



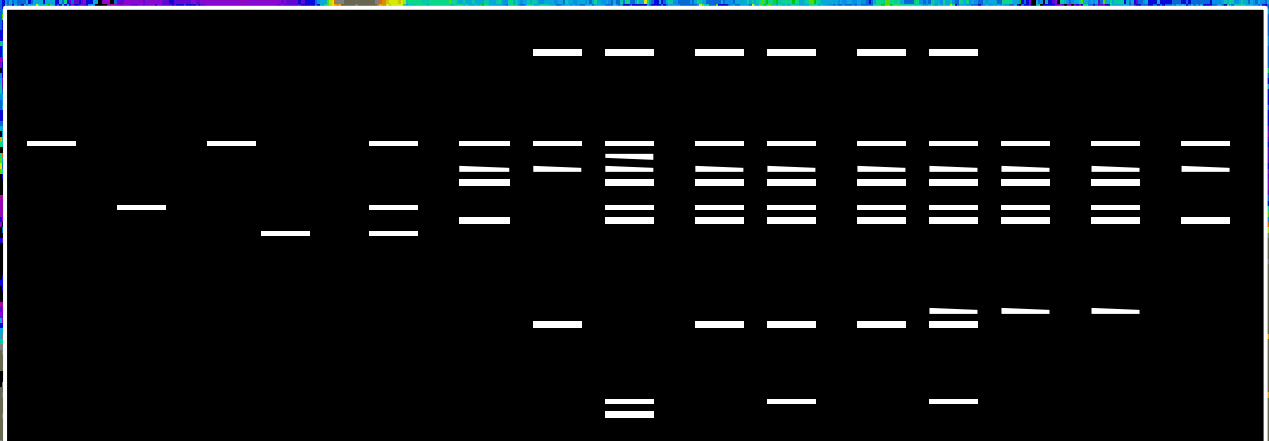
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EU WORKSHOP on

MICROBIAL DIVERSITY
IN AQUATIC SYSTEMS:
ENVIRONMENTAL GENE CLONING
AND ANALYSIS

ROSCOFF, FRANCE
JUNE 25th-27th 2000



Co-ordinator

**Dr Dave Scanlan
Dept. of Biological Sciences,
University of Warwick, UK**

Local Organiser

**Dr Daniel Vaultot,
Station Biologique,
Roscoff, France**

EU WORKSHOP SCHEDULE JUNE 25-27 2000

Sunday 25 June

09.00-09.10 **D. Scanlan** Opening Remarks

Session 1 Techniques for assessing diversity and community structure of microbial populations Chair Terry Marsh

09.10-10.00 **G. Muyzer** Use of DGGE in analysing microbial community structure

10.00-10.50 **M. Ferris** Insights into microbial community structure revealed by molecular analyses of population distributions along environmental gradients using cloning and DGGE

Coffee Break 1050-1120

11.20-12.10 **C. Tebbe** Use of PCR-SSCP (single strand conformation polymorphism) in Microbial Ecology

12.10-12.35 **B. Engelen** Fingerprinting bacterioplankton of the central Baltic by 16S rDNA community analysis

12.35-13.00 **P. Lebaron P. and C. Courties** Cell sorting and molecular biology for diversity assessment

Lunch 13.00-14.30

Chair Gerard Muyzer

14.30-15.20 **T.L. Marsh, F. Crocker, M.S. Riley, and B. Wade** Practical Aspects and Pitfalls of Microbial Community Analysis with T-RFLP

15.20-15.45 **S. Sjoling and D. Cowan** Diversity of prokaryotes and direct cloning of novel enzyme genes from Antarctic marine environment.

15.45-16.15 Tea Break

16.15-16.45 **C. Tebbe** 'Dry' demonstration of the SSCP technique

16.45 – Demonstration/ use of DGGE & HA Yellow/Red gels

Evening: Demonstration of the ARB database system

Monday June 26th

0900- 0935 **Visualisation of DGGE gel – DGGE results/comments/discussion**

Session 2 Diversity of Prokaryotic Picoplankton in Aquatic Environments: Chair Dave Scanlan

09.35 – 10.25 **B. Palenik**: Diversity of Marine Cyanobacteria

10.25-10.50 **F. Partensky , L. Garczarek and W.R. Hess** Molecular bases of ecotypic diversity in *Prochlorococcus*

10. 50-11.20 Coffee Break

11.20-11.45 **L. Charpy** Prokaryotic picophytoplankton in tropical coastal lagoons

11.45-12.10 **P. Rajaniemi, A. Rantala, K. Haukka, K. Sivonen** Diversity of cyanobacteria in the Finnish lake environment

12.10-12.35 **S. Benlloch, J. Garcia-Martinez, A. Lopez-Lopez, P. Lopez-Garcia and F. Rodriguez- Valera** Prokaryotic biodiversity in aquatic marine environments.

12.35-1300 **O. Narcessian** Assessment of microbial diversity in a deep hot oil reservoir from Western Siberia by molecular methods

Lunch 13.00- 14.30

Session 3 Molecular markers for identifying and exploring biodiversity amongst eukaryotic picophytoplankton Chair Brian Palenik

14.30-14.55 **L. Guillou, M. A. Cambon, & G. Barbier.** Genetic diversity of the French *Dinophysis*, *Alexandrium* and *Gymnodinium* genera based upon direct cells amplification on fixed cultures or natural samples.

14.55- 15.20 **B. Edvardsen, K. Shalchian-Tabrizi, S. Brubak, E. Dahl, K.S. Jakobsen, L.K. Medlin & E. Paasche** Genetic analyses of *Dinophysis* species isolated from Norwegian waters

15.20-15.45 **T. Castberg** Multi-method approach to study marine microbial ecology. Examples from a mesocosm experiment with an *Emiliana huxleyii* bloom terminated by virus.

15.45-16.15 Tea break

1615- Demonstration of FISH (introduction to confocal microscope etc)

1630- 1700 **N West** Use of tyramide signal amplification in fluorescent in situ hybridisation technology to detect marine cyanobacteria

1730 –1900 **N. Simon/F. Not** Use of FISH to detect eukaryotic picoplankton
I. Biegala Use of FISH in detecting bacteria associated with dinoflagellates

20.00 Workshop Banquet

Tues 27th June

Session 3 cont. Molecular markers for identifying and exploring biodiversity amongst eukaryotic picophytoplankton Chair Linda Medlin

09.00-09.25 **D. Vaultot & S.Y. Moon** Diversity of eukaryotic picoplankton in the Pacific

09.25-09.50 **W.H.C.F. Kooistra, and R.P.T. Koeman** Detection of Raphidophyceae, potentially harmful microalgae

09.50-10.15 **A.C. Barbrook and C.J. Howe** Minicircular plastid DNA markers in dinoflagellates.

10.15-10.45

10.45-11.15 Coffee Break

11.15-11.30 **A Wilmotte** MICROMAT: Workshop on DNA extraction methods

11.30-13.00 **Roundtable discussion on problems etc associated with environmental gene cloning**

Lunch 13.00 –14.30

14.30- Free time e.g. visit to Ile de Batz / opportunity for informal discussions

Application of PCR-DGGE in microbial ecology

Gerard Muyzer Netherlands Institute for Sea Research, Address: Den Burg, Texel, P.O. Box 59, The Netherlands

Microbial ecology is the study of interactions among microorganisms and between microorganisms and their environment. For a long time these studies were done using cultivation methods and microscopy. However, it is now well recognised among microbiologists that only a small part (ca. 1%) of all bacteria in nature can be isolated in pure cultures. The development of techniques to extract genomic DNA and ribosomal RNA from environmental samples has allowed new ways to study microbial communities independently from cultivation. So far, most of results in these studies have been obtained by cloning and sequencing of 16S rDNA fragments obtained after PCR amplification of bacterial genomic DNA extracted from environmental samples. However, microbial ecology mostly involves the study of microbial populations over a longer time frame. For this reason the cloning approach is not useful, because it is time-consuming, cumbersome and impractical for multiple sample analysis. For this purpose a quick and easy approach, which gives direct visualisation of predominant constituents of mixed microbial communities, and which allows multiple sample analysis is needed. DNA fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE), developed to detect sequence variation in DNA fragments, have been introduced into microbial ecology to determine the genetic diversity of complex microbial communities and to monitor population shifts after environmental perturbations. In this seminar I will briefly summarise the state-of-art of PCR-DGGE in microbial ecology and will present some our work.

Insights into microbial community structure revealed by molecular analyses of population distributions along environmental gradients using cloning and DGGE

Michael Ferris Montana State University, Microbiology Dept. 109 Lewis Hall, Bozeman, MT, USA 59715

An intriguing pattern revealed by many molecular surveys of bacterial populations in natural habitats is the occurrence of numerous closely related sequence types in individual environmental samples. The reasons for such small scale genetic variation among sequence types in the same sample is not well understood. A number of explanations are possible, including methodological artifacts in the PCR or sequencing processes. However, systematic analyses of microbial communities along environmental gradients have revealed population distribution patterns which indicate adaptations of closely related bacterial populations to macro- and micro scale environmental niches. As with all investigations of population biology, a more comprehensive understanding of the forces influencing the diversity of microbial populations will ultimately come from the recognition of patterns of distribution revealed by numerous investigators studying microbial populations in habitats worldwide.

