed that the OTU composition is geographically structured as each region has a more or less unique flora. One sample from the Transantarctic Mountains (TMLU4re) 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 (TMFO1, RIR8, RIR2 and VHAL) (**Fig. 6**).

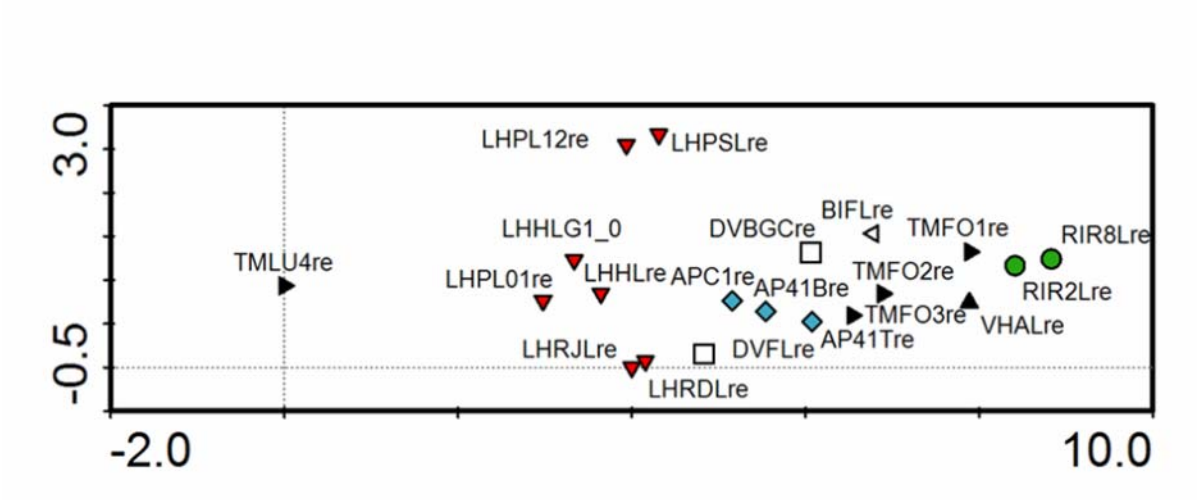

Fig. 6. Detrended Correspondence Analysis for samples of TM, EA, DV and Antarctic Peninsula showing that each region is more or less unique in terms of OTUs composition. Samples close to each other are similar, each region has a different symbol. LH: Larsemann Hills, BI: Bolingen Islands, VH: Vestfold Hills, RI; Rauer Islands, DV: Dry Valleys, AP: Antarctic Peninsula and TM: Transantarctic Mountains

3. Microalgal diversity

i. Isolation of microalgae, with focus on green algae and diatoms

In total 56 diatom strains and 139 green algal strains were successfully isolated. Microscopical screening of diatom cultures revealed eight different morphological species: *Navicula* cf. *gregaria*, *Craticula* sp, *Stauroneis* sp., *Nitzschia* sp., *Nitzschia* cf. *homburgiensis*, *Nitzschia perminuta*, *Hantzschia* sp. and *Pinnularia microstauron*. Twelve Antarctic *Pinnularia* strains were selected for a molecular genetic study using the chloroplast gene (*rbcL)* and the LSU rDNA gene. These strains were incorporated in a phylogenetic tree including members of this genus from 6 globally distributed regions (Souffreau *et al.* in prep.) and identified as *Pinnularia microstauron*. The ongoing analysis of strains from other regions will provide insights in the distribution of this genus.

The green algal strains belong to 14 different clades and point to a high diversity of this group. 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; In addition to the taxa showing high similarity to one of our isolates, there's the *'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).

ii. 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. Direct ordination (Fig. 5) 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 Antarctic green algae.

iii. Diatom counts.

Diatom analyses are nearing completion and will be analyzed in the same way as we did for the uncultivated diversity of bacteria and green algae.

