Progress Report AWI Oktober 2002

Workpackage 1 Cultures

Rough cultures were established every two months from April 2000 to May 2002. A total of 20 cultures have been deposited with Roscoff. There are now 424 cultures with visible growth and all of these can be processed now for SSCP. A total of 106 cultures have already been screened with SSCP and identified where unialgal. 104 Cultures have sufficient growth that they can be screened with flow cytometry, of these 20 have been screened. Ten cultures have had their pigments extracted for HPLC characterisation.

For future culture analysis an agreement is about to be signed with the Fraunhofer Institute in Stuttgart (FIS). The FIS will support us with the establishment of cultures and will screen cultures for economically interesting substances.

Workpackage 2 Environmental Clone Cultures

We have begun to establish full-length sequences for selected clones:

- 5 sequences for the "novel red algal clade"
- 2 sequences for novel stramenopiles (sent to Ramon)
- 2 sequences for alveolates (sent to Agnes)

Workpackage 3 Probe Development

We have finished all of our probe development but still outstanding are more FISH tests with the Hetero01 probe. This will take place in November when additional staff will be trained with this probe.

Workpackage 4 Probe measurement

Most of our efforts in this reporting period were concentrated on this workpackage.

DNA-Chips

1. Hybridisation of picoplankton-18S-DNA to a DNA-chip

In our last report we presented results of preliminary experiments in which the 18S-DNA from different algal classes served as a target for a hybridisation with probes that have been immobilised on a DNA-Chip (Fig.1). The signals observed in the

preliminary experiments appeared to be specific except for Dino E-12, which gives a weak signal for each of the tested algae. When the last report was written, only 18S-DNA from algae that are bigger than 3 µm had been hybridised to the DNA-chip. Because the chip is to be developed is dedicated to the monitoring of picoplankton-species it was also necessary to test if the DNA-chip works for the detection of picoplankton 18S-DNA. Therefore 18S-DNA of a Bolidophyceae and a Prasinophyceae, both isolated in the PICODIV-project have been hybridised to a DNA-chip. This was one of the first experiments which has been done following the last report. After the hybridisation of the Bolidophyceae to the DNA-chip it was possible to detect a specific signal for Boli 02, but there was no detectable signal for Boli 01. The 18S-DNA of the Prasinophyceae was bound by Chlo 02 but not by Chlo 01. Like in previous experiments Euk1209, the eukaryotic control gave in both experiments only very weak signals.

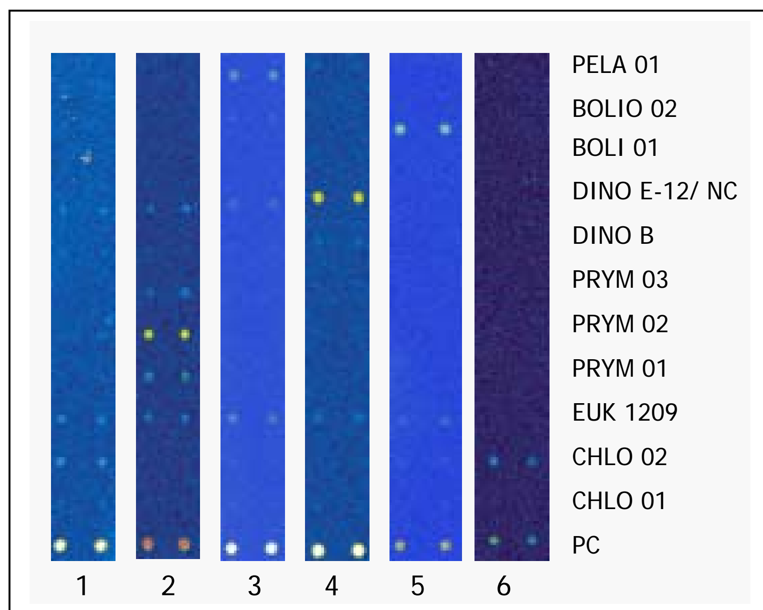

Fig. 1: Hybridisation of PCR-fragments from the 18S-rRNA of different algal classes to a DNA-Chip. 1. *Dunaniella salina* (Chlorophyceae), 2. *Prymnesium patelliferum* (Prymne-siophyceae), 3. *Coccoid pelagophyte* (Pelagophyceae), 4. *Alexandrium tamarense* (Dinophyceae), 5. PicoDiv Clone # 151 Bolido-phyceae, 6. PICODIV Clone # 53 (Prasinophyceae). Because Dino E-12 appeared to be too unspecific to be used in microarray-experiments.in lane 5 and 6 the space for Dino E-12 served as a negative control.