Use of PCR-SSCP (single strand conformation polymorphism) in Microbial Ecology
Christoph C. Tebbe Institute for Agroecology, FAL (Federal Research Center for Agriculture),
Bundesallee 50, 38116 Braunschweig, Germany.

SSCP is a rapid electrophoretic technique to differentiate between DNA molecules of the same size but of different nucleotide composition. Based on 16S rRNA gene sequences amplified from environmental DNA, SSCP can be utilized as an alternative to DGGE or TGGE. For such community profiles, it is important to choose the single stranded approach (removal of the non-coding DNA strand by exonuclease digestion). Bands of profiles can be identified by direct sequencing or subcloning and sequencing in analogy to the evaluation of DGGE patterns. Silver staining does not interfere with the reamplification and cloning procedure. SSCP can also be used to screen a large number of isolates, e.g., as an alternative to ARDRA or other PCR products. The importance of this technique for environmental gene cloning will be discussed.

Fingerprinting Bacterioplankton of the central Baltic by 16S rDNA community analysis
Bert Engelen & M. G. Hofle GBF, Division of Microbiology, Mascheroder Weg 1, D-31824
Braunschweig, Germany.

During a cruise on the research vessel R/V "Aranda" in fall 1998, bacterioplankton from different sites of the central Baltic was collected by filtration of seawater. The monitored physico-chemical parameters served as background data for environmental conditions. The samples were analysed by 16S rDNA fingerprinting on TGGE to assess the structure and genetic diversity of bacterioplankton without cultivation. Therefore, total nucleic acids were extracted from filters and purified for PCR amplification to be loaded on a TGGE gel. The resulting 16S rDNA fingerprints were digitised to determine band positions and intensities. A cluster analysis lead to a comparison of TGGE band patterns, indicating a similar composition of the communities in habitats of similar physico-chemical conditions. To get a better understanding of the TGGE community fingerprints, the complete 16S rDNA of the bacterial community from one surface water sample (Gotland deep, 5m) was cloned after PCR amplification with universal bacterial primers into a TA vector for further sequencing. The clonebank with the 16S rDNA inserts from different community members was screened on TGGE. In contrast to TGGE bands obtained from the DNA fingerprints that represent only a short fraction of the molecule with less sequence information, the access to the cloned complete 16S rDNA sequence leads to DNA with high purity and allows a more accurate phylogenetic affiliation.

Cell sorting and molecular biology for diversity assessment
Philippe Lebaron and Claude Courties Laboratoire ARAGO BP44 66651 Banyuls-sur-mer
Cedex France

Population ecology is an emerging theme in the aquatic environment to analyze temporal and spatial variations of specific populations of bacterioplankton. Recently, flow cytometry has proven very useful to investigate the relationships between taxonomic, genetic and functional components of the diversity of bacterial communities in both natural fresh- and marine-waters. We will emphasize these recent investigations and instrumentation limits. The combination of molecular techniques with cell sorting of labeled cells by flow cytometry allows the detection and identification of bacterial species in natural waters which are not culturable. This approach allows new investigations of the relationships between genetic, taxonomic and functional diversity. The diversity of species as determined from sorted fractions has sometimes little overlap with the diversity determined at the community level. The ecological relevance of diversity assessment will be discussed.

Practical Aspects and Pitfalls of Microbial Community Analysis with T-RFLP

Terence L. Marsh, Fiona Crocker, Merry S. Riley, and Brian Wade

Ctr. Microbial Ecology, 41 Giltner Hall, Michigan State University, East Lansing, Michigan, USA

Terminal restriction fragment length polymorphism (T-RFLP) has proven to be a rapid and high resolution technique for the comparative analysis of microbial communities. The sensitivity of the technique is such that minor changes in primers, template concentration, annealing temperatures, number of PCR cycles, and the amount of PCR product analyzed can influence the community profile. Moreover the phylogenetic distribution of restriction sites along the primary sequence of the target and the relative position of the labeled primer will determine the resolution of the profile. We have successfully applied this technology to more than ten different habitats. We report here on our progress in the dissection of these diverse microbial communities.

Diversity of prokaryotes and direct cloning of novel enzyme genes from the Antarctic marine environment

Sara Sjoling and Don Cowan University College London, Dept Biochem and Molbiol, Gower St, London WC1E 6BT

The Antarctic continent represents a relatively uncharacterised biological resource where the microbial biodiversity offers enormous potential for both species diversity and application-directed studies. We have used molecular approaches for phylogenetic characterization of micro-organisms from Antarctic aquatic sediment, using 16S rDNA sequence analysis. To exploit the in situ genomic diversity, we have developed methods for generating multigenomic libraries of both culturable and unculturable micro-organisms, by direct isolation and cloning of total community DNA. Libraries are screened for selected enzyme activities, positive clones are isolated and the enzymes will be expressed and characterised for use as novel biocatalysts.

Insights into cyanobacterial diversity from RNA polymerase (rpoC1) sequence data

Brian Palenik SIO/Univ. of Calif. San Diego, La Jolla, CA 92093-0202 U.S.

Environmental variables such as light, temperature, salinity, and nutrients seem to be influencing microbial genetic diversity in marine ecosystems, but to an extent that is still poorly characterized. In addition, biotic factors such as viruses and grazers are likely to affect microbial diversity as well. Various approaches for examining in situ diversity are being pursued. To examine cyanobacterial diversity specifically, we have been using sequence data from DNA-dependent RNA polymerase (rpoC1) genes. Cyanobacterial specific primers allow cyanobacterial rpoC1 sequences to be PCR amplified from DNA obtained from freshwater or marine samples, cyanobacterial symbiont (tunicate) hosts, and nonaxenic cultures. Our recent results on more than forty isolates indicate that multiple clades of *Synechococcus* can be found in marine systems. Many of the clades are also seen in rpoC1 libraries obtained from seawater samples. Some of these correlate with unique adaptations such as motility. Other clades are strongly associated with oligotrophic or neritic ecosystems. For example, whole cell antibody approaches in addition to rpoC1 data suggest that a unique clade of *Synechococcus* is adapted to the surface oligotrophic ocean. However, the specific adaptations of clades to their preferred ecosystem still remained to be determined.

Molecular bases of ecotypic diversity in *Prochlorococcus*

Frederic Partensky, Garczarek L. and Hess W.R. Station Biologique, Roscoff, Cedex, France

The occurrence of two ecotypes of *Prochlorococcus* in the field was recently demonstrated. Using representative isolates of these ecotypes, we found that they diverge by both the number of genes encoding their major antenna system and by the presence of a complete cluster of phycoerythrin genes in low light which is reduced to a single gene in high light adapted strains. Physiological implications are discussed.

Prokaryotic picophytoplankton in tropical coastal lagoons

Loic Charpy IRD, COM Rue de la Batterie des Lions, 13007 Marseille

In many tropical coastal regions, picocyanobacteria are a major component of biomass and productivity for most of the year. In most of the French Polynesian atoll lagoons, *Synechococcus* formed the predominant group in terms of abundance, carbon biomass and primary production. However, average lagoonal picoplankton abundance varied by a factor of 200 depending on the geomorphology of the atolls. One of the surprising discoveries is that the taxonomic composition of cyanobacteria in atolls change dramatically from coastal waters dominated by *Synechococcus* to open ocean dominated by *Prochlorococcus*. What are the environmental factors responsible for this shift in dominance such as light, nutrients, grazing pressure or viruses? Are lagoonal *Synechococcus* genetically different from the oceanic ones (Nif gene)? Responses to these questions are one of the objectives of the new IRD research Unit: Cyano.

Diversity of cyanobacteria in the Finnish lake environment

Pirjo Rajaniemi, Anne Rantala, Kaisa Haukka, Kaarina Sivonen Helsinki University, Department of Chemistry and Microbiology, P.O.Box. 56, FIN-00014 Helsinki University

In our research group we have various projects studying the cyanobacterial ecology, phylogeny and physiology, cyanobacterial toxins and other bioactive compounds and their biosynthesis. We have isolated, purified strains axenic and identified numerous planktic cyanobacteria. These isolates in our culture collection serve as model strains in the future cyanobacterial studies. We have used denaturing gradient gel electrophoreses (DGGE) to follow changes in the bacterial communities in the natural freshwaters and the Baltic Sea as well as in controlled enclosures.