IV. Conclusions and discussion

1. General Conclusions on the diversity of cyanobacteria

1) Isolation of cyanobacteria requires more time than for bacterial isolates. The isolations of strains from the TM allowed the findings of a new OTU not observed by cloning or DGGE techniques. The isolations are still in progress to obtain pure isolates.

2) Three OTUs included sequences of three regions (BB, TM, EA). This suggests a flux of microorganisms between the respective regions.

3) The sample WO4 littoral was submitted to cloning and DGGE. The cloning showed 2 OTUs, which correspond to the preliminary DGGE results (2 dominant bands, data not shown). In general, samples from the West Ongul region did not seem to harbor a large diversity (1 to 2 DGGE bands per sample).

5) The sequence BB164_5b represents a potential undiscovered diversity that was found by DGGE analysis. The corresponding sample should be cloned for further investigation of the new diversity.

6) The two lakes of the Transantarctic Mountains share only one OTU (OTU44) that is cosmopolitan and was found also outside Antarctica. Though the lakes share a similar

size and location, 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.

7) Our data show an impoverished diversity in TM, WO and BB samples, compared to the numbers of OTUs found in other regions of Antarctica. Indeed, an average of 8.5 OTUs per lake 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).

8) The OTUs 44 and 63 that include a quite large number of sequences from different regions are cosmopolitan and may have an increased tolerance to salinity because they include sequences of cyanobacteria from saline, hypersaline and desert areas of the world.

2. Diversity of microalgal isolates

The high diversity of the green algal isolates suggests that this group successfully colonized the Antarctic continent. Two taxa (II11, VI11), belonging to the genera *Chlorella* and *Scenedesmus*, were detected in most regions, suggesting that they are widely dispersed over Antarctica. More detailed data, e.g. ITS sequence data, is however required to elucidate whether these two taxa consist of different species or are the same two species that are easily dispersed around the Antarctic continent. Apart from these two taxa, most (10 out of 14) are only retrieved from one ice-free region, suggesting that a lot more taxa would be discovered if more samples and regions were studied.

Moreover, all Antarctic sequences differed by at least 3 basepairs from sequenced representatives from other regions and had widely differing branch lengths pointing to a unique Antarctic flora. These results clearly contrast with dominance of cosmopolitan species, which is suggested based on morphological work on terrestrial algae (Broady 1996).

While our phylogenetic tree is not calibrated due to the paucity of fossils, green algae are estimated to have originated roughly between 500 to 1500 million years ago (Hedges *et al.* 2004, Yoon *et al.* 2004, Berney & Pawlowski 2006, Cavalier-Smith 2006, Roger & Hug 2006). Using a range of 700 to 1200 million years, which is what most authors agree upon, we speculate that the Antarctic taxa have been isolated between 5 and 566 million years ago.

3. Diversity of bacterial isolates

Yield on different media: After plating the dilution series, there are between 351 and 741 isolates taken from the different incubation conditions. From our isolations on the different types of media, we observe that samples TM2 and LA3 seems to host more **halophilic organisms**. This is in line with expectations as from the sample description these samples were known to be more saline. Sample WO10, from the coastal West Ongul region, shows the highest growth on the marine media too.

Samples BB50, TM4 and PQ1 contain mainly **oligotrophic** organisms as the highest growth was observed on the oligotrophic media R2A and R2A/10. On the other hand, samples BB115 and SK5 contain mainly organisms with **specific nutrient requirements** as they show the most growth on the PYGV medium, a medium with added vitamins and sugars.

When considering total numbers of bacteria isolated, the gravel samples (BB50 and BB115) showed a much higher growth than the littoral samples, which can be explained by the fact that the littoral samples are more diluted. However, littoral sample WO10, also showed very high CFU/g, even higher than sample BB115.

Grouping by rep-PCR clustering: Among the more than 700 rep-clusters detected so far, we found very few that contained isolates from the same samples (4.3% contained isolates from two different samples; 0.4% contained isolates from three 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 needs to be established by, for example, 16S rDNA sequencing.

