ANNEX I

"DESCRIPTION OF WORK"

Monitoring biodiversity of picophytoplankton in marine waters

PICODIV

PROPOSAL: EVK2-1999-00119

CONTRACT:

COORDINATOR:

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CONTRACTORS:

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1 CONFIDENTIAL SUMMARY

Picophytoplankton (cells less than 3 µm) dominate primary productivity in most marine ecosystems. However very little is known of their biodiversity or how it is affected by ecosystem changes. The objective of this proposal is to develop molecular tools to quantify and monitor picophytoplankton biodiversity. These tools will rely on the gene coding for ribosomal RNA whose sequence allows to precisely determine the taxonomic position of an organism. We will assemble a very large sequence database for picophytoplankton both from cultures and from natural samples. Using this database, we will design probes against all major picophytoplankton taxonomic groups. In parallel, we will develop fast molecular methods to detect these probes, such as quantitative PCR or DNA arrays. These methods will be used to monitor changes in coastal picophytoplankton biodiversity and validated against other methods (pigment analyses, electron microscopy).

2 SCIENTIFIC/TECHNICAL OBJECTIVES AND INNOVATION

2.1 Objectives

2.1.1 Background

Picoplankton (defined operationally hereafter as cells that pass through a 3 µm filter) dominate the photosynthetic biomass in many marine ecosystems, not only in the very oligotrophic regions of the world oceans, such as the central Pacific gyre or the Eastern Mediterranean Sea, but also in mesotrophic areas, such as the high chlorophyll - low nutrient equatorial regions. However, picophytoplankton are clearly not exclusively restricted to pelagic environments. In many coastal regions, they are present throughout the year and constitute a 'background' population, onto which episodic phenomena such as the spring bloom develops. In some environments, such as coastal lagoons, picoplankton are a major component of biomass and productivity for most of the year. Picophytoplankton are also very relevant from the human point of view, since some bloom-forming picoplankters such as *Aureococcus* spp. are toxic.

Photosynthetic picoplankton encompass both prokaryotic and eukaryotic species:

- Prokaryotes. Only two major genera are important for the picoplanktonic community in marine waters: *Synechococcus* (Waterbury et al. 1979) and *Prochlorococcus* (Chisholm et al. 1988). Whereas *Prochlorococcus* dominates over *Synechococcus* in most oligotrophic regions, except at high latitudes, the reverse is true under meso- and eutrophic conditions. With such wide ecological distributions, these two genera display a large genetic and phenotypic variability, that is just beginning to be assessed.
- Eukaryotes. In contrast to prokaryotes, the taxonomic diversity of picophytoplanktonic eukaryotes is much broader. In fact, nearly every algal phylum has picoplanktonic representatives. Still, a vast number of taxa remain unknown and undescribed.

To date fewer than 30 species of picophytoplankton have been described. This number pales in comparison to the more than 4,000 marine phytoplankton species that have been described to date and to the over 100,000 that are believed to exist. A clear proof of our poor knowledge of picophytoplankton diversity is revealed by the discovery of three novel algal classes in the last ten years described from picophytoplanktonic taxa (to put this into perspective, to ignore an algal class corresponds to ignoring the existence of mammals or birds among vertebrates):

1991 class Pedinophyceae based on *Resultor mikron* 2 µm
1993 class Pelagophyceae based on *Pelagomonas calceolata* 2 µm
1999 class Bolidophyceae based on *Bolidomonas pacifica* 1.5 µm

Because so little is known about the taxonomy and systematics of picophytoplankton we have very little data to estimate the levels of its biodiversity under natural conditions and how the picophytoplankton is affected by environmental variability linked to either anthropogenic influence or to larger scale phenomena such as those

linked to climate change or global warming. However we have some indications that picophytoplankton species (and therefore picophytoplankton biodiversity) may react sharply to changes in marine systems:

- The prokaryote *Prochlorococcus* consists of at least two different genotypes/phenotypes, each one dominates under different environmental conditions: i.e., one is present under the high light/low nutrient conditions of the marine surface layer, and the other under the low light/higher nutrient conditions of the bottom of the euphotic zone. Thus *Prochlorococcus* is able to partition its niche genetically so that it is phenotypically adapted to its environment.
- The abundance of *Synechococcus* in the equatorial Pacific responds to climatic El Niño events.
- The potentially toxic brown picoplanktonic alga *Aureococcus* was unknown before 1985, but since then it has bloomed repeatedly in US coastal waters.

Our ignorance concerning picophytoplankton diversity is mostly explained by the fact that, because of their very small size, pico-phytoplankton cells most often lack any distinguishing features and are very difficult to identify by classical methods. In fact many have evolved to small "green or brown ball" morphotypes that mask a broad taxonomic diversity. Our present state of knowledge regarding picophytoplanktonic biodiversity is in fact analogous to that prevailing ten years ago for aquatic bacteria. Until the early 1990's, the taxonomy and understanding of bacterial diversity was based primarily on species isolated into culture. No-one could have predicted the vast diversity of these organisms in nature.

2.1.2 Objectives

In order to remedy to this very poor state of knowledge concerning a group that, in many ecosystems, accounts for 60 to 80% of photosynthetic biomass and production, there is a very urgent need to develop efficient monitoring tools of picophytoplankton diversity. This problem is in fact very analogous to that encountered by microbiologists that cannot tell apart bacteria based on their shape or even on their metabolic requirements. The latter have relied more and more in recent years on molecular biology techniques to identify and detect bacteria in the environment. We plan during the course of this project to expand this very successful approach to picophytoplankton.

Therefore the major objective of this project is to:

Develop, test and validate probing methods based on molecular biology techniques that allow for routine and extensive assessment of picophytoplankton diversity (species composition and relative contribution of taxa to total community) in the marine environment.

The method we will develop can be streamlined as follows:

- Take a marine sample
- Filter cells and eventually extract DNA or RNA.
- Apply a set of probes (i = 1…N) each recognizing a specific picophytoplankton taxon, e.g., class, genus or species
- Measure fraction of population recognized by each probe (f_i) using high throughput detection methods
- Compute picophytoplankton biodiversity.

2.2 Innovation

The greatest difficulty with identifying picophytoplankton in culture, and even more critically in the marine environment, lies in their very small size and their absence of notable features. A number of methods (Table 1) are available to assess picophytoplankton taxonomical composition from which biodiversity can be computed (as a reminder, biodiversity estimates must rely on sound taxonomy).

Table 1: Methodologies available for the assessment of picophytoplankton diversity in marine samples

Methodology	Output	Taxonomic resolution	Analysis speed	Remark
Culture	List of major cultivable taxa	Species	Very slow	Very strong bias due to selectivity of culturing media and conditions
Electron microscopy	Cell number for each identifiable taxa	Species	Slow	Sample preparation and analysis very long Subject to loss Some cells cannot be identified (coccoid)
Pigment analysis	% of Chlorophyll a for each taxa	Class	Medium	Depends on hard to verify hypotheses (carotenoid/Chl a ratio must be known for each class)
Flow cytometry	Cell number	Domain	High	Very low taxonomic resolution
SSU rDNA direct sequencing	List of taxa	Species	Slow	Biases linked to PCR and cloning
SSU rDNA DGGE	Number of taxa	???	Medium	Gives only a fingerprint of diversity
SSU rDNA probes	% of total DNA or cell number for each target taxa	Species	High	Very flexible Fully developed below

All these methodologies have their advantages and drawbacks. Some have low taxonomic resolution (flow cytometry, pigment analysis) but allow to process a large number of samples quickly. Others offer very high taxonomic resolution but only an extremely small number of samples can be processed for a given study (electron microscopy). Molecular techniques appear clearly to offer the best solution. As will be detailed in the next paragraph, they offer an excellent taxonomic resolution and even more interestingly one can choose the taxonomic level targeted. Among the different molecular techniques, the oligonucleotide probe approach seems by far the most appropriate, especially since recent technological developments derived from the push for large scale genome sequencing promise to increase dramatically sample throughput as well as quantification aspects.

The small sub-unit ribosomal RNA gene (**SSU rDNA**) has become the universal taxonomic yardstick for bacteria and eukaryotes alike, especially because of the huge data base available to date (6205 sequences for prokaryotes and 2054 for eukaryotes in the latest release from mid 1998 of the Ribosomal Data Base project). A comparison of their sequences allows to unambiguously place an unknown organism at its correct taxonomic position.
Once the SSU rDNA sequence of existing or novel taxa has been established, one can design small oligonucleotide probes (between 15 and 20 nucleotides long) that recognize only certain taxa. The vast amount of rapidly accumulating sequence data provides an enormous base from which oligonucleotide sequences of varying specificity can be found. The beauty of it is that one may target virtually any level of taxonomy, from the class down to the species. Because probes can be coupled with a variety of reporter molecules (fluorescent, chemi-luminescent or radioactive), detection can be achieved in many different ways (fluorescent microscopy, flow cytometry or membrane hybridization). This approach has revolutionized the field of microbiology. For phytoplankton, only a limited numbers of probes are available, some of them at the class level (e.g. Prymnesiophyceae) and some of them at the species level (e.g. for toxic species). For the picophytoplankton size fraction, this approach has begun to be applied to both eukaryotes and prokaryotes. For example, sequence data of the numerically dominant prokaryote *Prochlorococcus*, SSU rRNA has already been used to identify genotype-specific oligonucleotides. Using such specific probes a niche-partitioning of *Prochlorococcus* genotypes from environmental samples has been observed, either adapted to high-light/low-nutrient surface waters or low-light/ high-nutrient deeper waters. However these studies remain very marginal.