In the EU project called MidiChip we are developing DNA array technology for rapid detection of different genotypes of cyanobacteria present in lake waters. In the MidiChip project we will monitor cyanobacterial diversity over two years in the lake Tuusulanjärvi, a eutrophic Finnish lake. We will use molecular techniques, e.g. cloning, DGGE, sequencing of 16S RNA and other marker genes and in situ hybridisation. Furthermore, we will utilize different isolation and culturing techniques to diversity studies. The results of our study will be linked together with the results from counting and physico-chemical parameters to form a broad picture of cyanobacterial diversity in the Finnish freshwaters. Based to these studies the DNA microarray for rapid monitoring water quality will be developed.

Prokaryotic biodiversity in aquatic marine environments

Susana Benlloch, Jesus Garcia-Martinez, Arantxa Lopez-Lopez, Purificacion Lopez-Garcia and Francisco Rodriguez-Valera Universidad Miguel Hernandez. Division Microbiologia, Facultad de Medicina. Ctra Valencia, km 87. San Juan 03550. Alicante.Spain

We are interested in exploring bacterial biodiversity in natural environments, specifically aquatic marine environments such as the sea, coastal lagoons and hypersaline environments derived from evaporation of seawater. For this purpose, RISA and RFLP have been used (as molecular fingerprinting techniques). 16S rDNA libraries from total DNA extracted directly from natural samples have been constructed and analysed also. We have studied the microdiversity and distribution of the SAR11 cluster and Group I of marine Archaea from Mediterranean and Antarctic waters, at different locations and depth. There was a high concordance in both DNA region chosen for the analysis, even for the extremely variable ITS, where potential probes have been proposed for the identification and isolation of these microorganisms. We have also studied the prokaryotic biodiversity in the water column through a transect from the Weddell Sea (Antarctica) and Patagonia (Argentina).

Assessment of microbial diversity in a deep hot oil reservoir from Western Siberia by molecular methods

Nercessian, O.¹, Corre, E.¹, Lysov, Y.², Bulygina, E.² and C. Jeanthon¹

¹ LEMAR-CNRS UMR 6539, Institut Universitaire Européen de la Mer (IUEM), Université de Bretagne Occidentale (UBO), Place Nicolas Copernic, Technopole Brest-Iroise, 29280 Plouzané, FRANCE ² Engelhardt Institute of Molecular Biology, Moscow, RUSSIA

The microbial diversity in a deep hot oil reservoir from Western Siberia has been assessed through phylogenetic analysis of 16S SSU-RNA genes and DNA microchip technology. For phylogenetic analysis, different combinations of archaeal and bacterial specific primers were used to amplify 16S SSU-RNA genes. Among the 138 archaeal and 311 bacterial clones that were categorized by Amplified Ribosomal DNA Restriction Analysis (ARDRA), 26 archaeal and 57 bacterial clones were partially sequenced and compared with sequences available in databases.

In an attempt to develop new, accurate and powerful molecular tools for microbial ecological studies, DNA microchip technology was evaluated. After designing new oligonucleotide probes complementary to 16S rRNA of archaeal and bacterial thermophilic genera, optimization of hybridization conditions were performed. The validated probes were then evaluated on 3 samples of production fluids.

The results of both strategies were found complementary: both archaeal and bacterial sequences were detected. While phylogenetic analysis of 16S ssu-RNA genes identified mainly methanogens (genera *Methanocalculus*, *Methanosaeta*, *Methanococcus*), sulfate-reducers (*Archaeoglobus*-like, *Desulfotomaculum*) and fermentative organisms (genera *Moorella*), DNA microchip experiments detected thermophilic genera known to thrive in oil reservoirs but also in marine and terrestrial environments (genera *Thermococcus*, *Pyrococcus*, *Thermotoga*, *Petrotoga*, *Geotoga*, *Thermoanaerobacter*, *Desulfotomaculum*). Surprisingly, the latter experiments allowed the detection of other genera which was only encountered, up to now, in marine and terrestrial hydrothermal environments but not in oil reservoir environments (genera *Desulfurococcus*).

In conclusion, microorganisms detected in the oil reservoir of Western Siberia seemed to be adapted to the *in situ* reservoir temperature. Moreover, although the reservoir was located thousand kilometers from any oceans or hydrothermal areas, we detected sequences closely related to ubiquitous microorganisms originating from marine and terrestrial volcanic environments. Therefore, the results obtained support the existence of deep subterranean biosphere.

Genetic diversity of the French *Dinophysis*, *Alexandrium* and *Gymnodinium* genera based upon direct cells amplification on fixed cultures or natural samples
Laure Guillou Cambon, M. A. & Barbier, G. IFREMER Centre de Brest, DRV/VP/CMM, BP 70, 29 280 Plouzané, France.

Dinophysis, *Alexandrium* or *Gymnodinium* genera (dinoflagellates) contains species which are toxic for human or fish by producing diarrhetic, paralytic or neurotoxic toxins. These three genera occur along the French coast during most part of the year (during spring, summer and autumn). These species can be toxic under 100 Cells/L (case of *Dinophysis acuminata*), or difficult to recognize by microscopy (case of *Alexandrium* or *Gymnodinium* spp). The aim of this study is to develop molecular diagnostics to increase the sensibility and the security of the detection of toxic algae in natural samples and the integration of these tools in the routine phytoplankton monitoring programs managed by IFREMER on the French coast.

An easy method which allowed the amplification directly on the entire cells fixed with lugol is described. Pre-treatment, washing and denaturation time were optimized in order to obtain a single amplification band by PCR of both intergenic and D1D2 domain of the large ribosomal subunit (about 1500 bp). This method allow to amplify single cell from both culture or natural samples. Results on the genetic diversity of *Dinophysis*, *Alexandrium* and *Gymnodinium* on French coast are presented. Further studies include the detection by specific probes. Variability found inside SSU rDNA, LSU rDNA and intergenic part (ITS1 and ITS2) are discussed. Two different detection methods will be compared: high-density membranes and nested PCR, and the first results obtained will be shown.

Genetic analyses of *Dinophysis* species isolated from Norwegian waters

¹**Bente Edvardsen**, ²Kamran Shalchian-Tabrizi, ¹Sissel Brubak, ³Einar Dahl, ²Kjetill S. Jakobsen, ⁴Linda K. Medlin & ¹Eystein Paasche

¹Section for Marine Botany & ²Section for General Genetics, University of Oslo, P.O. Box 1031 Blindern, 0316 Oslo, Norway, ³Institute of Marine Research, Flødevigen Marine Research Station, N-4817 His, Norway. ⁴AWI, Am Handelshaven 12, D-27570 Bremerhaven, Germany.

Dinophysis species may contain Diarrhetic Shellfish Toxins (DST). They occur all along the Norwegian coast and may in some regions prevent harvesting of mussels several months each year. The content of toxins seems to vary considerably between and within species. Also the morphology is very variable within some species and the species-delineation can at times be unclear. We have examined the phylogenetic relationship of four *Dinophysis/Phalacroma* species (*D. acuminata*, *D. acuta*, *D. norvegica* and *P. rotundatum*) isolated from Norwegian waters inferred from the 18S ribosomal RNA gene. The genetic variability within the species *D. acuminata* and *D. norvegica* was examined by analysing the first internal transcribed spacer (ITS1) in 5 isolates per species collected at different times of the year and from two localities off the coast of southern Norway. The three photosynthetic species *D. acuta*, *D. acuminata*, and *D. norvegica* were very similar within the 18S rRNA gene and differed in only 5-8 out of 1802 bp. The non-photosynthetic *P. rotundatum*, however, differed in ca. 60 bp compared to the three photosynthetic species. This supports the original distinction between *Dinophysis* and *Phalacroma*. In the phylogenetic analyses the *Dinophysis/Phalacroma* (dinophysoid) species fall into a common clade that are associated to a branch composed of gymnodinioid, prorocentroid and peridinioid species (GPP complex). Despite differences in morphology between the five isolates of *D. acuminata*, they all had almost identical ITS1 sequences. Similarly, all 5 isolates of *D. norvegica* were identical in this non-coding region. The ITS1-sequences in *D. acuminata* and *D. norvegica* were very similar, differing in < 10 bp. We are presently developing oligonucleotide probes for *Dinophysis* species based on the sequence differences within the rRNA operon.

Multi-method approach to study marine microbial ecology. Examples from a mesocosm experiment with an *Emiliana huxleyii* bloom terminated by virus
Tonje Castberg Dept. of Microbiology PO Box 7800, University of Bergen, 5020 Bergen, Norway

To study viruses and the population dynamics of their potential hosts in natural marine environments we have combined a range of methods to observe interactions between microalgae, prokaryotes and viruses. In a mesocosm experiment in May 1999 we applied flow cytometry (FCM) to survey the dominating algal, bacterial and viral populations and microscope (LM) for identification of major algal species. Pulse field gel electrophoresis (PFGE) was used to analyze the viral community in terms of genome size distribution. The prokaryote and eukaryote communities were analyzed by denaturant gradient gel electrophoresis (DGGEs) of PCR amplified 16S and 18S rDNA fragments. A selection of major bands from the eucaryote DGGEs was sequenced. Standard nutrient (N;P;Si), primary production and chlorophyll measurements were also done. The complexity of microbial ecosystems makes it necessary to use a wide range of different methods to describe its structure and understand its function. Each of the methods used provided detailed information on the various populations but it was necessary to combine results from all methods to obtain a more complete picture of community dynamics and to discern any possible interactions between populations. By combining and comparing the data obtained with FCM, LM, PFGE and DGGE we could overcome the inherent limitations of each method and thus interpret the results much further.