In most samples around 15% of isolates did not cluster with others and had a unique rep profile. However in PQ1 and LA3, 32% and 21% of isolates, respectively, had a unique rep-profile. These samples seem particularly diverse. It should be noted that from samples WO10 and SO4 not all isolations have been finished and data are still coming in.

Preliminary identification by partial 16S rDNA sequencing: Our preliminary data illustrate the diversity of the samples, with *Proteobacteria*, *Bacteroidetes*, *Deinococci*, *Actinobacteria* and *Firmicutes* being recovered from all samples, but in different relative amounts. Considering the two saline samples (TM2 and LA3), we did not observe particular classes significantly more present in these saline samples than in the other samples.

In several of the groups, part of the isolates showed low similarity values with neighbouring sequences in the EMBL-database were observed. This points to the presence of important numbers of potentially new taxa in all of the phyla that we recovered and particularly in the *Bacteroidetes*, one of the groups we plan to focus on in

phase 2. Also important was the finding of potentially new representatives of the phylum *Deinococci*, a remarkable group of particularly extremophilic and mostly radiation-resistant bacteria with relatively few cultivated representatives. Our collection of isolates thus represents a valuable resource for future research of potentially new substances and functionalities.

4. Non-cultivated diversity

As the full analysis of the DGGE results is not finished yet, the following conclusions should be regarded as preliminary. When completed, we expect that our results will enable us to assess (i) the importance of geographical and local environmental variables, and (ii) life history characteristics (e.g. sexual vs. asexual life cycle, the formation of resting stages) in shaping the microbial communities in Antarctic lakes. For the bacteria, the DGGE results seem to point to a minor importance of geographical variables although the DGGE resolution may be too limited in order to reveal patterns in the bacterial community composition. For cyanobacteria, different distribution patterns are observed, but more data is needed to refine the analysis. The first DGGE results for the green algae suggest that regional factors may be more important than for bacteria. Further analysis of these data is needed to integrate the results for the different groups studied.

References

Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic Identification and In Situ Detection of Individual Microbial Cells without Cultivation. Microbiol. Rev. 59:143-169

Berney C, Pawlowski J (2006) A molecular time-scale for eukaryote evolution recalibrated with the continuous microfossil record. Proceedings of the Royal Society B-Biological Sciences 273:1867-1872

Boutte C, Grubisic S, Balthasart P, Wilmotte A (2006) Testing of primers for the study of cyanobacterial molecular diversity by DGGE. Journal of Microbiological Methods 65 : 542-550

Broady PA (1996) Diversity, distribution and dispersal of Antarctic terrestrial algae. Biodiversity and Conservation 5:1307-1335

Cavalier-Smith T (2006) Cell evolution and Earth history: stasis and revolution. Philosophical Transactions of the Royal Society B-Biological Sciences 361:969-1006

Corinaldesi C, Danovaro R, Dell'Anno A (2005) Simultaneous recovery of extracellular and intracellular DNA suitable for molecular studies from marine sediments. Appl. Environ. Microbiol. 71:46-50

Cowan D A, Tow L A (2004) Endangered Antarctic Environments. Ann. Rev. Microbiol. 58:649-90

Diez B, Pedros-Alio C, Marsh TL, Massana R (2001) Application of Denaturing Gradient Gel Electrophoresis (DGGE) to Study the Diversity of Marine Picoeucaryotic Assemblages and Comparison of DGGE with Other Molecular Techniques. Appl. Environ. Microbiol. 67:2942-2951

Ellis-Evans JC (1996) Microbial Diversity and Function in Antarctic Freshwater Ecosystems. Biodivers. Conserv. 5:1395-1431

Gibson JAE, Wilmotte A, Taton A, Van De Vijver B, Beyens L, Dartnall HJG (2006) Biogeographic trends in Antarctic lake communities. In 'Trends in Antarctic Terrestrial and Limnetic Ecosystems. Bergstrom DM, Convey P, Huiskes AHL (eds.) Springer. p 71-99.