There are several very novel aspects in this project. One of them is the focus on picophytoplankton that has received so little attention until now. Another is the design of a large number of probes for eukaryotes, something not done yet at least to our knowledge. However we feel that the major innovation of the project will consist in developing techniques that allow us to use oligonucleotides probes on a much wider scale than has been done before.
The use of probes to assess the biodiversity of natural samples requires the use of very efficient, very sensitive and very quantitative methods of detection. Table 2 summarizes some of the aspects of the methods we intend to develop and use in this project. DNA chips for example will be very suitable to detect a very large number of taxa in a limited number of samples, allowing an extremely precise description of the community present. Moreover, DNA chips are probably the most suited tool to assess biodiversity from a monitoring point of view by environmental agencies. On the other hand, if one is interested to detect a specific organism (for example a toxic species) in a very large number of samples, then quantitative PCR will be most appropriate.

Table 2: Techniques that will be used in the project for probe detection.

Characteristics in terms of number of probes (small ˜ 10; large ˜ 100; very large ˜ up to 10,000) and number of samples (medium ˜ 100; large ˜ 1000) that can be measured simultaneously and possibility to quantify the result (low means presence vs. absence, while very high means a large dynamical range (1 to 1000) .

Methodology	Probes #	Samples #	Quantification	Cost	Availability
Fluorescent *in situ* hybrization	Small	Medium	Low	Low to medium	Available now
Probe arrays					
High density membranes	Large	Medium	Medium	Low to medium	Available now
DNA chips	Very large	Medium	High	High	To be developed
Quantitative PCR	Small	Large	Very high	Medium	To be developed

3 PROJECT WORKPLAN

3.1 Introduction

3.1.1 General strategy

Our strategy to meet the objectives outlined above is encapsulated in the following four steps:

1. Obtain SSU rDNA sequences for as many as possible picophytoplankton taxa from both cultures and natural samples (Wpk 1 and 2)
2. Using this sequence database, develop hierarchical probes recognizing each taxonomic group having picophytoplanktonic representatives (Wpk 3)
3. Develop fast and efficient techniques to quantify the fraction of the pico-phytoplankton recognized by the probes in natural samples (Wpk 4)
4. Test and validate these probes on time series of picophytoplankton biodiversity (Wpk 5)

3.1.2 Detailed strategy

In this project, we will focus on the picophytoplankton from **coastal European waters** that have been much less studied in comparison to those of offshore oligotrophic waters. For this purpose we have selected three sites located in the following regions:

- English Channel (Roscoff)
- North Sea (Helgoland)
- Western Mediterranean Sea (Bay of Blanès)

These sites have been carefully selected as offering a wide range of environmental conditions representative of EU coastal waters. Moreover, all have been extensively monitored in the past and abundant background information is available on environmental conditions as well as phytoplankton populations. One of them (North Sea site) has been designated as a flagship site for long-term and large-scale marine biodiversity research at a recent European meeting on biodiversity.

Although these three sites will serve as focal points for our project, we will also take advantage of oceanographic cruises planned outside this project to examine the diversity of picophytoplankton in other environments. In particular we plan to sample the following ecosystems:

- Mediterranean Sea
- Red Sea
- North Atlantic Ocean
- Antarctic Ocean
- Arctic Basins

First, for probe design we need to obtain SSU rDNA sequences covering the full taxonomic spectrum of picophytoplankton. For this purpose, we will adopt a two pronged approach:

a - We will obtain sequences from **fully characterized laboratory strains (Wpk. 1)**. We will secure all picoplanktonic strains available from international culture collections such as the CCMP (Center for Cultures of Marine Phytoplankton, Bigelow USA). However, we know that such collections only feature a limited number of picoplanktonic strains, because very little effort has been devoted to this size class to date. Therefore we need to embark on a very strong effort of strain isolation. For this purpose, we will establish cultures (see next part for method description) of both prokaryotic and eukaryotic picophytoplankton from the environments listed above using methods that have already proved very successful for this purpose (prefiltration of natural samples, monitoring of cultures by flow cytometry). Once established, the cultures will be screened by a variety of techniques (flow cytometry, electron microscopy, pigment analysis, molecular methods) to assess their taxonomic position. Those that obviously contain novel taxa will be further purified by dilution or plating and more fully studied (electron microscopy sections), sequenced and described formally.

b - As we know that a large number of planktonic organisms still escape culture due to the lack of optimum culture conditions, we will also use the molecular approach that has been so successful for bacteria, i.e. **environmental ribosomal RNA gene cloning** and sequencing **(Wpk. 2)**. These sequences will be obtained from the same environments from which we obtained cultures (Wpk. 1). It is highly likely that this will reveal novel groups that we can then target for culturing.

Second, using the sequence database obtained both from cultures and natural samples, we will design **hierarchical probes (Wpk. 3)** for each taxonomic level containing picophytoplanktonic representatives (e.g.

classes such as the Pelagophyceae or species such as *Micromonas pusilla*). These probes will be validated against strains obtained in Wpk 1 and 2.

Third, we will develop **methods** (**Wpk. 4**) to assess the fraction of the marine pico-phytoplankton recognized by a given probe. We will focus on very recent techniques allowing quantitative and extensive probe measurements (fluorescent in situ hybridization, probe array, quantitative PCR).

Fourth, we will apply these methods to determine **time series of picophytoplankton diversity** (**Wpk. 5**) during a full year at our three coastal sites (English Channel, North Sea, Mediterranean Sea). At the same time, the composition and abundance of the picophytoplankton will be studied with more conventional techniques, such as electron microscopy or pigment analysis, and alternate molecular methods (DGGE). These data will permit to validate the data obtained by the molecular probe approach. We will interpret then the biodiversity patterns as a function of the other environmental parameters of the site sampled. We will determine in particular whether there is a succession of groups and species (as is the case for the larger nano and micro-phytoplankton) or whether a small group of ubiquitous species are always present and merely change their abundance (but not their diversity) in response to environmental changes.

3.1.3 Methods to be used in project: rationale and description

3.1.3.1 Cultures.

In order to describe new taxa it is absolutely necessary to establish cultures of the species in question. Moreover, unialgal cultures are crucial for the study of their physiology as well as their potential use for biotechnological applications. Although media for phytoplankton cultures are well established (e.g. K medium for oceanic phytoplankton, Keller et al. 1987), cultivation approaches for picophytoplankton require very specific precautions, such as pre-filtration of sea water, low levels of initial enrichment and continuous monitoring of cultures with methods, such as flow cytometry (see next paragraph) and microscopy.
In this project we will use three types of cultures:

- **Pre-cultures**. This is the first stage established directly from the natural sample. The aim here is to maximize the culture conditions (media, light etc…) to obtain the largest spectrum of organisms.
- **Cultures**. After a few weeks to a few months growth, pre-cultures are screened quickly to find interesting organisms. Pre-cultures are then purified to establish long-term cultures. The aim here is to obtain uni-algal cultures that are stable over time.
- **Strains**. After a few months of reliable growth, cultures are screened again but this time with more sophisticated tools (in particular HPLC, molecular methods - see below) to find novel organisms. At this stage, one tries to establish strains with a pure genotype starting from a single cell either by infinite dilution or plating on solid medium. Strains will serve as the basis for the formal description of novel taxa and will be deposited in reference culture collections.

3.1.3.2 Flow cytometry (FCM)

Flow cytometry was initially designed for cell biology and clinical applications. It allows to determine the abundance and parameters such as size or fluorescence of cell populations at a very high rate (typically several hundred cells per second). Due to the very small size of cells, FCM is in general the only way to monitor efficiently and reliably cells both in the marine environment and in culture. However, when used alone, i.e. without labeling cells with probes, it has a very limited taxonomic resolution for picophytoplankton, separating at best 2 types of prokaryotes (*Prochlorococcus* vs. *Synechococcus*) and 3-4 types of eukaryotes. FCM will be used in this project to assess picoplankton populations in the field and in culture but also to physically sort cells of interest to obtain cultures and novel organisms for sequencing.
We will use FACSort/FACScan flow cytometers already available in SBR and CSIC laboratories, as well as a FACSvantage to be purchased by AWI. We will use standard detection techniques used in oceanography based on photosynthetic pigment fluorescence detection as well as nucleic acid staining (e.g. Olson et al. 1991)

3.1.3.3 Electron microscopy

Electron microscopy is critical at two stages. First, preparation of **whole mounts** (Moestrup and Thomsen 1980; Moestrup 1984) allows for rapid screening of cultures at an initial stage (presence/absence of scales and flagella, type of flagella if present). When the cultures are established routine examination at certain intervals is desirable to observe possible changes of stage in the life cycle and contaminants in the cultures. Whole mounts are also the only way to identify the morphology of picophytoplankton in the field. Second, **thin section** preparations (e.g. Birkhead and Pienaar 1994) are crucial when describing a new taxon for the

revelation of organelles and characters of taxonomic importance (eg. number of membranes surrounding the chloroplast, flagellar apparatus).

3.1.3.4 Pigment analysis by HPLC

Pigment signature is a key taxonomic tool to assess the class to which a photosynthetic organism belongs. For prokaryotes pigment analysis is critical to assess the phenotype of genera such as *Prochlorococcus* (Chl *b/a* ratio). In this project, pigment analysis will be used as a screening tool. Also, detailed pigment identification will be absolutely necessary for the final description of novel taxa. Pigment analysis will also be used to obtain an estimate of biodiversity in field samples (Wpk. 5) at the class level.

