

# AWI PICODIV report, Feb 2002

## Analysing cultures by SSCP

We have worked on the large analysis of picoplankton cultures by SSCP and subsequent sequencing. First the cultures are photographed. Then a small aliquot (1.5 ml) is centrifuged, resuspended in TE and boiled for 10 min. The supernatant is then directly used for a 528F/926R SSCP PCR. Products are checked on a gel (usually 50% of the reactions are ok) and purified with quiagen columns. An aliquot is taken for sequencing. Then the single strand digest is carried out, checked again on a gel and the loaded to an SSCP gel. The gel holds minimum 20 samples. An example is below right.

As can be seen, some of the reactions produced single bands (lanes 1, 2, 5, and 6) indicating unialgal cultures, others multiple bands (lane 4).

We sequenced some of the products (528F primer), and a control product from *Skeletonema*. The *Skeletonema* product gave a clean sequence identical with *Skeletonema*.

The SSCP product in lane 1 which, on the first glance, looks like a single band, produced minor double bands in the sequencing reaction. A closer look shows a second band in the smear on top.

The SSCP product in lane 2 produced a clear sequence.

The SSCP product in lane 4 produced many double bands in the sequencing reaction as was to be expected because of the many band visible in the gel and the culture is likely not unialgal.

Because the combined SSCP/sequencing analysis looks promising we have begun to screen all cultures with this method. To date about 70 cultures have been analysed by SSCP and of these 10-15% of the cultures produce single bands. We plan to continue with approx. 20-40 analyses per week.

One sequence analyzed from the SSCP band shows high affinity to the Prasinophytes so we plan full length sequencing of this culture to fully identify the alga.

At the moment we have 380 cultures that grow visibly. More than 980 pre-cultures are still in 24 well plates. Only those cultures producing a clear major band in the SSCP analysis will in the future be screened with flow cytometry.

We have established a collaboration with the Fraunhofer Institute in Stuttgart, Germany. They wish to analyse our cultures for commercially interesting compounds and are developing algal reactors. Therefore we will continue to start new pre-cultures.



## Using SSCP to analyse environmental Biodiversity

We are about to establish the SSCP method to study biodiversity in the  $< 3\mu\text{m}$  fraction from Helgoland. The first results look promising:



The gel shows SSCP profiles of DNAs isolated between January (1) and July (7) 2001. Because the SSCP PCR was done with 528F/926R we can directly cut out bands, sequence them and compare them to the PICODIV databank. We will use the method to analyse the entire time series we have.

### Current status of the DNA-Chip approach

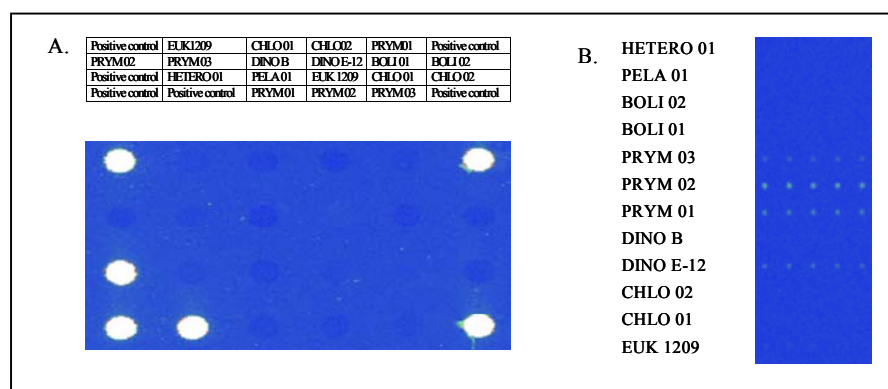
PCR-products from nine different species have now been tested in hybridizations on DNA-Chips containing higher-level probes (Tab.1).