Fluorescent In Situ Hybridisation of Marine Cyanobacteria Using 16S rRNA-Targeted Oligonucleotides in Combination With Tyramide Signal Amplification

N. J. West¹, W. Schönhuber^{2,3}, R. Rippka², R. Amann³ & D. J. Scanlan¹. ¹ Dept. of Biological Sciences, University of Warwick, Coventry, UK; ² Unité de Physiologie Microbienne, Institut Pasteur, Paris, France; ³ Max-Planck-Institut für Marine Mikrobiologie, Bremen, Germany.

The marine photosynthetic prokaryotes *Prochlorococcus* and *Synechococcus* are dominant components of the picophytoplankton in the world's oligotrophic regions. *Prochlorococcus* exists as multiple ecotypes that are adapted to high or low light environments (HL and LL). A comparison of 16S rRNA sequences of cultured *Prochlorococcus* isolated from HL and LL environments allowed 16S rRNA oligonucleotides to be designed which target 2 separate HL clusters and 2 separate LL clusters of these strains. Hybridisations with cultured *Prochlorococcus* cells using CY3-labeled probes were unsuccessful since positive signals were masked by the autofluorescence of the photosynthetic pigments. Subsequent hybridisations with horse-radish peroxidase (HRP)-labeled probes, detected by incubation with the substrate fluorescein-tyramide, gave extremely bright signals with both *Prochlorococcus* and *Synechococcus* cells. Hybridisations with natural samples from the N. Atlantic and Mediterranean Sea have been carried out and have revealed the spatial distribution of *Prochlorococcus* genotypes. This seminar will describe the technical aspects of the TSA method for detection of 16S rRNA hybridisations, and the results of hybridisations with cultured and natural *Prochlorococcus* cells will be presented.

Identification of bacteria associated with dinoflagellates using TSA-FISH and confocal microscopy

Isabelle C. Biegala, N.Simon, J.-F.Lennon, E.Alverca, G.Kenaway, D.Vaulot Station Biologique de Roscoff, Place Georges Teissier, 29680 Roscoff, France.

TSA-FISH (Tyramide Signal Amplification - Fluorescent In Situ Hybridization) associated with confocal microscopy have been developed in order to study interactions between dinoflagellates and bacteria in the production of PSP (Paralytic Shellfish Poisoning). Those techniques allow clear identification of group specific bacteria whatever their kind of association with dinoflagellates: 1) free, living in the close environment of the dinoflagellate; 2) attached to the dinoflagellate theca; or 3) endocellular (endocyttoplasmic and endonuclear).

Oceanic 18S rDNA sequences from picoplankton reveal new eukaryotic lineages

Seung Yeo Moon-van der Staay and **Daniel Vaulot** Station Biologique BP74, Roscoff, France.

Picoeukaryotes are major contributors to total phytoplankton biomass in open oceanic waters. However, their diversity is poorly known. We investigated eukaryotes in a picoplankton sample collected from the equatorial Pacific Ocean by random sequencing of 18S ribosomal RNA gene clones. Total genomic DNA was isolated from a 3 µm-filtered sample, and 18S rRNA genes were amplified using universal eukaryotic primers. Environmental sequences of thirty-five representative variants were compared with other known 18S rRNA gene sequences representing a range of eukaryotic evolutionary lineages. The result demonstrates the high diversity of picoeukaryotes. Most of the sequences, which are largely assigned to haptophytes, green algae, dinoflagellates, stramenopiles, choanoflagellates, and acantharians, were previously unknown. Clones representing a lineage which is apparently a close relative of the dinoflagellates were detected. We suggest that they represent a novel group, not described so far, that might be one of the potentially important groups in oceanic picoeukaryotes.

Detection of Raphidophyceae, potentially harmful microalgae

Wiebe Kooistra and Koeman, R.P.T. Marine Biology, RuG Postbox 14, 9750 AA Haren (GN), The Netherlands

Since the early 1990's HAB-forming Raphidophycean genera *Heterosigma*, *Chattonella* and *Fibrocapsa* have become a part of the phytoplankton in the North Sea. We use PCR with specific primers to detect these species in water samples from the Dutch part of the North Sea. PCR results are compared with those of visual examinations. We also assess specificity and detection limits of the method.

Minicircular plastid DNA markers in dinoflagellates

Adrian Barbrook and Christopher J. Howe Dept of Biochemistry, Building O, Downing Site, Tennis Court Road, Cambridge, CB2 1QW, UK

We have characterised the plastid DNA from dinoflagellates in an attempt to provide markers for the identification of different species and strains. We have discovered a number of plastid genes encoded on DNA minicircles in the dinoflagellate *Amphidinium operculatum*. In each case a single gene is encoded on a circle of approximately 2.4 kb. We believe that they will provide useful markers for identification.

MICROMAT: Workshop on DNA extraction methods

Evelyne Brambilla (DSMZ, DE), Sylvie Cousin (RUG, BE), Bert Gerrits van den Ende (CBS, NL), Ignacio Gonzalez (Merck-Sharp-Dohme Espana), Stana Grubisic (Ulg, BE), Blair Lawley (BAS, UK), Regine Neumann (DSMZ, DE), Ulrike Steiner (DSMZ, DE), **Annick Wilmotte** (Ulg, BE)

Algology Laboratory, Dept Botany B22, Univ Liege, B-4000 Liege, Belgium
email: awilmotte@ulg.ac.be Phone: +32 4 366 38 56 Fax: +32 4 366 28 53

Mat samples of Lake Fryxell and Ace Lake (Antarctica) were used to compare 3 different extraction methods, using different mechanical disruption tools (Bead-beater (Braun Biotech), Stomacher (Omnilab), Fast DNA-Kit (BIO101) and purification methods (Prep-A-Gene (BioRad), Wizard (Promega), GeneClean (BIO101)). The quantity and quality of the genomic DNAs were compared by agarose gel electrophoresis and different PCRs. A 'standard' PCR with specific primers for bacteria, cyanobacteria, protists and fungi was carried out to verify that the obtained DNA could be amplified. Then, a DGGE analysis was performed after a PCR reaction giving a small amplicon with a GC-Clamp at one end. This analysis gave an idea of how representative the tested taxonomic groups (bacteria, cyanobacteria, protists) are in the extracted DNA.

DISCUSSION SESSION: Tues 27th June 11.15-13.00

This session is intended to be an open forum to discuss issues considered to be important in environmental gene cloning. Please come willing to contribute either an overhead or just spoken input to any of the specific topics that have been listed. Also, feel free to bring up additional questions/problems etc that you think should be addressed when constructing and analysing environmental clone libraries or performing community structure analysis using molecular techniques but which are not mentioned below:

- Sample collection: volume, filtration method, flow cytometry sorting, extraction on board ship, preservation
- Sample treatment once back in the lab.
- Storage time
- Choice of gene (only SSU or SSU + other: LSU, *rpoC*, *rbcL* etc...)
- Use of DNA vs RNA
- PCR or not PCR
- Cloning strategy (choice of vector)
- Clone selection (random, use of probes, RFLP, DGGE, TRFLP, single base sequencing, SSCP)
- Sequencing strategy (automatic sequencer)
- Sequence analysis
- How is the clone library representative of the initial diversity?
- Chimeras : How to detect them? Can one design experiments to test that?
- Effect of rRNA gene numbers (important for eukaryotes such as dinoflagellates).

PRACTICAL PROTOCOLS

These protocols supplement the practical demonstrations/discussion sessions on DGGE, HA Yellow/Red gels and FISH techniques. Nucleic acid extraction techniques are included as supplementary information.

It is intended that the practical sessions be used not only to observe specific techniques 'in action' but also to discuss with participants any practical problems or issues that you yourself may have had when using the technique

Nucleic acid extraction from seawater samples (Dave Scanlan)

Filter 4 - 10 litres of seawater onto a 0.2 µm pore size Gelman Supor-200 47 mm diameter filter on a vacuum of approx. 5-8 in Hg. Use of a 0.45 µm pore size Gelman Supor-450 47mm diameter filter improves rate of filtration significantly and is fine for both *Synechococcus* and *Prochlorococcus*. The Supor filters are advantageous because they have:

- i. low level of contaminants;
- ii. high filtration rates;
- iii. dissolve entirely in phenol during the extraction process.