In this project, picophytoplankton pigments extracted in 90% acetone will be chromatographically separated using the standard HPLC methods. Individual pigments will be identified and quantified by comparing their chromatographic retention time and spectral characteristics with those of commercially available standards (Water Quality Institute, Denmark).

3.1.3.5 Gene sequencing

Gene sequencing will be a central technique in this project since it will allow to obtain the large sequence database of the SSU rDNA genes necessary for the project. We will apply this method to cultures and also directly to field samples in order to discover taxonomic entities that, until now, have escaped detection by culture or direct observation.

In this project, DNA will be extracted from cultures and natural samples using standard protocols (Fuhrman et al. 1988). DNA will be amplified using universal eukaryotic or prokaryotic primers targeted to the 5' and 3' prime ends of the gene of interest (in most cases SSU rDNA). Amplicons will be cloned in suitable vectors and colonies will be screened using either of several techniques (single base sequencing, DGGE, RFLP). Unique clones will be sequenced in both directions using an automatic sequencer. Sequence will be aligned and inserted in a data base maintained by SBR and available to all participants.

3.1.3.6 DGGE of SSU rDNA

Although SSU rDNA sequencing is the most comprehensive approach for phylogenetic analysis, rapid screening of isolates or rapid assessment of diversity in natural samples requires a different approach. Denaturing gradient gel electrophoresis (DGGE) is a method that permits the separation of gene fragments of the same size but with different base composition. Bands of interest can be eluted from the gel and sequenced for quantitative assessment. It is an extremely useful technique for detecting qualitative changes in diversity. It has been extensively used for prokaryotes (Muyzer and Smalla 1998), including photosynthetic prokaryotes (West and Scanlan 1999), but again is virtually unexplored for eukaryotes (van Hannen et al. 1998). We will use DGGE to screen culture, to assess their purity (Wpk. 1), and to obtain an independent estimate of biodiversity in field samples (Wpk. 5)

In this project, DNA extracted from cultures and natural samples will be used as template in PCR reactions using both bacterial and eukaryotic primers. Priming regions will be selected to amplify a region of up to 500 bp of the SSU rDNA, the larger DNA fragment easily separated in a DGGE gel. PCR products will be loaded on a polyacrylamide gel with an increasing concentration of the denaturing agents urea and formamide. After electrophoresis, the gel will be stained with GelStar (FMC Bioproducts) and the image documented with a Polaroid camera or with an image analysis system. When necessary, the band can be cut from the gel, re-amplified with the same primers, and sequenced. Band patterns of natural communities will be used as fingerprints of those assemblages, and both the band position and intensity will be used to compare communities by cluster analysis.

3.1.3.7 Design of oligonucleotide probes against SSU rDNA and RNA

The successful design of rRNA-targeted probes for groups or species is subject to a few rules. First, only monophyletic groups should be targeted. It is impossible to find a probe specific for taxa such as *Chlorella* or *Chlamydomonas*, that are polyphyletic. Thus, a thorough knowledge of the phylogeny of the organisms of interest is a prerequisite. Second, sequence databases upon which the probes are designed should be representative of the complexity of the community to be investigated to insure that the probe is specific. The specificity of the probe can then be tested on cultures.

In this project, once phylogenetically consistent groups will be defined, probe design will be performed in practice, with the help of software packages, such as the ARB program package (http://www.biol.chemie.tu-muechen.de). These packages allow rapid screening of databases to identify phylogenetic "signatures". Localization of potential target sites in other sequences will be checked using the "PROBE_MATCH" function of ARB or the "CHECK_PROBE" function of RDP. Target site suitability will be checked by eye from partial sequence alignments provided by the output from these programs: the number, quality and localization of

mismatches between the target and non-target sequences are of prime importance. For best discrimination, probes should be 100% complementary to their target sites for the species selected as targets. Mismatches should ideally be placed in the center of the nucleotide probe. At least 2 mismatches are necessary for the probe to discriminate target from non target sequences (Amann et al. 1995). Because the number of sequences in databases is increasing rapidly, the suitability of the probes will be checked periodically. Probes will be validated against available cultures by dot blot hybridization.

3.1.3.8 Detection of oligonucleotide probes against SSU rDNA and RNA

Several approaches will be developed and used in parallel (see Table 2).

- **Fluorescent in situ hybrization (FISH).** In this approach, probes are labeled with a fluorescent molecule (fluorescein FITC, Cy3) and introduced directly into whole cells. Cells recognized by the probe fluoresce and can be detected either by microscopy or flow cytometry. The major advantages are the ability to visualize the morphology of target cells, and circumventing the need to use the PCR, and the biases associated with this method. Among the disadvantages are the necessity to permeate the cell membrane/wall to allow probe penetration and the difficulty to detect slowly growing cells that have few ribosomes. These characteristics make FISH suitable for prokaryotic picophytoplankton that has few cell types, allowing for relatively easy protocol development, but it is much harder to use for eukaryotes for which each class has a different cell wall structure and requires a specific protocol. In this project, we will restrict FISH use in field samples to prokaryotes using a recently developed fluorescent signal amplification method (TSA), that has proved successful for cyanobacteria. We will also test it on eukaryotes in cultures.
- **Probe arrays**
  - **Dot blot membranes**. The DNA dot blot technique is a quick and simple way to analyze the specificity of oligonucleotide probes. Denatured DNA, i.e. of PCR-amplified SSU genes, is blotted onto and fixed to a nylon membrane. The oligonucleotide probes can be labeled non-radioactively, i.e. with a digoxigenin (DIG) molecule, and hybridized to this membrane under stringent conditions. By applying a DIG-specific antibody with a covalently bound phosphatase enzyme and adjacent transformation of a chemiluminescent substrate, positive signals are only given by probes that bind specifically to the DNA. With this well-established detection system it is possible to analyze newly developed probes and to test their specificity against their target and non- target species efficiently. Using automated blotting machine, a large number of samples (50-60) can be blotted onto a membrane. The membranes can be challenged with a DIG- or radioactively- labeled probe, quantified for its relative chemiluminescent intensity, the probe stripped on the target RNA or DNA and re-probed with a different probe. Quantification is likely to be only relative but nevertheless distinct patterns in distribution can be revealed.
  - **DNA microchips**. This technology, only introduced in the last couple of years has rapidly spread to all domains of molecular biology in particular for gene expression studies. It consists of depositing a high density array (100 to 10,000 elements) of either oligonucleotides or DNA pieces onto a microscope slide. The DNA to be probed is then fluorescently labeled and hybridized to the chip. The fluorescence of each individual probe/target hybrid is then read by a laser scanning device. It has recently been applied to oligonucleotide probes directed against SSU rDNA of bacteria. The major advantages of this method are the possibility to assess several hundred probes at the same time with a small sample size required for hybridization. In this project, we do not plan to use extensively this novel and still expensive approach, but we will investigate its potential application to our problem.
- **Quantitative PCR with energy transfer-labeled primers**. This recently developed technique consists of using one specific probe as a PCR primer and labeling it with a fluorescent molecule and a quencher. When the probe is unbound, the fluorophore is quenched and does not fluoresce. When the probe is bound to amplified products the quencher is physically separated from the fluorophore which is then free to emit fluorescence. Alternatively, the DNA produced by the PCR reaction can be stained with a fluorescent dye such as SYBR I. The main advantages of this method are (1) the speed of the measurement (bulk fluorescence), which is made easier by the use of a relatively inexpensive PCR machine that can monitor fluorescence in real time, (2) its excellent linearity and (3) its larger dynamical range. It has already been tested using phylogenetic probes.

3.2 Detailed project description

3.2.1 Workpackage list

Wpk #	Workpackage title	Lead contractor	Start month	End month	Del. No
1	Obtain rDNA sequences from picophytoplankton cultures	1	1	36	1, 2, 3
2	Obtain rDNA sequences from picoplankton environmental samples	2	1	30	4, 5
3	Develop hierarchical molecular probes against all major picophytoplankton taxa	3	1	30	6
4	Develop methods of molecular probe quantification	3	1	30	7, 8, 9
5	Validate molecular probes on coastal biodiversity time series	4	12	36	10, 11, 12
	Total				

3.2.2 Deliverable list

Deliverable	Deliverable title	Delivery date	Nature[1]
1	Picophytoplankton strains from coastal and oceanic regions	24	Da
2	SSU rDNA sequence from picophytoplankton strains	24	Da
3	Characterization and description of picophytoplankton strains	36	Re
4	Gene Clones libraries from coastal and oceanic regions	18	Da
5	SSU rDNA sequences from coastal and oceanic regions	24	Da
6	Hierarchical probes against all major picophytoplankton taxa	30	Da
7	FISH methodology for prokaryotic picophytoplankton	24	Me
8	Probe array methodology for picophytoplankton	30	Me
9	Quantitative PCR methodology for picophytoplankton	30	Me
10	Estimation of picophytoplankton biodiversity with classical methods	36	Da
11	Molecular estimation of picophytoplankton biodiversity	36	Da
12	Validation of probe methodology for biodiversity	36	Re

1 Abbreviations: Da: Data; Re: Report; Me: Method.

3.2.3 Workpackage description

3.2.3.1 Workpackage number: 1

Participants: All
Lead contractor: SBR

Objectives

The objective of Wpk. 1 is to obtain a large number of SSU rDNA sequences spanning the whole taxonomic diversity of picophytoplankton by isolating a large number of cultures from a variety of environments. These cultures will be screened by a variety of techniques suitable to the very small size of the cells, purified and sequenced. Novel taxa will be described.