Gordon DA, Priscu J. Giovannoni S (2000) Origin and Phylogeny of Microbes Living in Permanent Antarctic Lake Ice. Microb. Ecol. 39:197-202

Hanagata N (1998) Phylogeny of the subfamily Scotiellocystoideae (Chlorophyceae, Chlorophyta) and related taxa inferred from 18S ribosomal RNA gene sequence data. J. Phycol. 34:1049-1054

Hedges SB, Blair JE, Venturi ML, Shoe JL (2004) A molecular timescale of eukaryote evolution and the rise of complex multicellular life. Bmc Evol Biol 4

Hodgson DA, Verleyen E, Sabbe K, Squier AH, Keely BJ, Leng MJ, Saunders KM, Vyverman W (2005) Late Quaternary climate-driven environmental change in the Larsemann Hills, East Antarctica, multi-proxy evidence from a lake sediment core. Quaternary Research 64**:** 83-99

Hodgson DA, Vyverman W, Verleyen E, Leavitt PR, Sabbe K, Squier AH, Keely BJ (2005) Late Pleistocene record of elevated UV radiation in an Antarctic lake. Earth and Planetary Science Letters 236**:** 765-772

Hughes KA, McCartney HA, Lachlan-Cope TA, Pearce DA (2004) A Preliminary Study of Airborne Microbial Biodiversity over Peninsular Antarctica. Cell. Mol. Biol. 50:537-542

Komárek J, Anagnostidis K (2005) Cyanoprokaryota 2. Teil Oscillatoriales. Elsevier GmbH, Spektrum Akademischer Verlag, Heidelberg, pp. 1-759.

Lawley B, Ripley S, Bridge P, Convey P (2004) Molecular analysis of geographic patterns of eukaryotic diversity in Antarctic soils. Appl. Env. Microbiol. 70**:**5963-5972

Lesser MP, Barry TM, Banaszak AT (2002) Effects of UV radiation on a chlorophyte alga (Scenedesmus sp.) isolated from the fumarole fields of Mt. Erebus, Antarctica. J. Phycol. 38:473-481

Loisel P, Harmand J, Zemb O, Latrille E, Lobry C, Delgenes JP, Godon JJ (2006) Denaturing gradient electrophoresis (DGE) and single-strand conformation polymorphism (SSCP) molecular fingerprintings revisited by simulation and used as a tool to measure microbial diversity. Environ. Microbiol. 8:720-731

Morgan-Kiss RM, Ivanov AG, Modla S, Czymmek K, Huner NPA, Priscu JC, Lisle JT, Hanson TE (2008) Identity and physiology of a new psychrophilic eukaryotic green alga, Chlorella sp., strain BI, isolated from a transitory pond near Bratina Island, Antarctica. Extremophiles 12:701-711

Muyzer G, Brinkhoff T, Nübel U, Santegoeds C, Schäfer H, Wawer C (1998) Denaturing Gradient Gel Electrophoresis (DGGE) in Microbial Ecology. Molecular Microbial Ecology Manual 3.4.4:1-27

Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. Bioinformatics 14:817-818

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

Robinson SA, Wasley J, Tobin AK (2003) Living on the edge - plants and global change in continental and maritime Antarctica. Global Change Biology 9**:**1681-1717

Roger AJ, Hug LA (2006) The origin and diversification of eukaryotes: problems with molecular phylogenetics and molecular clock estimation. Philosophical Transactions of the Royal Society B-Biological Sciences 361:1039-1054

Sabbe K, Verleyen E, Hodgson DA, Vanhoutte K, Vyverman W (2003) Benthic diatom flora of freshwater and saline lakes in the Larsemann Hills and Rauer Islands, East Antarctica. Antarctic Science 15**:**227-248