2. Hybridisation of a mix of different target DNAs to a DNA-chip

DNA-microarray technology has been designed for the analysis of complex samples that contain a mixture of different target-DNAs. A hybridisation with a mix of three species to a DNA-Chip has been carried out to mimic the situation of a complex sample. The mix contained PCR-fragments from *Prymnesium patelliferum* (Prymnesiophycea), PICODIV-clone 151 (Bolidophyceae) and PICODIV-clone 53 (Prasinophyceae). The results from this hybridisation were compared to hybridisations in which equal amounts of a single 18S-PCR fragment were hybridised to a DNA-chip. The signal-intensities from the hybridisation have been analysed visually and quantitatively (Fig. 2). The target-DNA in the experiment was labeled with biotin and the detection of the biotinylated target-DNA was carried out with Streptavidin-Cy5. The signals were acquired with a PMT of 750 with the Axon 4000B-Scanner. The quantitative analysis of the hybridisation-signals showed, that the signal-intensities after the hybridisation of the mix of target-DNAs were equal to those gained from the hybridisation of single target-DNAs. This experiment indicates that 18S-PCR fragments in a complex sample can be specifically detected with a DNA-chip.

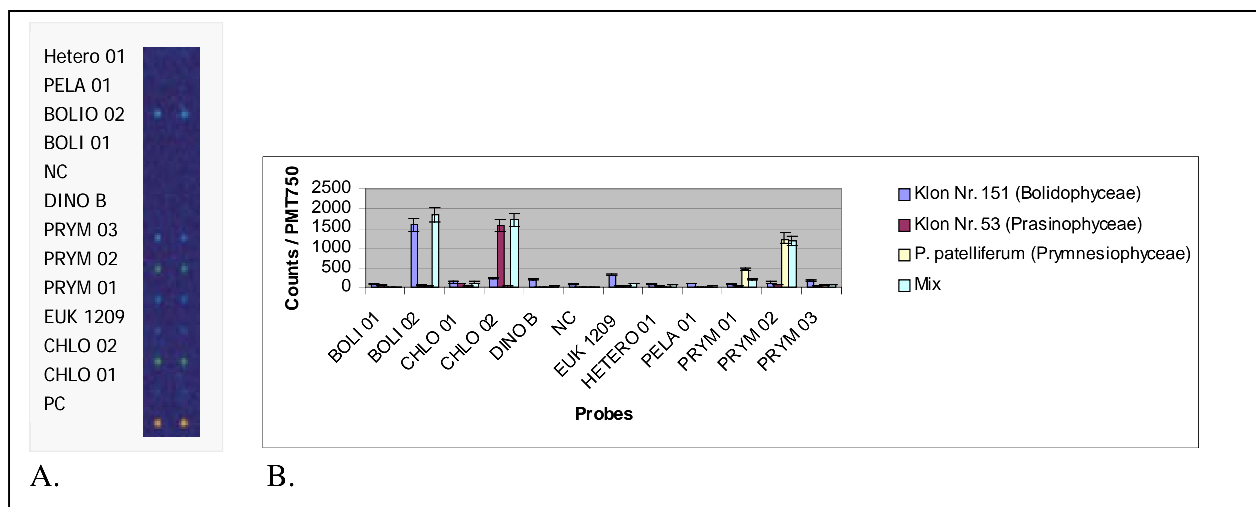

Fig. 2.: A hybridisation of a mix of 18S-PCR-fragments to a DNA-chip has been carried out. A. Image of the DNA-chip. B. Quantitative analysis of the signal-intensities from the different hybridisation.

3. Hybridsation of different target-concentrations to a DNA-chip

One of the aims of the PICODIV-project is to monitor the picoplankton-composition at certain sampling-sites over a longer time-period. As a consequence the DNA-chip has to be suited to detect different amounts of 18S-DNA amplified from complex samples. Again, we mimicked the situation of a complex sample by using a mix of three different 18S-PCR fragments for the hybridisation. Two hybridisations with different target-concentrations have been carried out. In the first hybridisation-mix the concentration of the Bolidophyceae and the Prasinophyceae was 30 nM, whereas the concentration of the Prymnesiophyceae was 10 nM. In the second mix the concentration of the Bolidophyceae and the Prasionphyceae was half of the concentration like in the first hybridisation-mix and the concentration of the Prymnesiophyceae was doubled in comparison to the first mix. The quantitative analysis of the two hybridisations shows that the ratios of the signal-intensities equal very closely the ratios of the different target-concentrations (Fig. 3 and Tabl. 1.)

Tab.1: Quantitative analysis of the hybridisation-signals shown in Fig.3. The indicated probes in lane 4 were supposed to bind to the indicated targets in lane 1.