Description

We will first collect all picophytoplankton strains from international culture collections.
We will **sample** for cultures at two types of sites (see details above **General strategy**):

- European coastal sites four times a year at each season Winter, Spring, Summer, Fall
    - English Channel (Roscoff *SBR*)
    - North Sea (Helgoland *AWI*)
    - Western Mediterranean Sea (Bay of Blanes *CSIC*)
- Oceanic sites from various areas adjacent to Europe (*SBR, UW, AWI, CSIC*)
    - Mediterranean Sea
    - Red Sea
    - North Atlantic Ocean
    - Antarctic Ocean

- Arctic Basins

To establish **pre-cultures**, samples will be first pre-filtered through 3 µm (Eukaryotes) and 0.6 µm (Prokaryotes) pore size filters. This water will be inoculated into either 10 mL tubes or 50 mL culture flasks to which medium at 1/10 or 1/100 dilution will be added. We will test a variety of algal media (K, f/2, PCR-S11, IMR) and will vary some of the components (in particular N and P source, trace metals). Some cultures will also be established by flow cytometry sorting. Typically we plan to establish about 20 pre-cultures per sample and of the order of 1,000 for the whole project. These pre-cultures will be incubated at the temperature prevailing *in situ* at sampling time and at different light intensities. For cruise samples, pre-cultures will be established on board and transferred to their final laboratory destination very rapidly at the end of the cruise (ideally within 24 hr.). After about one month growth, pre-cultures will be screened by flow cytometry and microscopy. This will allow to check tubes and flasks where significant growth has taken place, to discriminate prokaryotes from eukaryotes, coccoid from flagellate forms.

All pre-cultures with significant growth will be further purified over a period of 3 to 12 months by either serial dilution, filtration or flow cytometry sorting to establish **cultures**. These cultures will be screened by more sophisticated methods (Flow cytometry, whole mount electron microscopy - *UO*, DGGE of SSU rDNA - *CSIC* HPLC - *CSIC*) to determine those that correspond to novel taxa (class, clade, genus, species).

In the final phase, cultures from novel taxa will be cloned by serial dilution or growth on solid medium to establish **strains** to be deposited in the culture collection. These strains will be fully characterized using in addition to the previous methods: detailed pigment identification (*CSIC*), thin sections for electron microscopy (*UO*). The SSU rDNA of each strain will be sequenced (*CSIC*). Each novel taxon will be fully described and published in the peer-reviewed literature.

Deliverables

Number	Description	Input to Wpk
1	Picophytoplankton strains from coastal and oceanic regions	3
2	SSU rDNA sequence from picophytoplankton strains	3
3	Characterization and description of picophytoplankton strains	3

Milestones and expected results

Month 1 Sampling and pre-cultures from coastal sites begins (note: cruise samples will be processed according to a similar schedule, but delayed from the actual date of the cruise)
Month 12 Sampling and pre-cultures from coastal sites achieved
Month 13 All coastal pre-cultures screened
Month 16 All coastal cultures screened
Month 24 All coastal strains established
Month 24 All coastal and collection culture sequences obtained
Month 36 Taxonomy of all coastal strains finalized and novel taxa described
Results. We expect to establish a large collection of picophytoplankton cultures including many major new taxa. This collection and the derived sequences will serve as the basis for probe design and testing. Comparing the results from Wpk. 1 and 2 will also give precious information on the biases associated with culturing. Finally, this collection will be a resource on which we expect future end-users such as biotechnology companies to draw on.

3.2.3.2 Workpackage number: 2

Participants: SBR, UW, AWI, CSIC
Lead contractor: UW

Objectives

The objective of Wpk. 2 is to obtain SSU rDNA sequences representative of the *in situ* diversity of picophytoplankton by the construction of clone libraries from a variety of marine environments. Individual clones will be screened using various techniques, and novel gene sequences determined.

Description

Seawater for the extraction of environmental DNA will be collected at two types of sites (see details above **General strategy**):

- European coastal sites four times a year at each season Winter, Spring, Summer, Fall (*SBR, AWI, CSIC*)
- Oceanic sites from various areas adjacent to Europe (*SBR, UW, AWI, CSIC*)

Seawater samples (10 l) pre-filtered through a 3 µm membrane will be collected from discrete depths and filtered onto 47 mm 0.45-µm-pore-size polysulfone filters (Supor®-450) under gentle vacuum. DNA buffer (10

mM Tris·HCl pH 8.0, 100 mM EDTA, 0.5 M NaCl) 1 ml will be drawn through each filter before storage at -20 °C prior to DNA extraction. Total nucleic acids will then be extracted from the filters using lysozyme and sodium dodecyl sulfate (SDS) to lyse cells, extraction of cell lysates with phenol-chloroform-isoamyl alcohol (25:24:1; pH 8.0) and once with chloroform-isoamyl alcohol (24:1) before precipitating nucleic acids with 1 vol isopropanol and 0.4 vol 7.5 M ammonium acetate at room temperature. Total environmental DNA will be diluted prior to use. In addition to construction of SSU **rDNA** libraries a comparison will be made with a clone library constructed via a RT-PCR approach using **rRNA** as template. This potentially will allow identification of genotypes that are actively growing since the cell content of rRNA provides an index of the metabolic activity and growth rate of a population, information which is obviously ecologically relevant. We will use prokaryotic oxygenic-phototroph specific or eukaryote specific primers to amplify SSU rDNAs using the PCR and clone the products using high efficiency PCR cloning kits (Clontech, Invitrogen). These clone libraries will be transformed into a suitable *Escherichia coli* host strain and screened using several techniques:

- RFLP analysis
- Single base screening (e.g. T-tracking)
- DGGE analysis.

A minimum of two clones from all RFLP types containing two or more clones and all clones with unique banding patterns (RFLP or DGGE) will be sequenced. Identification and phylogenetic analysis of novel sequences will be carried out. An estimation of the coverage of the clone libraries constructed will also be made using the equation: $\mathbf{C = 1 - (N_1/N)}$ where N_1 is the number of clone types which occurred only once in the actual library (frequency =1) and N is the total number of clones examined. For this calculation we will assume that clones which are >97% similar are identical.

Deliverables

Number	Description	Input to Wpk
4	Gene Clones libraries from coastal and oceanic regions	3
5	SSU rDNA sequences from coastal and oceanic regions	3

Milestones and expected results:

Month 1 Begin extraction of environmental DNA
Begin Construction of SSU rDNA/rRNA clone libraries

Month 18 Clone libraries complete

Month 20 Environmental sequences available

Month 24 Full sequence analysis complete

Results. Wpk. 2 will produce gene sequences of taxonomic value which will be particularly helpful in the identification of novel picophytoplankton groups for which cultured counterparts do not exist. Thus, the sequence data produced by this workpackage is critical for the design of phylogenetical probes (Wpk 3) but also for comparison of the results of Wpk 1.

3.2.3.3 Workpackage number: 3

Participants: UW, AWI
Lead contractor: AWI

Objectives

Design hierarchical taxonomical probes against SSU rDNA for picophytoplankton

Description

We will develop probes against all major taxa of picophytoplankton for which sequences either are already available or will be determined in Wkp. 1 and 2. If some probes are already available and tested, we will of course use them. For eukaryotes (*AWI*) we will target the following levels: Class (e.g. Prasinophyceae, Pelagophycae); Clades; Species (e.g. *Micromonas pusilla*). For prokaryotes (*UW*), we will target all major cluster of *Prochlorococcus* and *Synechococcus* (e.g. high vs. low phycourobilin types). We anticipate all together to develop between 40 and 100 probes.

Probe design will be performed with the ARB program package (http://www.biol.chemie.tu-muechen.de). Localization of potential target sites in other sequences will be checked using the "PROBE_MATCH" function of ARB or the "CHECK_PROBE" function of RDP. Target site suitability will be checked by eye from partial sequence alignments provided by the output from these programs. Because the number of sequences in

databases is increasing rapidly, the probes will be checked periodically. Probes will be validated against available cultures as well as those resulting from Wpk. 1 or clones from Wpk. 2 by dot blot hybridization.

Deliverables (input to next workpackage):

Number	Description	Input to Wpk
6	Probes against all major picophytoplankton genera	5

Milestones and expected results:

Month 1	Probe design begins based on sequences available in public databases
Month 6	Probe design begins based on sequences acquired during project (Wpk. 1 and 2)
Month 30	Probes all designed and validated
Results.	Wpk. 3 will provide an extensive set of phylogenetic probes to assess the biodiversity of picophytoplankton in the marine environments. These probes will receive immediate application in Wpk. 5.

3.2.3.4 Workpackage number: 4

Participants: SBR, UW, AWI
Lead contractor: AWI

Objectives

Develop probe measurement technologies for high density sample throughput and high sensitivity

Description

A variety of hybridization techniques are available for using probes: the choice depends on the type of information or resolution required and the number and type of samples to be analyzed. Within this workpackage we will develop three methodologies, all with their advantages and disadvantages (see Table 2)

FISH for prokaryotes (*UW*). Genotype-specific oligonucleotides designed for specific *Prochlorococcus* and *Synechococcus* genotypes will be labeled with horse-radish peroxidase (HRP). We will then test the use of a tyramide signal amplification (TSA) system (Schonhuber et al. 1999) to perform FISH on field samples and enumerate specific genotypes *in situ* using epifluorescence microscopy.