Probe	Sequence	Class	Positive Control Species
Euk1209	GGGCATCACAGACCTG		▪ all eukaryotic species tested
HeterO1	ACGACTTCACCTTCCTCT	Heterokonta	▪ <i>Thalassiosira eccentrica</i> ▪ <i>Skeletonema costatum</i>
DINO B DINO E-12	CCTCAAACCTCCTTGCCITTA CGGAAGCTGATAGGTCAGAA	Dinophyceae	▪ <i>Alexandrium tamarense</i> ▪ <i>Prorocentrum minimum</i>
PRYM01 PRYM02 PRYM03	ACATCCCTGGCAAATGCT GGAATACGAGTGCCCCTGAC GTCAGATTTCGGGCAATT	Prymnesiophyceae	▪ <i>Prymnesium patelliferum</i>
CHLO01 CHLO02	GCTCCACGCCTGGTGGTG CTTCGAGCCCCCAACTTT	Chlorophyceae	▪ <i>Dunaniella salina</i> ▪ <i>Pyramimonas obovata</i>
PELA01	ACGTCCTTGTTGACGCT	Pelagophyceae	▪ <i>Coccolid pelagophyte</i> ▪ <i>Pulvinaria spec.</i>
BOLI01 BOLI02	CAGTCTGATTGAACTGCGT TACCTAGGTACGCAAACC	Bolidophyceae	▪ OLI Clones .

**Tab. 1:** List of the probes, the related sequences, the class which is recognized by the probe and the species which have been tested currently in hybridization-experiments on the DNA-chip.

It was possible to detect specific and some non-specific signals for all nine species. In order to be able to detect hybridization-signals for certain probes, modifications of the hybridization-protocol had to be carried out. In a very first experiment the probes Prym01, Prym02 and Prym03 did not hybridize to *Prymnesium patelliferum*. Neither was it possible to detect a hybridization-signal for a hybridization of DinoB and Euk1209 to their matching targets. The addition of a helper-oligonucleotide to the hybridization-solution that binds in close proximity to PRYM 02 led to significant signals for Prym01, Prym02 and Prym03 if *Prymnesium patelliferum* was hybridized to the DNA-Chip (Fig. 1).

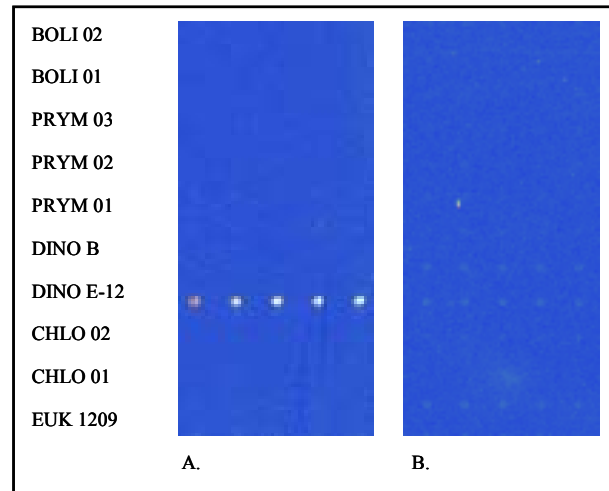
**Fig.1.:** The application of a helper-oligonucleotide that binds in close proximity to a binding-site of PRYM 02 leads to significant hybridization-signals if *Prymnesium patelliferum* is hybridized to the DNA-chip. A.: *Prymnesium patelliferum* hybridized without the helper-oligonucleotide to the DNA-chip containing the indicated probes. B.: *Prymnesium patelliferum* hybridized in presence of the helper-oligonucleotide to the DNA-chip.



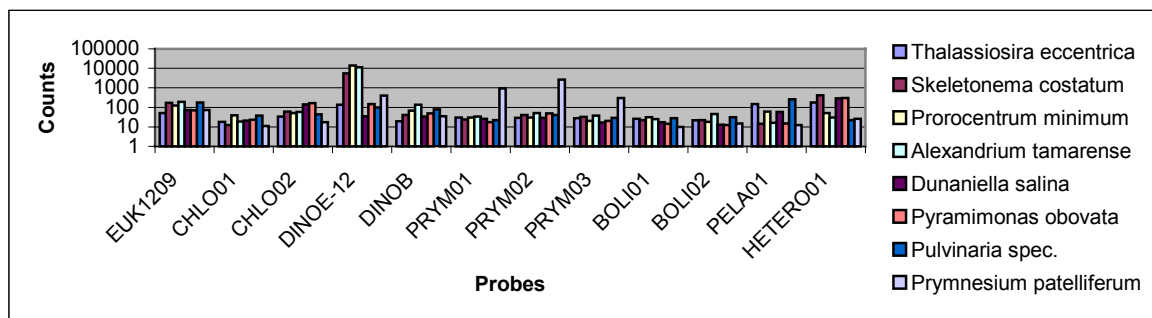
In contrast to the stimulating effect of the helper-oligonucleotides for the Prym-probes the application of helper-oligonucleotides that bind in close proximity to the probes DinoB and Euk1209 did not improve the outcome of the hybridization-experiment. Even though the helper-oligonucleotide were added, it was not possible to detect any signal for the probes