Speksnijder AGCL, Kowalchuk GA, De Jong S, Kline E, Stephen JR, Laanbroek HJ (2001) Microvariation Artefacts Introduced by PCR Cloning of Closely Related 16S RNA Gene Sequences. Appl. Environ. Microbiol. 67:469-472

Swofford DL (1999) PAUP* Phylogenetic analysis using parsimony (* and other methods), version 4. Sinauer Associates, Sunderland

Taton A (2006c) Diversité des cyanobactéries dans les tapis microbiens des lacs antarctiques. PhD thesis, Faculty of Sciences, University of Liège, Belgium. 354 pages

Taton A, Grubisic S, Balthasart P, Hodgson D A, Laybourn-Parry J, Wilmotte A (2006a) Biological Distribution and Ecological Ranges of Benthic Cyanobacteria in East Antarctic Lakes. FEMS Microbiol. Ecol. 57:272-289

Taton A, Grubisic S, Brambilla E, De Wit R, Wilmotte A (2003) Cyanobacterial Diversity in Natural and artificial Microbial Mats of Lake Fryxell (Mc Murdo Dry Valleys, Antarctica): a Morphological and Molecular Approach. Appl. Environ. Microbiol. 69:5157-5169

Taton A, Grubisic S, Ertz D, Hodgson D A, Piccardi R, Biondi N, Tredici M R, Mainini M, Losi D, Marinelli F, Wilmotte A (2006b) Polyphasic Study of Antarctic Cyanobacterial Strains. J. Phycol 42:1257-1270

Van de Peer Y, De Watcher R (1997) Construction of Evolutionary Distance Trees with TREECON for Windows: Accounting for Variation in Nucleotide Substitution Rate among Sites. Comput. Appl. Biosc. 13:227-30

Van der Gucht K, Sabbe K, De Meester L, Vloemans N, Zwart G, Gillis M, Vyverman W (2001) Contrasting Bacterioplankton Community Composition and Seasonal Dynamics in Two Neighbouring Hypertrophic Freshwater Lakes. Environ. Microbiol. 3:680-690

Van Trappen S, Mergaert J, Van Eygen S, Dawyndt P, Cnockaert MC, Swings J (2002) Diversity of 746 Heterotrophic Bacteria Isolated from Microbial Mats in Antarctic Lakes. Syst. Appl. Microbiol. 25:603-10

Verleyen E, Hodgson DA, Sabbe K, Vanhoutte K, Vyverman W (2004) Coastal oceanographic conditions in the Prydz Bay region (East Antarctica) during the Holocene recorded in an isolation basin. Holocene 14**:**246-257

Verleyen E, Hodgson DA, Sabbe K, Vyverman W (2004) Late Quaternary deglaciation and climate history of the Larsemann Hills (East Antarctica). Journal of Quaternary Science 19**:** 361-375

Vetriani C, Tran HV, Kerkhof LJ (2003) Fingerprinting microbial assemblages from the oxic/anoxic chemocline of the Black Sea. Appl. Environ. Microbiol. 69:6481-6488

Vincent W F (1988) *Microbial Ecosystems of Antarctica*. Cambridge UK: Cambridge Univ. Press. 304pp.

Vyverman W, Hodgson DA, Hoshino T, Imura S, Kudoh S, Kanda H, Verleyen E (2007) MERLIN, A joint research expedition between the Japanese REGAL project, the BAS CACHE-PEP program and the Belgian HOLANT and AMBIO projects. Field report 2007.

Yoon HS, Hackett JD, Ciniglia C, Pinto G, Bhattacharya D (2004) A molecular timeline for the origin of photosynthetic eukaryotes. Molecular Biology and Evolution 21:809-818

Zhu F, Massana R, Not F, Marie D, Vaulot D (2005) Mapping of picoeucaryotes in marine ecosystems with quantitative PCR of the 18S rRNA gene. Fems Microbiol. Ecol. 52:79-92