Probe arrays (*AWI*). We will explore the development of a platform that allows for a high throughput of samples with the maximum sensitivity of detection. The first methodology that we will utilize is high density sample arrays on nylon membranes with chemiluminescent detection. Another possibility that we will investigate will be the construction of DNA microchips which involves the robotic deposition of DNA probes at indexed positions on microscope slides which are then scanned with a confocal scanning microscope. These probes can hybridize with fluorescently labeled target DNA or RNA. The signal output from the hybridization is very sensitive and is highly quantitative. Micro-array technology can be used at high densities and pair-wise comparisons. This system requires optimization for environmental samples but can be started immediately with existing databases to develop quantitative comparisons.

Quantitative PCR (*SBR*). We will either use directly the probes designed in Wpk. 3 in conjunction with the nucleic acid stain SYBR Green I or we will modify the probes to incorporate fluorogenic 5' nuclease activity. In either case, the increase in fluorescence during the PCR reaction will be monitored continuously in a specific 96-well PCR machine such as the Perkin Elmer GeneAmp 5700.

These three techniques will be first extensively tested on the picophytoplankton cultures obtained as part of Wpk. 1 and then refined on field samples which offer the challenges of large diversity, relatively low cell concentrations and rRNA levels. We will especially concentrate on the issue of accurate quantification of the abundance of each probe target.

Deliverables (input to next workpackage):

Number	Description	Input to Wpk
7	FISH methodology for prokaryotic picophytoplankton	5
8	Probe array methodology for picophytoplankton	5
9	Quantitative PCR methodology for picophytoplankton	5

Milestones and expected results:

Month 1 Development of probe assessment methodologies begins
Month 30 Probe assessment methodologies ready
Results. Wpk. 4 will provide an extensive set of practical tools to assess the biodiversity of picophytoplankton in the marine environments. These tools will receive immediate application in Wpk. 5 but will also be major assets for transfer of the project results to end-users such as environment monitoring agencies

3.2.3.5 Workpackage number: 5

Participants: All
Lead contractor: CSIC

Objectives

Apply and validate the probe methodologies to determine the extent of spatial and temporal variability of photosynthetic picoplankton biodiversity at three European coastal sites.

Description

This workpackage will focus on the three European coastal sites (see Wpk. 1). A preliminary knowledge of picophytoplankton composition will be obtained from Wpk. 1, in which these systems will be sampled four times during the first year of the project.

Sampling will be performed **once a month** during the second year of the project in each of the three systems (*SBR, AWI, CSIC*). Several liters of surface seawater will be collected and processed as needed for each technique. **Environmental parameters** (temperature, salinity, nutrients) will be measured by standard oceanographic techniques. An aliquot will be fixed for whole mount **electron microscopy** (*UO*), which gives taxonomic information about some groups of picophytoplankton. An aliquot will be fixed to carry out **flow cytometry** measurements (*SBR, AWI, CSIC*) providing abundance of the most important groups of photosynthetic picoplankton, namely *Synechococcus*, *Prochlorococcus* and picoeukaryotes, as well as heterotrophic bacteria. Two samples for **pigment analysis** (*CSIC*) will be collected onto glass fiber filters, one corresponding to the whole assemblage and a second corresponding to the assemblage that pass through a 3 µm filter. Total and fractionated samples will be analyzed by HPLC.

For **DNA**, several liters of seawater will be sequentially filtered through a 3 µm and a 0.2 µm filter. Extraction of DNA from the filter containing the 0.2 – 3 µm fraction will be performed by conventional techniques. The quality of DNA will be checked by agarose gel electrophoresis, and the concentration of DNA determined by fluorescence. The overall composition of the community will be investigated by **DGGE** (*UW/Prok and CSIC/Euk*). The presence and intensity of bands for each sample will be evaluated and compared among different samples. A cluster analysis of communities will be performed with this information.

The composition of picophytoplankton assemblages will be determined by the probing methods developed in Wpk. 4. **FISH** (*UW/Prok*) will be used to detect the taxonomic status of individual cells. However, due to inherent methodological limitations (see above), it will be applied only to prokaryotic picophytoplankton (*Synechococcus* and *Prochlorococcus*). Extracted DNA will be analyzed by **probe array** (*Par 2/Prok and 3/Euk*), with the whole set of probes designed in Wpk. 3. This will be done by blotting the DNA on membranes or by the most innovative DNA microchip technology. In addition, the use of **quantitative PCR** (*SBR*) with energy transfer labeled primers, with primers specific for different groups, will be investigated to compare presence and abundance of certain groups.

The different estimates of biodiversity achieved by the diverse set of methods used will be compared, allowing a full validation of the probe methodologies. Moreover change in picophytoplankton composition will be interpreted in the light of the environmental parameters measured.

Deliverables

Number	Description	Input to Wpk
10	Estimation of picophytoplankton biodiversity with classical methods	
11	Molecular estimation of picophytoplankton biodiversity	
12	Validation of probe methodology for biodiversity	

Milestones and expected results:

Month 12 Sampling the coastal sites begins.
Month 24 Sampling the coastal sites achieved.
Month 36 All measurements of biodiversity achieved.
Month 36 Comparison and interpretation of biodiversity estimates achieved

Results. Wpk. 5 will provide a full validation of the probe methodologies developed and detailed knowledge of the biodiversity of photosynthetic picoplankton at contrasted coastal sites. This will provide a framework that is necessary for future studies of diversity (when and where are we most likely to retrieve new organisms?), and for monitoring purposes (does the natural assemblage change because of human influence or of natural seasonal changes?).

3.3 Role of each partner

The next table summarizes the major roles of each partner (to be detailed below). We tried to have each partner responsible for one of the major techniques used in the project on the basis of its past experience.

Partner	Acronym	Major responsibilities	Wpk.
1	SBR	▪ Sampling English Channel (Roscoff)	1, 2, 5
		▪ Sampling Mediterranean Sea cruise	1, 2
		▪ Culture screening	1
		▪ Culture collection	1
		▪ Sequencing of eukaryotic genes	2
		▪ Quantitative PCR technology	4, 5
2	UW	▪ All molecular work on photosynthetic prokaryotes (sequencing, DGGE, FISH, probes)	1, 2, 3, 4, 5
		▪ Sampling Red Sea cruise	1, 2
3	AWI	▪ Sampling North Sea (Helgoland)	1, 2, 5
		▪ Sampling N. Atlantic/Arctic and Antarctic cruises	1, 2
		▪ Culture screening	1
		▪ Probe design	3
		▪ Probe array	4, 5
4	CSIC	▪ Sampling Mediterranean Sea (Catalan Sea)	1, 2, 5
		▪ Sampling Mediterranean Sea cruises	1, 2
		▪ HPLC	1, 5
		▪ DGGE and sequencing of bands	1, 5
		▪ Sequencing of eukaryotic genes	2
5	UO	▪ Electron microscopy	1, 5

3.3.1 Partner 1: SBR

Partner 1 (SBR) will coordinate the project. It will be responsible for all interim progress reports, as well as for scheduling yearly meetings and special workshops. It will also produce a brochure on the project for the general scientific public and set up a web site for the project.
SBR will be responsible for sampling at the **English Channel coastal site** (Estacade station in Roscoff, France):

- four times during the first year for cultures (**Wkp. 1)** and gene clone library (**Wkp. 2**)
- once a month during the second year for biodiversity assessment (**Wkp. 5**).

The English Channel site off Roscoff has been studied for over 10 years with in particular studies of large phytoplankton and pigments.
SBR will also be responsible for sampling for cultures (**Wkp. 1)** and gene clone library (**Wkp. 2**) during the Mediterranean Sea PROSOPE (Summer 1999) cruise, provided it is scheduled as planned.
SBR will lead Wpk. 1 centered around **cultures**. In particular, it will act as a repository site (Roscoff Culture Collection) for all final strains obtained during the course of the project.
SBR will also be responsible for all **SSU rDNA gene sequencing** on strains (**Wpk. 1**) and natural samples (**Wkp. 2**) for eukaryotic picophytoplankton.
Finally, SBR will develop **quantitative PCR** (**Wpk. 4**) to assess the presence and abundance of specific taxa based on probes developed by AWI and, if development is successful, apply this technique to the annual time series samples from the three coastal sites (**Wpk. 5**).

3.3.2 Partner 2: UW

Partner 2 (UW) will be responsible for the characterization of biodiversity among the **prokaryotic picophytoplankton**. It will be responsible for sampling during one cruise in the **Red Sea** (**Wpk. 1 and 2**). It will co-ordinate **Wkp 2** which will construct **prokaryotic clone libraries** from coastal sites and cruises of opportunity. It will **sequence** all novel prokaryotic SSU rDNAs (**Wkp. 2**) and develop **probes** (**Wkp. 3**), probe

arrays and the **FISH** technology for prokaryotes (**Wkp. 4**). It will perform DGGE and FISH for analyzing the prokaryotic picophytoplankton community on coastal sites **time series** samples (**Wkp. 5**).
The UW will be responsible for the organization of the **workshop** to be held in Warwick on environmental gene sequencing.

3.3.3 Partner 3: AWI

The AWI will be responsible for sampling at the **North Sea site** (Helgoland):

- four times during the first year for cultures (**Wkp. 1)** and gene clone library (**Wkp. 2**)
- once a month during the second year for biodiversity assessment (**Wkp. 5**).