Protocol I: Mainly as set out in Kerkoff and Ward (1993). AEM 59:1303-1309

- i) Distribute 0.5 ml of 0.5 M EDTA over filter. Store filter at -70 C in a petri dish.
- ii) Roll filter into a 10 ml liquid tight sterile test tube (resistant to phenol and chloroform) so they uncurl on the sides of the tube.
- iii) Add 600 µl of cold 50 mM glucose-10 mM EDTA-25 mM Tris pH8.0 (GTE buffer), 8 µl of 250 mg/ml lysozyme, and 200 µl 0.5 M EDTA pH8.0.
- iv) Incubate on side (so that filter is immersed) at RT for 10 mins rolling tube occasionally.
- v) Lyse cells with 100 µl of 10% SDS and vortex well.
- vi) Extract immediately in 1.5 ml of phenol-chloroform- isoamyl alcohol (25:24:1). Such a high volume of phenol/chloroform is required for complete dissolution of the filters into the organic phase. Vortex well. Aliquot into Eppendorf tubes.
- vi) Centrifuge on maximum for 5 min at RT. Transfer aqueous (upper) phase to fresh tubes.
- vii) Extract with chloroform/isoamyl alcohol, and spin as above. Transfer aqueous phase to fresh tubes.
- viii) Precipitate with 1 volume of isopropanol and 0.4 vol of 7.5 M ammonium acetate.
- ix) Incubate at RT for 10 minutes. [For Red Sea water, we find that precipitation with ethanol causes a massive salt precipitation - so we avoid it.]
- x) Centrifuge on maximum for 15 min at RT.
- xi) Wash pellets in ice cold 70% ethanol.
- xii) Allow pellets to dry on table top for about 10 min.
- xiii) Resuspend in a volume of 50 µl TE2 (10 mM Tris, 0.1 mM EDTA, pH8.0).

Yield: 1 - 4 µg DNA depending on site and season.

Protocol II: Mainly as set out in Gordan and Giovannoni (1996). AEM: 62(4): 1171-1177.

- i) Place filter in 5 ml cryovial. Add 3 ml lysis buffer (20 mM EDTA, 400 mM NaCl, 0.75 M sucrose, 50 mM Tris pH9.0) to fully immerse filter. Freeze in liquid nitrogen and store at -70 C. The authors freeze their filters at -20 C as well.
- ii) Thaw sample on ice.
- iii) Add a) 350 μ l 10% SDS and mix. b) 33.5 μ l 10 mg.ml⁻¹ Proteinase K and mix.
- iv) Incubate at 37 C for 30 min ensuring that filter is fully immersed.
- v) Incubate at 55 C for 10 min.
- vi) Transfer lysate and filter to centrifuge tube that is resistant to phenol and chloroform. (I use 4 x 2 ml Eppendorfs, cutting the filter into 4 before placing into the 5 ml cryovial above.)
- vii) Extract once in 3 ml phenol:chloroform:isoamyl alcohol (25:24:1)
- viii) Extract once in chloroform:isoamyl alcohol (24:1)
- ix) Precipitate with 1 volume of isopropanol and 0.4 vol of 7.5 M ammonium acetate. Incubate at RT for 10 minutes. [For Red Sea water, we found that precipitation with ethanol causes a massive salt precipitation - so we avoid it.]
- x) Centrifuge on maximum for 15 min at RT.
- xi) Wash pellets in ice cold 70% ethanol.
- xii) Allow pellets to dry on table top for about 10 min.
- xiii) Resuspend in a volume of 50 μ l TE2 (10 mM Tris, 0.1 mM EDTA, pH8.0).

Collecting biomass for marine DNA extraction:

- i) Collect around 5 liters of surface seawater
- ii) Filter sample with a peristaltic pump, having two filters in sequence, first a 47 mm polycarbonate filter of 2/3/5 μ m poresize, and then a Sterivex unit (Millipore) of 0.2 μ m pore size.
- iii) Cover both filters with lysis buffer (40 mM EDTA, 50 mM Tris-HCl and 0.75 M sucrose) and store at -20°C.

Nucleic acid extraction

- i) Add lysozyme (1 mg ml⁻¹) to the Sterivex filter and incubate at 37°C for 30 min.
- ii) Add proteinase K (0.5 mg ml⁻¹) and SDS (1%) to the filter and incubate at 55°C for 2 hrs.
- iii) Extract the lysate twice with phenol: chloroform: isoamyl alcohol (25:24:1, pH 8.0) and once with chloroform: isoamyl alcohol (24:1).
- iv) Wash and concentrate the aqueous phase in a microconcentrator (Centricon 100, Millipore) and obtain a final volume of DNA extract of 200 μ l.
- v) Quantify DNA extract by a Hoescht dye fluorescence assay and check the integrity by agarose gel electrophoresis. Store extracts at -70°C until analysis.

DGGE protocol for bacteria associated to dinoflagellates (Sébastien Colin, Roscoff)

Extraction protocol : DNA extraction using simple cell lysis :

The classical DNA extraction protocol using phenol/chloroform from (Somerville and *al.* (1989) has been used.

- Samples (50/100 ml of culture) are harvested onto 47 mm diameter 0.2µm pore-size filters (Supor-200, GelmanSciences) in sterile conditions. Filters are immediately frozen (-20°C) until further processing.
- Filters are split into several fragments.
- Filters are then soaked them in 500µl of lysis buffer (SDS 0.0002% p/v, DTT 4mM, 40µl *Taq*PCRbuffer 10x, Mgfree).
- Add 10 glassbeads (0.75mm diameter).
- Strongly vortex for 1 min.
- Operate 3 cycles of freezing-thawing (between liquid nitrogen and 55°C water bath).
- Add proteinase K to 0.2µg/ml and incubate 1hr at 55°C.
- Store lysates at -20°C.

PCR conditions

- Primers:

Name	Sequences (5' - 3')									
358F-GC	CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG									
926R	CCG TCA ATT C(c/a)T TT(a/g) AGT TT									
Name	E.coli position	Specificity	Length	#A	#T	#G	#C	%GC	4GC+2AT	MW
358F-GC	341-358	eubacteria (DGGE)	57 17	5	1	32	19	89.5	216 58	19810
926R	907-926	eubacteria	20	4	9	2.5	4.5	35.0	54	6647

- PCR program :
 - Denaturate template for 5 min at 95°C.
 - 10 first cycles : denaturation step 1 mn at 95°C, annealing step 1 mn with touch-down 65°C to 55°C (one degree decreasing per cycle), and polymerisation step 1mn at 72°C (incremented of 5sec/cycles).
 - 15 to 20 last cycles in same conditions excepted for annealing step what is fixed at 55°C during 1mn.
- Enzyme :

We use to PCR beads ReadyToGo (Amersham Pharmacia Biotech) wich contain a *Taq*DNAPolymerase. Primers working concentration is 0.4 µM. With the second extration protocole, the volume of lysat we use, is 1/5 of total volume reaction (5µl for 25µl here).

DGGE:

- Gel : 6% polyacrylamide (acrylamide-*N,N'*-methylenebisacrylamide, 37.5:1).
- Denaturing gradient 20-50%v/v (100% denaturant solution contains urea 7M and 40%v/v formamide).
- Run at 60°C during 5 h at 150 V on BioRad DCode system
- Stain with SYBR Gold (1/10 000 final) and analyze on STORM imager (BioRad).

Protocols for PCR and DGGE. Institut de Ciències del Mar, Barcelona

PCR

Use 1 ng of whole microbial DNA as template for Polymerase Chain Reaction (PCR) amplification of bacterial or eukaryal SSU rDNA. The reactions contain 200 μ M of each of the deoxynucleoside triphosphates, 0.3 μ M of each of the primers, 1.5 mM MgCl₂, 1x PCR-buffer and 1 Unit of *Taq* DNA Polymerase in a final volume of 50 μ l.

Primers

Bacteria: Bacterial specific forward primer 358f with a 40 bp GC-clamp, and universal reverse primer 907r, which amplify a 550 bp DNA fragment of bacterial 16S rDNA (Muyzer et al. 1997).
Eukarya: Eukaryal specific forward primer EukA (Medlin et al. 1988) and universal reverse primer 516r (ACC AGA CTT GCC CTC C) with a 40 bp GC-clamp, which amplify a 560 bp DNA fragment of eukaryal 18S rDNA.

PCR program

Initial denaturation at 94°C for 5 min; 10 touchdown cycles of denaturation (at 94°C for 1 min), annealing (at 65 to 55°C for 1 min, decreasing 1°C each cycle) and extension (at 72°C for 3 min); 20 standard cycles of denaturation (at 94°C for 1 min), annealing (at 55°C for 1 min) and extension (at 72°C for 3 min), and a final extension at 72°C for 5 min.

PCR product check

Four μ l of the PCR product are verified by electrophoresis on a 0.8% agarose gel stained with ethidium bromide, and the DNA yield of the PCR is quantified loading a standard in the same gel (Low DNA Mass Ladder, GIBCO BRL).