DinoB and Euk1209. Therefore a different approach to improve the hybridization-result was carried out. The probes DinoB and Euk1209 were attached to a linker consisting of twelve adenine-residues at the 5'-end of the probe. This was done in order to increase the accessibility of the probes for the target-DNA by increasing the distance between the probe and the chip-surface. The application of linkers to the probes DinoB and Euk1209 led to weak signals when they were hybridized to the matching target-DNAs (Fig.2).

**Fig.2:** Effect of the application of a linker consisting of twelve adenine residues on the hybridization of *Prorocentrum minimum* (Dinophyceae) to the DNA-chip. A.: Probes DinoB and Euk1209 have been attached via a C6/MMT- modification at the 5'-end directly onto the chip-surface. B.: A linker consisting of 12 adenine residues has been put between the 5'-end of the probe and the C6/MMT-modification.



A quantitative analysis of the hybridization-experiments has been carried out. The absolute values of the hybridization-signals ranged from around 100 count to 11.000 counts. Therefore the signal values have been put together in a diagram with a logarithmic scale (Fig.2).



**Fig.2:** This diagram summarizes the number of counts that have been measured for the hybridizations of the indicated species to DNA-chips containing the indicated probes. The measurement was done with an Axon 4000-Scanner (Axon Instruments, Inc.). The setting of the PMT was 750.

The values of the absolute signal-intensities indicate a background-noise of 50 counts for those probes which don't match the target-DNA, whereas the signal-intensities for probes that perfectly match the target-sequence are at least 1.5 fold higher than the average background noise. In later experiments this low signal to noise-ratio is probably not sufficient to be able to reliably distinguish between specific signals and those which are unspecific. Therefore next

experiments should focus on increasing specific signals and eliminate unspecific signals. This could be done by the application of more stringent hybridization-conditions, whereas there are two strategies that could contribute to an increase of signal-intensities. Because of the labelling of the target-DNA by PCR-amplification there is currently only one fluorophore attached to a target-molecule. The signal intensity could be increased by attaching more than one fluorophore to the target-molecule. One approach would be to incorporate the fluorescent dye directly into the PCR-product by using fluorescently labelled nucleotides, since this would lead to the incorporation of several fluorophores into the target-molecule. A second approach would be, to biotinylate the target-DNA by PCR using biotinylated nucleotides or primer. In this case the hybridization could be detected via an antibody directed against biotin conjugated directly to Cy3 or Cy5 or via a sandwich that consists of a primary antibody directed against biotin and secondary, Cy3 or Cy5-conjugated antibody directed against the primary antibody. The use of polyclonal antibodies for the detection of the hybridization-signal would lead to strongly increased signal intensities, because a polyclonal antibody itself is conjugated to more than one fluorophore per molecule. In addition to that the antibody recognizes several epitops on the antigen and thereby attaches even more fluorophores to the target-molecule, which would lead to an increased hybridization signal.

Even though the hybridization-protocol has to be modified in order to optimise signal to noise ratios, the preliminary results indicate that there is a good chance to use the probes listed above (Tab.1) for the detection of microalgae at class-level on the monthly samples taken in the third year.

Identification of the Helgoland clade in the red algae. Partial sequences of the red alga *Cyanidium* have been downloaded from Genbank and put into the ARB alignment. These sequences cluster with the full length sequences of the environmental clones of the Helgoland clade. We have completed the sequencing of 6 more environmental clones from this clade and they await assembly. Thus this is not a new algal clade but is the unicellular red algal clade. *Porphyridium* is a highly redrived species that clusters with more complex Bangiophycidae to the exclusion of the lower red unicells, which lead us to the first interpretation that we had a new algal clade.