The North Sea sampling site will be located close to the island of Helgoland (54° 10.4´ N, 7° 53,1´ O). At this site a phytoplankton time-series with daily sampling has been conducted since 36 years. Parameters such as temperature, salinity and inorganic nutrient concentrations are measured; qualitative and quantitative phytoplankton composition is routinely investigated by inverted microscopy. Chlorophyll measurements will soon be incorporated into the sampling routine. These time-series data-set will serve as a support for the planned investigations.
It will also be responsible for sampling for cultures (**Wkp. 1)** and gene clone library (**Wkp. 2**) during the following Polarstern cruises (provided they are scheduled as planned):

- Summer 2000: North Atlantic/Arctic Ocean
- Fall 2000: Antarctic Ocean

With the samples obtained from the first year at the North sea site and from the two cruises, the AWI will establish, screen and purify to strain prokaryotic and eukaryotic picophytoplankton cultures (**Wkp. 1**).
AWI will be responsible for the design and testing of the oligonucleotide **eukaryotic probes** (**Wkp. 3**) and will be responsible for the development of a **probe array** platform upon which to utilize the probes in rapid and sensitive fashion (**Wkp. 4**). It will also use the probe system to analyze the ***in situ*** **diversity** of eukaryotic picophytoplankton during the whole year in the three sites (**Wkp 5**).
The AWI will be responsible for the organization of the **workshop** to be held in Bremerhaven about probe technologies.

3.3.4 Partner 4: CSIC

The CSIC group will be responsible for sampling at the **Mediterranean coastal site** (Blanes Bay, 42 18’ N, 3 18’ E)

- four times during the first year for cultures (**Wkp. 1)** and gene clone library (**Wkp. 2**)
- once a month during the second year for biodiversity assessment (**Wkp. 5**).

It will also be responsible for sampling for cultures (**Wkp. 1)** and gene clone library (**Wkp. 2**) during the following cruises (provided they are scheduled as planned):

- Fall 1999: Alboran Sea (MATER cruise)
- Feb 2000: Western Mediterranean Sea (HIVERN cruise)

With the samples obtained from the first year at the Mediterranean coastal site and from the two cruises, the first stages of establishment of prokaryotic and eukaryotic picophytoplankton cultures will be performed (**Wkp. 1**). These cultures will be transferred to SBR for purification. The CSIC group will be responsible for screening eukaryotic cultures from all participants by **HPLC** and **DGGE** (**Wkp 1**). Finally, the CSIC group will analyze the ***in situ*** **diversity** of eukaryotic picophytoplankton during the whole year in the three sites by using HPLC and DGGE and sequencing of bands (**Wkp 5**).
The CSIC group will be responsible for the organization of the **workshop** to be held in Barcelona about HPLC photosynthetic pigment analysis.

3.3.5 Partner 5: UO

The UO group will be responsible for the identification of species as well as the ultrastructural characterization and description of new taxa.

- It will screen all strains growing in culture (**Wkp. 1**) by transmission electron microscopy of stained and shadow-cast whole-mounts
- New taxa will be fixed, embedded and sectioned for transmission electron microscopy. When necessary (in the cases of e.g. cryptomonads and diatoms), they will be fixed, critical point-dried and coated for scanning electron microscopy (**Wkp. 1**).This will provide morphological and micro-anatomical characteristics for the comparison with gene sequences, and make it possible to produce a comprehensive account of the systematical affiliations of the picoplankton species investigated.
- It will measure the diversity of eukaryotic picoplankton species in natural samples (**Wkp. 5**). The diverse complexity of morphotypes present in natural material will be investigated in detail by electron microscopy. Routinely transmission electron microscopy of shadow-cast whole-mounts will be used, but the

methods will be adjusted to the type of organisms encountered and supplemented by studies of thin sections of embedded material and scanning electron microscopy whenever necessary.

4 CONTRIBUTION TO OBJECTIVES OF PROGRAMME/CALL

4.1.1 Biodiversity

Phytoplankton diversity is specifically targeted in international biodiversity research initiatives such as BIOMAR (1994). Clearly among phytoplankton, it is the picoplanktonic fraction for which taxonomy and diversity is the less known with fewer than 30 species described to date. This contrasts sharply to the dominance of picophytoplankton cells in virtually all marine systems. Therefore a huge effort is required in this domain since we have no idea how pico-phytoplankton is affected by environmental changes.

The aim of this proposal fits very precisely with the aims of Programme 4, namely to develop indicators of biodiversity for picophytoplankton. Moreover, since we will use and validate these tools at coastal sites over a full annual cycle, we will also provide data that will allow to understand and quantify biodiversity dynamics, as well as to monitor the decrease in biodiversity and the invasion of foreign species (cf. the example of the brown tide alga *Aureococcus*).

4.1.2 Functioning of marine ecosystems

Pico-phytoplankton constitute the major primary producers in most marine systems and as such form the basis of the food web in these systems. Depending on the exact structure of the picoplanktonic community and its diversity, the whole food web of the marine system may shift from one state to another. For example, the dominance of the very small *Prochlorococcus* (0.6 µm) may induce a microbial loop that is very efficient at recycling mineral elements, whilst the dominance of the larger *Synechococcus* may cascade to larger grazers that induce more efficient export towards the higher trophic levels. The data we will collect should provide baseline data to be input into models that will predict how coastal ecosystems vary at the seasonal scale.

5 COMMUNITY ADDED VALUE AND CONTRIBUTION TO EU POLICIES

In 1997, the European Science Foundation sponsored a workshop devoted to the drafting of an initiative formulated "A European Science Plan on Marine Biodiversity". In the executive summary of the report published in 1998, it is clearly stated that the scale of research efforts needed to remedy the lack of understanding of the extent and the role of marine biodiversity demands European scale collaboration. Our proposed research is fully in tune with such a recommendation.

Picophytoplankton are major primary producers in many oceanic areas that are adjacent to Europe. One of the best example is the Mediterranean Sea whose eastern part is the most oligotrophic area in the world and where picoplankton are in fact the only primary producers. In coastal areas, so prevalent among European marine systems and so important from the environmental and commercial points of view, the role of picophytoplankton has been much less studied, but we have strong indication that the smallest size class of microalgae are also very important. Another feature of picophytoplankton is that it contains many taxa that are very ubiquitous, such as *Synechococcus* or *Micromonas*. This contrasts very strongly with larger microalgae, such as diatoms for which individual species exhibit often restricted habitats and geographical ranges. Therefore, chances are very high that a better understanding of picophytoplankton biodiversity and a methodology for its assessment will be applicable to virtually all European marine systems.

Despite their key role, picophytoplankton are studied by a rather limited number of teams at the international level. Therefore it is clear that the type of project we propose cannot be tackled by a single national community. While some communities have a stronger expertise in classical taxonomy, other are more advanced in the use of molecular tools to address environmental questions. In our proposed research we have drawn together some of the leading experts in the assessment of both pro- and eukaryotic picophytoplankton biodiversity, mastering a variety of powerful techniques, ranging from pigment analysis by HPLC to flow cytometry, to form a consortium whose members complement each other. In fact, because of lack of critical mass at the national level, members of this consortium have already a long history of collaboration, both in and outside formal research programs.

Another clear advantage of performing this research at the European level, is the possibility of comparing picophytoplankton biodiversity in a variety of environments. The three coastal sites we have chosen as focal

points for our project provide a very good representation of European coastal waters, from the Mediterranean to the North Sea. By combining, cruises planned among the different partners, we should be able to provide basic biodiversity data (i.e. inventory the picoplankton flora) in an even much wider variety of marine systems ranging from the Red Sea to the Arctic.
With respect to EU policies, because our focus is on the development of monitoring tools, we anticipate that the methodology and probes we will be providing at the end of the project can be used in the medium term by agencies in charge of implementing EU policies on both biodiversity and long term and climatic issues. As stated earlier, picophytoplankton are one of the most important compartment at the basis of all food webs in marine systems. It responds clearly to changes in the environment. It is composed of species with a very wide range. Therefore it has many advantages to serve as a basis for any long term oceanic observation system. Also the molecular tools we focus on are very amenable to routine monitoring because once developed and packaged by third parties, they can be used in the field and the data acquisition do not require extensive expertise.

6 CONTRIBUTION TO COMMUNITY SOCIAL OBJECTIVES

The area where our project will contribute most concerns the quality of the environment. Marine areas, especially coastal ones, are key ecosystems in this context. Management and exploitation of these systems can only be conducted if one have a thorough knowledge of their functioning. Picophytoplankton is at the base of all oceanic food webs but the understanding of its diversity and dynamics is severely lagging behind. The methods and tools we will provide should considerably help in devising knowledgeable policies. As an example, we know that the cyanobacterium *Synechococcus* is good indicator of the eutrophication level of a marine system. In particular, low phycoerythrobilin types are clearly associated with high nutrient areas. Probes targeted against these organism could be used as routine monitoring tools to evaluate the nutrient loading of coastal areas.
Moreover, we need to establish baseline values for picophytoplankton diversity and to understand the magnitude of genetic change that can occur through space and time before we can make predictions about the role of anthropogenic input into marine ecosystems. Collecting biodiversity data on picophytoplankton at coastal sites during yearly cycles, as planned in this project, is relevant for building better models of marine systems that will help considerably in devising better policies for protecting and exploiting the marine environment.
Another aspect of our proposal concerns the isolation and maintenance of a very large collection of picophytoplankton strains. Clearly picophytoplankton constitute an immense and yet untapped reservoir of novel species with a very wide potential for completely novel biosynthetic pathways. We view this strain collection as an invaluable resource that companies interested in bio-products could screen for a variety of compounds. This could induce the creation of new companies or the development of existing ones in areas such as cosmetics, where some companies are already using bio-compounds extracted from microalgae.