DGGE

Denaturing Gradient Gel Electrophoresis (DGGE) is performed with the DGGE-2000 system (C.B.S. Scientific Company) as described in Muyzer et al. (1997).

Gel casting

A 6% polyacrylamide gel is casted by mixing two stock solutions of acrylamide (37.5:1 acrylamide: bisacrylamide) containing different amounts of DNA denaturant agents: 40 and 80% for bacterial PCR products and 45 and 65% for eukaryal PCR products (100% denaturant agent is defined as 7 M urea and 40% deionized formamide). The reproducibility of the gradient is obtained by using a two chambers gradient maker and controlling the flow of the acrylamide into the plates with a peristaltic pump at around 5 ml min⁻¹. The gradient is overlaid with nondenaturant acrylamide in order to obtain well polymerized slots. The gel is casted several hours before loading.

Gel running

800 ng of PCR product (typically 40 μ l of the PCR product) are loaded in the gel slots with a Hamilton syringe for each sample (a maximum of 18 samples per gel). The gel is run at 100 V and 60°C for 16 h in 1x TAE buffer (40 mM Tris base [pH 7.4], 20 mM sodium acetate, 1 mM EDTA).

Gel staining

We used the DNA stains GelStar (FMC BioProducts) or SYBRGold (Molecular Probes). One of the glass plates is detached from the gel and 15 ml of the stain solution (15 ml of 1x TAE with 3 μ l of the stain stock solution) are added covering the whole gel. The gel is stained for 30 minutes in the dark, rinsed with a large volume (around 500 ml) of 1x TAE buffer, removed from the glassplate and transferred carefully to a UV transparent gel scoop (Sigma). The gel is visualized with U.V. in the Fluor-S MultiImager (Bio-Rad) with the Multi-Analyst software (Bio-Rad).

High-resolution images (1312 x 1034 pixels, 12-bits dynamic range) are saved as computer files (4.6 Mb).

Quantitative analysis of DGGE fingerprints

The computer image of the gel is analyzed with the Diversity Database software (Bio-Rad) as explained in Schauer et al. (2000). The software performs a light intensity profile through each lane, detects the bands, and calculates the relative contribution of each band to the total band signal in the lane after applying a detailed rolling disk as background subtraction. Intensity values are recalculated for all the bands of one lane to sum up to 100% relative intensity.

The different lanes from the same gel are compared and the bands occupying the same position in the gel are identified.

A matrix is constructed for all lanes, taking into account the presence or absence of individual bands, and the relative contribution of the band to the total intensity of the lane. This matrix was used to calculate a distance matrix using Euclidean distances (Systat 5.2.1). A dendrogram relating all samples is obtained with the unweighted pair group average linkage method (UPGMA) in cluster analysis (Systat).

The number of bands and the intensity of each band can be used to calculate the Shannon diversity index (H') with the following formulae:

$$H' = - \sum_{i=1}^{i=n} p_i \ln p_i$$

where n is the number of bands in the sample and p_i the relative intensity of the i th band.

References

Muyzer, G., T. Brinkhoff, U. Nübel, C. Santegoeds, H. Schäfer, and C. Wawer. 1997. Denaturing gradient gel electrophoresis (DGGE) in microbial ecology, 3.4.4:1-27. *In* A. D. L. Akkermans, J. D. van Elsas, and F. J. de Bruijn (eds.), *Molecular Microbial Ecology Manual*.

Medlin, L., H.J. Elwood, S. Stickel and M.L. Sogin 1988. The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene* 71, 491-499.

Schauer, M., R. Massana and C. Pedrós-Alió. 2000. Spatial differences in bacterioplankton composition along the Catalan coast (NW Mediterranean) assessed by molecular fingerprinting. *FEMS Microbiol. Ecol.* (in press).

Two FISH protocols are described below which use the tyramide signal amplification system to detect either cyanobacteria or bacteria associated with dinoflagellates

TSA-FISH protocol for bacteria (I Biegala and N. Simon)

(from Amann 1995 and modified from Schönhuber et al. 1997)

Note: This protocol is currently (March 2000) being modified for picoplankton by F. Not and N. Simon

A. Cells preparation

A1. Fix the cells

i) Add 1 vol. of ice cold 10% paraformaldehyde (made fresh with 1X PBS, aliquoted and stored at -20°C) to 9 vol. of cell culture (1% final PFA concentration).

ii) Incubate 1h at 4°C in dark or overnight. The fixed cell culture can be stored 24h at 4°C.

A2. Filtration and alteration of cell wall with alcohol and lysozyme

- i) Use clean filtration devices (Tulip and support washed with 1% HCl and sterilized).
- ii) Filtrate cell sample on (25 mm, 0.2 µm pore size) inorganic membrane filter (Anodisc, Whatman, Maidstone, UK), with a 10 mHg pump.
- iii) Add 2 ml of 50% EtOH, leave 3 min, and filter.
- iv) Add 2 ml of 80% EtOH, leave 3 min, and filter.
- v) Add 2 ml of 100% EtOH, leave 3 min, and filter.
- vi) After this step the filter can be kept in cleaned plastic box at room T°C in the dark, stored for several months. Or immediately processed further to alter the cell wall.
- vii) Put the filter in 5 ml plastic well
- viii) Add on top 1 to 2 ml of 5 mg/ml lysozyme in 0.1 M Tris 0.05 M EDTA buffer pH 7.5-8, incubate for 30 min at 37°C (*).
- ix) Rinse the filter 3 times 1' in 5ml H₂O
- x) Add 2 ml of 50% EtOH, leave 3 min, and filter.
- xi) Add 2 ml of 80% EtOH, leave 3 min, and filter.
- xii) Add 2 ml of 100% EtOH, leave 3 min, and filter.
- xiii) After this step the filter can be stored in cleaned plastic box at room T°C in dark for 3 months at least.
- xiv) The filters can be cut in 14 fragments with fine scalpel keeping for each fragments the plastic end. The plastic end is then colored with Tipex and a black pen in order to recognize easily the right side from the reverse.

(*). This step has been modified later on to 100 µl/ml lysozyme in 0.1 M Tris 0.05 EDTA pH 7.5-8, 5 min at room temperature.

B. In situ hybridization

B1. Hybridization

The stringency of the hybridization must be empirically optimized (range between 37-55°C) higher stringency can be achieved by the addition of formamide to the hybridization buffer.

Hybridization is done in a sealed moisture chamber to prevent evaporation of the hybridization solution, which can result in non-specific probe binding to the cell.

Soak a piece of Whatman 3MM paper (2.6 cm/6 cm) in 0.8 ml of hybridization buffer (0.01% SDS 50% Formamide for GAMA and BETA probes; 0.01% SDS 36% Formamide for EUB and ALFA) and place humid chamber (50 ml polypropylene screw top tube, FISHER). Allow to equilibrate several minutes at 35°C.

Put 10 µl of hybridization buffer on coated slide.

Add 1µl of oligonucleotide probe HRP labeled and 1µl of non HRP labeled competitor probes when necessary (probes concentration: approx. 50 ng/µl).

Cover with filter fragment (cells on top, they should not be in contact with the buffer, to avoid swimming around)

Quickly transfer the slide in the pre-warmed humid chamber. Incubate 2h to 3h at 35°C.

B2. Wash

Immerse the filter (cells on top) in plastic wells in 5ml wash buffer (0.01% SDS with a concentration of NaCl (0.08 M) which will give an equivalent stringency as the formamide concentration (35 %).

Cover with plastic lid and incubate in dark, 2 times 30 min at 37°C.

B3. Equilibrate

Immerse the filter(cells on top) in plastic wells (with flat bottom) in 5ml TNT buffer
Cover with plastic lid and incubate 15 min room temperature, in dark

B4. TSA reaction (Tyramide Signal Amplification)

Thaw an aliquot of dextran sulfate (Sigma) 40%
Mix 1:1 with amplification diluent (Kit: NEN Life Science Products)
Mix 1:50 fluorescein tyramide or tetramethylrhodamine tyramide (Kit: NEN Life Science Products) with the mixture of dextran sulfate and amplification diluent
Add 10µl of the fluorochrome tyramide on the filter (cells on top)
Incubate at room temperature in dark for 30 min

B5. Wash

Immerse the filter (cells on top) in plastic wells in 5ml TNT buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05 % TWEEN 20)
Incubate 2 times 20 min at 55°C

At this step either wash the filters once in plastic wells with 5ml double distilled H₂O and allow them to dry at 37°C until complete dehydration and repeat a second hybridization with the other fluorochrome or follow the protocol.

B6. DAPI labeling

Put 10µl of DAPI <5µg/ml on coated slide on filter, cells on top
Incubate for 15 min at room temperature in the dark

B7. Wash

Immerse the filter (cells on top) in plastic wells in 5ml double distilled H₂O
Incubate 10 min at room temperature in dark
Allow the filter to dry on slide at 37°C

B8. Mount slide for epifluorescence observations

Cover filter (cells on top) with 20 µl of antifading Citifluor agent.
Cover with cover slip and seal with varnish

The slides are then kept at 4°C in dark and can be further analyzed and observed after at least several weeks.