7 ECONOMIC DEVELOPMENT AND S&T PROSPECTS

7.1 Exploitation and dissemination of results, IPR

We intend to make the results of our project widely available in a variety of ways, some of which are detailed in our management plan. The web site that we will develop will be the focal point to present the results of our research. It will be updated regularly and will point to the different channels through which we intend to disseminate our results.

- **Publications.** As is usual for any scientific research, our results will be published in peer-reviewed journals. This will be especially important for the description of new taxa. We will be very careful to target a large variety of journals with fields ranging from oceanography to biotechnology, from phycology to global change of the environment, in order to increase exposure of the scientific community to the results of the project. Moreover we will make every possible effort to disseminate our major discoveries in a variety of media including the daily press, popular science magazines and the radio.

- **Attendance to meetings**. We plan to present our results not only to scientific meetings, but also to more technically oriented meetings, especially those dealing with biotechnology to popularize picoplanktonic microalgae.

- **Culture collection**. All strains obtained during this project will be deposited in a central culture collection hosted in Roscoff that will be available for screening by any interested party. Strains of all novel taxa will be also deposited once described in the CCMP culture collection in Bigelow, Maine USA that is serving as a central repository for marine microalgae.

- **Databases**. We plan to maintain a number of databases that will be shared among all partners during the project. At the end of the project and after publication in peer-reviewed journals, these data will be made available to end users. These databases include:

Database	**Partner responsible**	**Public location at the end of the project**
Coastal and oceanic sites environmental and biodiversity data	SBR	SBR Website
Cultures	SBR	SBR Website
Pigments	CSIC	CSIC Website
Gene sequences (Prokaryotes)	UW	GenBank
Gene sequences (Eukaryotes)	SBR	GenBank
Probes	AWI	Oligonucleotide Probe Database

- **Workshops**. We plan to hold three workshops on some of the techniques implemented during this project. These workshops will be widely advertised and opened to third-parties:
  - Gene clone libraries from natural samples
  - HPLC photosynthetic pigment analysis
  - Probe technologies

- **Probe technologies for the environment**. We anticipate that some of the techniques that we will develop such as DNA chips will interest commercial companies that will want to develop prototypes for either government monitoring agencies or scientific research.

7.2 Scientific and Technological prospects

7.2.1 Understanding marine systems

Picophytoplankton forms the base of the food web in most marine systems. The knowledge of its composition, especially on the eukaryotic side, is still very sketchy. The large scale picture that will result from our project should lead to the identification and isolation of some of the key organisms in this size class. Future studies could then focus on these taxa to understand their physiology, their genetic diversity, their population dynamics and more generally their exact role in the ecosystem. A good example of such key organism is *Prochlorococcus*, dominant in oligotrophic systems, such as the Mediterranean Sea, and that was still unknown only eleven years ago. Now an entire MAST III program (PROMOLEC) is devoted to the study of this single organism. In the framework of the present project, we expect to reveal organisms that may well have comparable importance in coastal systems.

The data we will obtain on picophytoplankton biodiversity in coastal systems should form the baseline for future studies of phytoplankton diversity on much longer temporal and wider spatial scales than those investigated in the present project, that should be very useful to understand what type of changes are occurring on a global scale.

Another potential use of these biodiversity time series data will be the validation of more realistic and sophisticated models of marine systems, that should, in particular:

- incorporate the picophytoplankton fraction into global models,
- take into account the different physiological types present among picophytoplankton (e.g. high nutrient requiring species vs. those that can thrive even under oligotrophic conditions),
- take into account the genetic variability of picophytoplankton populations.

7.2.2 Monitoring marine systems

Clearly there is a lack of appropriate tools to monitor the biological component of marine systems, in contrast to their physical and chemical characteristics that can be monitored in real time and even remotely. The tools

and methods we intend to develop and validate will offer near real-time capabilities because they rely on very recent developments in molecular biology such as the DNA chips or large scale gene sequencing. These techniques are driven by whole genome sequencing projects that have literally blossomed in the last year or so (e.g. a year ago, the rice genome was planned to take 5 years to be completed, and now one venture has announced that it will be achieved within six months). It is obvious that by the end of the project, better, faster, and less expensive instruments and technologies will be available to take advantage of the probes we will have developed.

Another aspect linked to the monitoring of marine systems, is the possibility that our project uncovers indicator species. Such species can only thrive under very specific ecophysiological conditions such as high nutrient or low salinity. As an example, the absence of *Prochlorococcus* in coastal waters has been hypothesized to be linked to its sensitivity to copper concentrations. Recording the absence or presence of such indicator species provides very important information on the marine environment and its evolution.

7.2.3 Biotechnology

Although still used on a very small scale, microalgae are clearly an excellent material for many future biotechnology applications. Among microalgae, the smaller size classes constituted by picophytoplankton are potentially very attractive targets for several reasons.

- Picophytoplankton contain many novel phyla at very high phylogenetic levels, such as the class level. These new phyla may contain novel compounds absent from previously known phyla, which may be of interest for applications such as pharmacology, food industry, or cosmetics. For example, the brown tide picophytoplanktonic alga, *Aureococcus anophagefferens*, discovered a few years ago contains an unidentified toxin with biological effects on bivalves. Picophytoplanktonic relatives of diatoms or dinoflagellates, may contain, as some species of the latter groups, compounds that could have therapeutic interest in cancer research.
- Picophytoplankton are interesting for biotechnology applications of microalgae because they can attain high cell densities. Their very small size makes their handling easy and very similar to that of heterotrophic bacteria, allowing the use of existing industrial systems for mass cultures and product extraction.

7.2.4 Research in biology

The smallest representatives of microalgae offer many fascinating aspects that could be targeted in the future for fundamental studies in many fields of biology:

- Picophytoplankton are minimalist cells. Many of them present the most basic blueprint for a photosynthetic organism. Many pico-eukaryotes have a single chloroplast and a single mitochondrion. Their genome size is very small (for example *Ostreococcus tauri* has a genome size of about 10 Mbp, i.e. less than yeast). They likely therefore contain the minimum number of genes necessary for metabolic processes such as photosynthesis. They may have also discarded all 'junk' (non-coding) elements from their genome. For example, the prokaryote *Prochlorococcus* possesses coding sequences overlapping one another on the two complementary DNA strands. These organisms therefore are potentially fascinating models for fundamental studies in genomics, molecular and cell biology.
- Many picophytoplankton species are very small coccoid cells that can grow easily on agar and that could constitute excellent models and tools for genetic studies. For example, the marine cyanobacterium *Synechococcus* is very easily transformable and has been already used for targeted mutagenesis
- Newly discovered phyla, of which picophytoplankton is very rich, are undoubtedly essential for our understanding of the evolution of algae and higher plants. As an example, the discovery of the Bolidophyceae, a very close relative of diatoms, sheds a new light on the evolution of the latter group that is so diverse in marine waters. We also expect that many of the uncultivable taxa that we will uncover by direct gene sequencing from environmental samples will be extremely useful to construct algal phylogenies.

Our project could also have technological applications for applied biomedical research. The use of molecular techniques in the marine environment offer challenges not found in other fields of classical genomics. These challenges include the necessity to detect and quantify precisely very low abundance of target organisms and/or molecules. Therefore, we expect that some of the improvements that we will make to the molecular techniques we will use, may be transferred back to the biomedical field.

8 THE CONSORTIUM

8.1 Participant 1: SBR

Station Biologique de Roscoff, CNRS UPR 9042 et Université Pierre et Marie Curie (Paris 6)
Oceanic Phytoplankton team
Place Georges Tessier, F29680 Roscoff France
Ph: Fax: +33 (2) 98 29 23 23 Fax: +33 (2) 98 29 23 24
Web site: www.sb-roscoff.fr

SBR has been pioneering the use of **flow cytometry** in oceanography in the last 15 years, in particular to study picoplankton. Some of its major contribution in this field include the direct determination of *Prochlorococcus* growth in the ocean or the detection of virus populations in oceanic waters. More recently, it has played a key role in the introduction of **molecular biology** into oceanography in France.

Scientific Personnel involved in the PICODIV project

Position	Expertise
Directeur de Recherche CNRS	Responsible for project
Chargé de Recherche CNRS	*Prochlorococcus* ecology
Maître de conférence UPMC	Phytoplankton taxonomy
Assistant- Ingénieur CNRS	Roscoff Culture Collection
Assistant- Ingénieur CNRS	Flow cytometry
Technician to be hired	Culture
	Sequencing
Post doctoral fellow to be hired	Gene cloning
	Sequence analysis
	PCR development

8.2 Participant 2: UW

University of Warwick, Department of Biological Sciences,
Phytoplankton Group, Gibbet Hill Road, Coventry, CV4 7AL, UK
Tel: +44 - 1203- 523523 Fax +44 - 1203- 523701
web site: http://www.bio.warwick.ac.uk/

UW has been studying the **molecular biology of marine cyanobacteria** for the last 10 years. It has been one of the first groups to utilize molecular techniques to address the factors that might control photosynthesis in these organisms, particularly the role of inorganic nutrients, and to study the structure of cyanobacterial communities.