FISH/TSA Protocol for marine cyanobacteria (method taken from 'Identification and Enumeration of *Prochlorococcus* sp. in Natural Communities by In Situ Hybridization Using 16S rRNA-Targeted Oligonucleotides and Tyramide Signal Amplification' Nyree J. West, Wilhelm A. Schönhuber, Rudolf I. Amann, Rosmarie Rippka and David J. Scanlan submitted).

Cell fixation of cultured isolates.

Prochlorococcus and *Synechococcus* cells (approximately 2 ml of liquid cultures) are pelleted by centrifugation (10,000 × g, 5 min) and resuspended in 200 µl PBS (130 mM sodium chloride, 10 mM sodium phosphate buffer [pH 7.2]). Fixation is performed either by mixing immediately with 1 vol ethanol (96%) or by adding paraformaldehyde (4% in

PBS) to a final concentration of 3% v/v and incubation for 1 hr on ice. In the latter case, the cells are washed once with PBS, resuspended in the same buffer and then mixed with 1 vol of ethanol (96%). The fixed samples are stored at -20°C .

Sampling and fixation of natural samples.

Water samples are collected from discrete depths and aliquots (1-50 ml) were immediately fixed by adding glutaraldehyde to a final concentration of 0.1% prior to freezing in liquid nitrogen.

In situ hybridization.

Cultured isolates of the genera *Prochlorococcus* and *Synechococcus* are prepared for whole cell hybridization as described in Schönhuber, W., B. Zarda, S. Eix, R. Rippka, M. Herdman, W. Ludwig, and R. Amann. 1999. In situ identification of cyanobacteria with horseradish peroxidase-labeled, rRNA-targeted oligonucleotide probes. *Appl. Environ. Microbiol.* **65**:1259-1267, including incubation with different concentrations (1 and 5 mg/ml) of lysozyme for 30 min at 37°C . For in situ hybridization of natural communities, aliquots (400 μl) of the glutaraldehyde fixed samples are filtered onto polycarbonate filters using a filter tower with a diameter of 5 mm; the filters are then washed once with 1 ml PBS and divided into quarters. Further treatment is as described for cultured strains (see ref. above), except that the first ethanol dehydration step is omitted. Hybridization of HRP-labeled probes was detected by incubation with either the non-fluorescent substrate TETON or with the fluorescent substrate fluorescein-tyramide for 10-60 min. For hybridization of a mixture of two different strains, hybridizations are performed successively with the appropriate probes. Detection was achieved by incubation for different periods of time using fluorescein-tyramide as the first substrate (40 min incubation) and tetramethylrhodamine-tyramide as the secondary substrate (90 min incubation) since the signal from the former is greater. Slides are mounted in a 4:1 mixture respectively of Citifluor and Vectashield. Slides are examined using a Zeiss Axiophot2 microscope equipped for epifluorescence with a high pressure mercury bulb (100W) and specific filter sets 01, 09 and 15 (Carl Zeiss) and Chroma HQ 41007 (Chroma Tech. Corp, Brattleboro, Vermont).

LIST OF PARTICIPANTS

Didier ALAZARD

IRD 163 Avenue de Luminy, 13288 Marseille cedex 9, Marseilles France

email: Didier.alazard@esil.univ-mrs.fr

Phone: +33 4 91 82 85 73 Fax: +33 4 91 82 85 70

Adrian BARBROOK

Dept of Biochemistry, University of Cambridge, Building O, Downing Site, Tennis Court Road, Cambridge, CB2 1QW, UK

email: acb18@mole.bio.cam.ac.uk

Phone: +44 1223 333687 Fax: +44 1223 333345

Talk title:

Susana BENLLOCH

Universidad Miguel Hernandez. Division Microbiologia Ctra Valencia, km 87. San Juan 03550. Alicante.Spain

email: susana.benlloch@umh.es

Phone: +34 96 590 9313 Fax: +34 96 590 9457

Isabelle BIEGALA

Station Biologique de Roscoff Place Georges Teissier, 29680 Roscoff, France

email: biegala@sb-roscoff.fr

Phone: +33 2 98 29 23 70 Fax: +33 2 98 29 23 24

Jean BLANCHOT

Antenne IRD (ex ORSTOM)

Station Biologique de Roscoff Place Georges Teissier, 29680 Roscoff, France

Email: blanchot@sb-roscoff.fr

Phone +33 2 98 29 23 14 Fax +33 2 98 29 23 24

Tonje CASTBERG

Dept of Microbiology, Box 7800, University of Bergen, 5020 BERGEN,, Norway

email: tonje.castberg@im.uib.no

Phone: +47 55 58 46 44 Fax: +47 55 58 96 71

Henry CAUCHIE

CRP-GL CREBS, 162a avenue de la Faiencerie

email: cauchie@crp.gl.lu

Phone: +35 24 70 26 14 30 Fax: +35 24 66 64 44 13

Loic CHARPY

IRD, COM, rue de la Batterie des Lions, 13007 Marseille, France

email: lcharpy@com.univ-mrs.fr

Phone: +33 4 91 04 16 50 Fax: +33 4 91 04 16 35

Claude COURTIES

Laboratoire ARAGO BP 44 66651 Banyuls-sur-mer Cedex France

email: courties@obs-banyuls.fr

Phone: +33 (0)4 68 88 73 53 Fax: +33 (0)4 68 88 73 98

Frida Lise DAAE

Dept. of Microbiology, University of Bergen, Jahnebakken 5, PB 7800, N-5020 Bergen, Norway.

email: frida.daae@im.uib.no

Phone: +47 55 58 26 63 Fax: +47 55 58 96 71

Beatriz DÍEZ

Institut de Ciències del Mar, CSIC Passeig Joan de Borbó S/N 08039 Barcelona, Catalonia, Spain

email: bdiez@icm.csic.es

Phone: +34 39 32 21 64 16 Fax: +34 39 32 21 73 40

Bente EDVARDSEN

Section for Marine Botany, Univ. of Oslo, P.O. Box 1069 Blindern, 0316 Oslo, Norway

email: bente.edvardsen@bio.uio.no

Phone: +47 22 85 45 31 Fax: +47 22 85 44 38

Wenche EIKREM

Section for Marine Botany, University of Oslo, Department of Biology P.O. Box 1069, Blindern, 0315 Oslo, Norway

email: wenche.eikrem@bio.uio.no

Phone: +47 22 85 45 31 Fax: +47 22 85 44 38

Bert ENGELN

GBF - Division of Microbiology, Mascheroder Weg 1, D-31824 Braunschweig, Germany.

email: ben@gbf.de

Phone: +49 53 16 18 14 40 Fax: +49 53 16 18 14 11

Marie-Laure FARDEAU

IRD Marseilles, 163 Avenue de Luminy, case 925, 13288 Marseille cedex 9 France

email: fardeau@esil.univ-mrs.fr

Phone: +33 (0) 4 91 82 85 76 Fax: +33 (0)4 91 82 85 70

Héloïse FELMAN

Station Biologique de Roscoff Place Georges Teissier, 29680 Roscoff, France

Email: felman@sb-roscoff.fr

Phone +33 2 98 29 23 70 Fax +33 2 98 29 23 24

Michael FERRIS

Dept. Microbiol., Montana State University, 109 Lewis Hall, Bozeman, MT, 59715 USA

email: mferris@montana.edu

Phone: (406) 994 3412 Fax: (406) 994 4926

Laure Guillou

IFREMER Centre de Brest, DRV/VP/CMM, BP 70, 29 280 Plouzané, France.

email: Laure.Guillou@ifremer.fr

Phone: +33 2 98 22 45 53 Fax: +33 2 98 22 47 57

Richard HOWARTH

Dept. of Biological Sciences, University of Warwick, Gibbet Hill Road, Coventry, CV4 7AL, UK.