Scientific Personnel involved in the PICODIV project

Position	Role
Lecturer/ Research Scientist	Clone libraries; management duties.
Postdoctoral Researcher	Gene cloning; Sequence analysis;
to be appointed	FISH; Dot blot hybridisation
	DGGE of prokaryotic picophytoplankton

8.3 Participant 3: AWI

Alfred Wegener Institute for Polar Research
Am Handelshafen 12
D-27570 Bremerhaven Germany
Tel. 49-471-4831-443
Fax. 49-471-4831-425
AWI has pioneered the use of PCR to obtain **SSU rRNA genes for molecular systematics** and has expanded into **probe development** for marine phytoplankton, in particular toxic micro-algae.

Scientific Personnel involved in the PICODIV project

Position	Role
Senior Research Scientist	Responsible for project
Junior Research Scientist	Helgoland sampling Phytoplankton taxonomy
Post-doctoral Assistant	Probe development
Technician-AWI	AWI Culture Collection
Technician to be hired	Flow cytometry Culture maintenance
Post Doc to be hired	Gene cloning Sequence analysis probe development

8.4 Participant 4: CSIC

Institut de Ciències del Mar, CSIC, Department of Marine Biology and Oceanography
Passeig Joan de Borbó s/n, E-08039 Barcelona, Spain.
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CISC-ICM has an extended expertise in **HPLC analysis of pigments** applied to the field of taxonomy. The ICM group has applied **molecular techniques to characterize the diversity** of marine picoplankton, including bacteria, archaea and eukarya.

Scientific Personnel involved in the PICODIV project

Position	Role
Research Scientist	Responsible for sampling
Research Professor	Phytoplankton cultures
Research Scientist	HPLC analyses
Posdoctoral researcher	Molecular analyses
Technician to be hired	Phytoplankton cultures
Technician to be hired	Molecular analyses

8.5 Participant 5: UO

Dept. of Biology - Section for Marine Biology, University of Oslo
Blindern, Oslo, Norway
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Web site:http://biologi.uio.no/

UO has 30 years of experience with **ultrastructural studies** of algae, including the **description** of several pico and smaller nanoplankton taxa. During the last four years the group has combined morphological and molecular results.

Scientific personnel involved in the PICODIV project

Position	Role
Professor	Phytoflagellate taxonomy, responsible for UO
Researcher	Molecular phylogeny and detection of algae
Post.doc.	Picophytoplankton taxonomy, fine structure, EM
Technician	Culture maintenance

9 PROJECT ADMINISTRATION

9.1 Project management

The overall management of the project will ensure:

(a) the communication between partners and with the EU office,
(b) the timely production of deliverables,
(c) the integration of the work of partners and work packages.

The data management structure is organized so as to be able to follow the development of the project closely, distribute data and results to the partners and provide the deliverables of the work performed under the contract.

The dissemination of the results is planned to ensure proper distribution of the results and deliverables to the outside community.

9.1.1 Management structure and reporting

9.1.1.1 Steering Committee

The project will be managed by a steering committee consisting of the principal scientists and the co-ordinator. The steering committee works to the following terms of reference:

- to provide advice to the co-ordinator on the execution of the project,
- to assess the fulfillment of the tasks and deliverables,
- to summarize and synthesize the scientific results,
- to decide on regulations to ensure that the work of the partners is performed according to the "Description of Work".

9.1.1.2 Co-ordinator, project secretariat, progress monitoring.

The co-ordinator will provide a project secretariat to:

- oversee the day to day business of the project,
- set up data exchange and information routines for the project,
- keep track of the activities and ensure the flow of information,
- provide oversight of plans and logistics (e.g. newsletters).

The project secretariat will establish within the first 6 months a project home page on a World Wide Web (WWW) server accessible by Internet. In an area restricted to project partners and the European Commission (password protected), it will provide:

- the "Description of Work" for the contract,
- work schedules and logistics information,
- updates on deliverables, news and project reports,
- a forum for scientific exchange and discussion,
- data inventories and data handling guidelines,
- issues regarding dissemination of results, also including abstracts of papers and presentations.

The WWW server will also have a "home page" to provide non-confidential information on the project for outsiders (e.g., project summary, published papers, products for users).

9.1.1.3 Meetings and workshops

Regular workshops will be organized, with mandatory participation of all partners (either PI or authorized representative), at least once per year. Additionally, smaller workshops of task groups will be used with flexibility during the project to plan, execute and summarize specific tasks. Three such workshops are planned.

Month	Location	Object
1	Warwick	Gene clone libraries from natural samples
12	Barcelona	HPLC photosynthetic pigment analysis
24	Bremerhaven	Probe technologies

9.1.1.4 Reports and planning

The project will be reporting regularly on its development and results on three different levels:

(a) Management reports: Every 6 months the co-ordinator will provide a concise management report following the meetings of the steering committee. The report will include:

- a summary of all activities conducted in the preceding period,
- a review of progress in respect to the details in the "Description of Work",
- plans for the following 12 months detailed on the task level including, if necessary, a proposal for adjustments,
- minutes of the steering committee meetings,

- an updated listing of all publications submitted and published and of presentations of project results made on scientific meetings (all project publications such as articles, posters, oral presentations must expressively acknowledge the EU funding by specifying the contract number),
- an updated listing of personnel paid partly or fully from project funds.

(b) Annual scientific reports: The annual report, produced by all participants and compiled by the co-ordinator into a coherent document, will provide the scientific results and deliverables produced in the preceding year. It will contain the 6-month management report, a summary of the scientific achievements, a listing of deliverables produced according to the "Description of Work" and a detailed description of the scientific results.
(c) Final report: A final project report will be delivered in compliance with the general conditions of contract and the Guidelines for the "Description of Work" of RTD projects. It will include a final management report, a detailed scientific and technical report, a synthesis of work, intended for a peer-reviewed journal, and a Technology Implementation Plan (TIP).

9.1.2 Data management

9.1.2.1 Data policy

All project data will be finally banked at a competent data centre (see 7.1 for location of databases),

- reporting issues (cruise summary reports, cruise reports, EDMED forms etc.) will go through the project/data manager who forwards these to the data centre chosen for the project,
- data submission, which includes meta-data, data documentation, information on calibration and procedures will go directly to the data centre, with report to the project/data manager who provides an inventory of all submitted data, available to the partners on the WWW server.
- data distribution requests will go directly to the data centre. Questionable requests are discussed with the data originator and co-ordinator who together decide on granting of the request. The WWW server will provide an inventory of the requests and data distribution available to the partners.
- quality control of the data is primarily a responsibility of the partner who shall ensure that the procedures set up by the data centre are followed. Additional control of data quality before banking of data will be done by the data centre.
- property rights on the data: In accordance with the EU code, data originating from the project are "foreground information" as defined in the general conditions of contract and this will determine the rules for sharing data within the project, between EU projects and with third parties. Data entering the data centre chosen for final banking of the project data will be subject to limited access in the form of "foreground information" until 6 months after the formal end of the project, when they will become public information.

9.1.2.2 Data and information system

A data and information management system based on Internet will be used for the project management. The data and information management system will enable the project co-ordinator to respond to the requirements of the EU data management code:

i) Cruise summary report forms ("ROSCOP forms"), including cruise reports, station lists etc., will be sent for each cruise to the co-ordinator within 1 month of the end of the field campaign. These will be forwarded to the chosen data centre and to ICES (which centrally records EU project related field campaigns).
ii) Inventories of continuous observations will be updated regularly by the responsible scientists and copied to the co-ordinator.
iii) The co-ordinator adds the copies of the cruise reports and inventories to his regular management reports.

These 3 elements will be managed on the WWW server and will provide a service to all project participants. The WWW server will contain details of the established procedures for instrument calibration and sampling to ensure that the merged data sets are both internally and externally compatible (quality assurance). The WWW server will be the basis for the internal news service of the project with areas for research news, newsletter articles, discussions, abstracts, papers and information from the co-ordinator.

9.2 Manpower matrix

Numbers are in person-month and only consider personnel for which budget is requested from the Community.

	SBR	Coordination	UW	AWI	ICM	UO	Total
Workpackage 1	32		5	36	35	29	**137**

Workpackage 2	32		12	12	11		**67**
Workpackage 3			4	9			**13**
Workpackage 4	6		5	9			**20**
Workpackage 5	26		10	6	79	7	**128**
Coordination		6					
Total	**96**	**6**	**36**	**72**	**125**	**36**	**371**

10 LIST OF REFERENCES AND RELATED PROJECTS

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10.2 Related projects

PROMOLEC (MAST III). This projects coordinated by SBR and to which UW participates deals with the molecular ecology of *Prochlorococcus*. It includes some of the approaches that will be taken in PICODIV (strain isolation, use of SSU rDNA gene to assess genetic diversity). Therefore PICODIV will benefit from the method developed in PROMOLEC. However, we expect to find very little *Prochlorococcus* at the major field sites studied during PICODIV, except probably at the Mediterranean site in summer. Therefore results from the two projects should have virtually no overlap.

AIMS (MAST III). This projects to which AWI participates is dedicated to the automatic detection of phytoplancton using flow cytometry. Phylogenetic rRNA probes are used to validate the detection algorithms. PICODIV will probably make use of some of the rRNA probes developed during AIMS, but we expect the picoplankton community to have a widely different taxonomic composition from the larger phytoplankton targeted by AIMS and that most of the probes developed during PICODIV will be unique to the project.