Email:

Phone +44 24 76 52 25 72 Fax +44 24 76 52 37 01

Jan KASTOVSKY

Faculty of Biological Sciences, University of South Bohemia, Branisovska 31, 370 05 Ceske Budejovice, Czech Republic

email: tichym@tb.bohem-net.cz

Phone: +42 03 33 72 11 01 Fax: +42 03 33 72 12 46

Wiebe KOOISTRA

Marine Biology, RuG Postbox 14, 9750 AA Haren (GN), The Netherlands

email: w.h.f.c.kooistra@biol.rug.nl

Phone: +31 50 36 32 25 8 Fax:

Mikel LATASA

Institut de Ciències del Mar, CSIC Passeig Joan de Borbó S/N 08039 Barcelona, Catalonia, Spain

email: latasa@icm.csic.es

Phone: +34 39 32 21 64 16 Fax: +34 39 32 21 73 40

Florence LE GALL

Station Biologique de Roscoff Place Georges Teissier, 29680 Roscoff, France

Email: felman@sb-roscoff.fr

Phone +33 2 98 29 23 70 Fax +33 2 98 29 23 24

Philippe LEBARON

Laboratoire ARAGO BP44 66651 Banyuls-sur-mer Cedex France

email: lebaron@arago.obs-banyuls.fr

Phone: +33 (0)4 68 88 73 53 Fax: +33 (0)4 68 88 73 95

Dominique MARIE

Station Biologique de Roscoff Place Georges Teissier - 29682 Roscoff Cedex, France

email: marie@sb-roscoff.fr

Phone: +33 2 98 29 23 72 Fax: +33 2 98 29 23 24

Terence MARSH

Ctr. Microbial Ecology, 41 Giltner Hall, Michigan State University, East Lansing, Michigan, USA

email: MARSHT@pilot.msu.edu

Phone: 517 432 1365 Fax: 517 432 3770

Ramon MASSANA
Institut de Ciències del Mar, CSIC Passeig Joan de Borbó S/N 08039 Barcelona,
Catalonia, Spain
email: ramonm@icm.csic.es
Phone: +34 9 32 21 64 16 Fax: +34 9 32 21 73 40

Linda MEDLIN
Alfred-Wegener-Institute for Polar and Marine Research. Am Handelshaven 12, D-27570
Bremerhaven, Germany.
email: imedlin@awi-bremerhaven.de
Phone: +49 471 4831 1443 Fax: +49 471 4831 1425

Helga MEHL
Alfred-Wegener-Institute for Polar and Marine Research. Am Handelshaven 12, D-27570
Bremerhaven, Germany.
email: hmehl@awi-bremerhaven.de
Phone: +49 471 4831 1556 Fax: +49 471 4831 1425

Maria Angela MUGNAI
Centro Studio Microrganismi Autotrofi-CNR, P. le Cascine, 27 50144 Florence, Italy
email: m.mugnai@csma.fi.cnr.it
Phone: +39 05 53 28 83 40 Fax: +39 05 53 30 431

Adrian MUNGUIA VEGA
Marine Phatology (CIBNOR), Km 1 Carretera San Juan de la Costa "El Comitán" A.P.
128. La Paz, B.C.S. 23000 Mexico
email: airdrian@cibnor.mx

Gerard MUYZER
Netherlands Institute for Sea Research (NIOZ) Den Burg, Texel, P.O. Box 59, The
Netherlands
email: gmuyzer@nioz.nl
Phone: +31 222 369 521 Fax: +31 222 319 674

Olivier NERCESSIAN
LEMAR-CNRS UMR 6539, Institut Universitaire Européen de la Mer (IUEM),
Université de Bretagne Occidentale (UBO), Place Nicolas Copernic, Technopole Brest-
Iroise, 29280 Plouzané, FRANCE
email: olivier.nercessian@univ-brest.fr
Phone: +33 2 98 49 87 79 Fax: +33 2 98 49 87 05

Fabrice NOT
Station Biologique de Roscoff Place Georges Teissier, 29680 Roscoff, France
email: not@sb-roscoff.fr
Phone: +33 2 98 29 23 70 Fax: +33 2 98 29 23 24

Luisa ORSINI
Marine Botany, Stazione Zoologica, Villa Comunale, 80121 Napoli, Italy

email: orsini@alpha.szn.it

Phone: +39 08 15 83 32 59 Fax: +39 08 17 64 13 55

Brian PALENIK

Scripps Institute of Oceanography, Univ. of Calif. San Diego, La Jolla, CA 92093-0202 USA.

email: bpalenik@ucsd.edu

Phone: (858) 534 7505 Fax: (858) 534 7313

Frederic PARTENSKY

Station Biologique de Roscoff Place Georges Teissier, 29680 Roscoff, France

email: partensk@sb-roscoff.fr

Phone: +33 2 98 29 23 14 Fax: +33 2 98 29 23 24

Carlos PEDRÓS-ALIÓ

Institut de Ciències del Mar, CSIC Passeig Joan de Borbó S/N 08039 Barcelona, Catalonia, Spain

email: cpedros@icm.csic.es

Phone: +34 9 32 21 64 16 Fax: +34 9 32 21 73 40

Anton POST

H. Steinitz Marine Biology Laboratory, The Interuniversity Institute for Marine Science, Coral Beach, P.O. Box 469, 88103 Eilat, ISRAEL

Email: anton@vms.huji.ac.il

Phone: (972) 76 36 01 22 Fax: (972) 76 37 43 29

Pirjo RAJANIEMI

Department of Chemistry and Microbiology, Helsinki University, P.O.Box. 56, FIN-00014 Helsinki, Finland.

email: pirjo.rajaniemi@helsinki.fi

Phone: +358 9 19 15 93 07 Fax: +358 9 19 15 93 22

Anne RANTALA

Department of Applied Chemistry and Microbiology, University of Helsinki, P.O.Box 56, Biocenter 1 A, Viikinkaari 9, FIN-00014

email: Anne.Rantala@helsinki.fi

Phone: +358 9 19 15 92 72 Fax: +358 9 19 15 93 22

Khadidja ROMARI

Station Biologique de Roscoff Place Georges Teissier, 29680 Roscoff, France

email: romari@arpb.univ-montp2.fr

Phone: +33 2 98 29 23 14 Fax: +33 2 98 29 23 24

Dave SCANLAN

Dept. of Biological Sciences, University of Warwick, Gibbet Hill Road, Coventry, CV4 7AL, UK.

email: dp@dna.bio.warwick.ac.uk

Phone: + 44 24 76 52 83 63 Fax +44 24 76 52 37 01

Renate SCHAREK

Alfred-Wegener-Institute, BAH am AWI Postfach 180, D-27483 Heligoland, Germany

email: rscharek@awi-bremerhaven.de

Phone: +49 47 25 81 92 68 Fax: +49 47 25 81 92 68

Nathalie SIMON

Station Biologique de Roscoff Place Georges Teissier, 29680 Roscoff, France

email: simon@sb-roscoff.fr

Phone: +33 2 98 29 23 70 Fax: +33 2 98 29 23 24

Sara SJOLING

Dept Biochem and Molbiol, University College London, Gower St, London WC1E 6BT

email: s.sjoling@ucl.ac.uk

Phone: +44 020 76 79 22 37 Fax: +44 020 76 79 71 93

Christoph TEBBE

Institute for Agroecology, FAL (Federal Research Center for Agriculture), Bundesallee 50, 38116 Braunschweig, Germany

email: tebbe@kepler.dv.fal.de

Phone: +49 531 59 67 36 Fax: +49 531 59 63 66

Jahn THRONDSSEN

Department of Biology, University of Oslo P. Box 1069 Blindern, 0316 Oslo, Norway

email: jahn.throndsen@bio.uio.no

Phone: +47 22 85 45 26 Fax: +47 22 85 44 38

Martin TICHY

Laboratory of Photosynthesis, Institute of Microbiology. Faculty of Biological Sciences, University of South Bohemia, Branisovska 31, 370 05 Ceske, Budejovice, Czech Republic

email: tichym@tb.bohem-net.cz

Phone: +42 03 33 72 11 01 Fax: +42 03 33 72 12 46

Klaus VALENTIN

Alfred-Wegener-Institute for Polar and Marine Research. Am Handelshaven 12, D-27570 Bremerhaven, Germany.

email: kvalentin@awi-bremerhaven.de

Phone: +49 471 48 31 14 52 Fax: +49 471 48 31 14 25

Daniel VAULOT

Station Biologique de Roscoff Place Georges Teissier, 29680 Roscoff, France

email: vaulot@sb-roscoff.fr

Phone: +33 2 98 29 23 34 Fax: +33 2 98 29 23 24

Ricardo VAZQUEZ JUAREZ

CIBNOR, Biotechnology of Marine Organisms, Mar Bermejo 195 Col. Playa Palo Santa Rita, La Paz, BCS, CP 23 090 Mexico

email: rvazquez@cibnor.mx

Phone: +52 112 5 36 33 x 3804 Fax: +52 112 5 47 10 or +52 112 5 36 25

Nyree WEST

Dept. of Biological Sciences, University of Warwick, Gibbet Hill Road, Coventry, CV4 7AL, UK.

email: mmqw@dna.bio.warwick.ac.uk

Phone: + 44 24 76 52 25 72 Fax +44 24 76 52 37 01

Annick WILMOTTE

Algology Laboratory, Dept Botany B22, Univ Liege, B-4000 Liege, Belgium

email: awilmotte@ulg.ac.be

Phone: +32 43 66 38 56 Fax: 32 43 66 28 53

Dorothea ZANDVLIET

St George's Hospital Medical School, Cranmer Terrace, Tooting Broadway, London

email: d.zandvliet@sghms.ac.uk

Phone: +44 20 87 25 26 83 Fax: +44 20 86 72 02 34