Target	Mix A	Mix B	Probe	Ratio A/B
Clone # 151 (Bolidophyceae)	30 nM	15 nM	Euk1209 Hetero 01 Boli 01 Boli 02	Signal too weak No signal No signal 1.8
Clone # 53 (Prasinophyceae)	30 nM	15 nM	Euk 1209 Chlo 01 Chlo 02	Signal too weak No signal 1.4
Prymnesium patelliferum	10 nM	20 nM	Euk 1209 Prym 01 Prym 02 Prym 03	Signal too weak 0.5 0.7 0.5

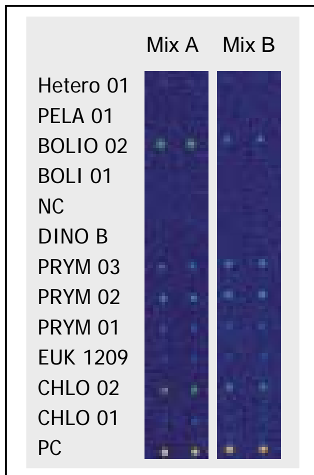

Fig. 3: Hybridisation of two hybridisation-cocktails with different target-concentrations. The hybridisation-cocktails contained target-DNA from a Bolidophyceae (PicoDiv-clone # 151), a Prasinophyceae (PICODIV-clone # 53) and a Prymensiophyceae (*Prymnesium patelliferum*) in two different concentrations.

4. Correlation of the hybridisation-signals with the location of the probes

The previously described results indicate that not all of the probes, which have been selected bind equally well to their 18S-targets. A determination of the binding-loci revealed that they are randomly distributed over the sequence of the 18S-rRNA. The binding-loci have been correlated with the signal-intensities of the hybridisation experiments and it appears that those probes that only generate poor signal-intensities are located all at a distance > 1000 bp from the 5'-End of the 18S-Gene (Tab. 2). This result indicates that the probes which are located closer to the 3'-End of the target are not suitable to be used on a DNA-chip. Future probes should be located in a maximum distance of 1000 bp from the 5'-End of the 18S-DNA. This observation indicates that the target molecule might be too long to bind to a ~20 bp oligonucleotide that has been immobilised on the surface of the DNA-chip.

Probe	Loci in the 18S-Gene	Tm	Signal
Boli 02	~ 300 bp	54°C	+
Dino E-12	~ 350 bp	60°C	+
Prym 03	~ 450 bp	50°C	+
Pela 01	~ 900 bp	56°C	+
Prym 01	~ 950 bp	54°C	+
Prym 02	~ 950 bp	64°C	+

Chlo 02	~ 950 bp	56°C	+
Chlo 01	~ 1350 bp	62°C	-
Dino B	~ 1400 bp	56 °C	-
Euk 1209	~ 1400 bp	52 °C	-
Boli 01	~ 1450 bp	60 °C	-
Hetero 01	~ 1700 bp	54 °C	-

Tab. 2.: Correlation of the location of the probes to the signals obtained in the hybridisation-experiments.

4. Fragmentation of the target-DNA leads to an improved signal-intensity of Euk 1209

The correlation of the probe-loci with the signal-intensities obtained from hybridisations of a ~ 1800 bp PCR-fragment to a DNA-chip containing probes with a length of ~ 20 bp indicated that 1800 bp might be too long to obtain a decent hybridisation-signals on the chip. To test this hypotheses, a fragmented 18S-PCR-product was hybridised to the DNA-chip. To obtain fragments of DNA that all carry a label, that can be detected on the DNA-chip, it was necessary to label the target-DNA with Psoralen-Biotin in this experiment. As a side-effect of a rough labelling-protocol for the Psoralen-Biotin-labelling the target-DNA gets degraded during this process. After the labelling the 18S-target DNA is fragmented from the original size of ~1800 bp down to maximal ~ 900 bp (Fig. 4).

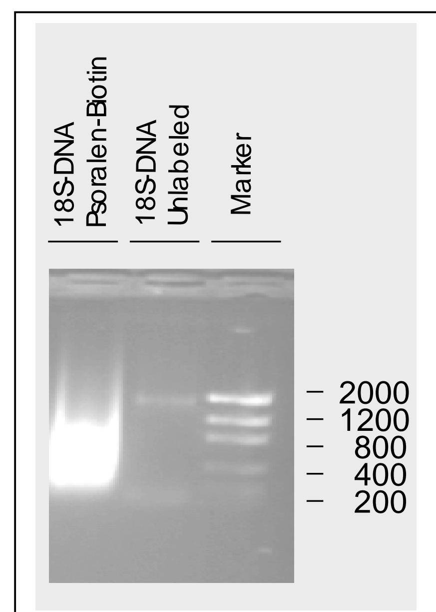

Fig. 4.: 18S-target-DNA after the labelling with Psoralen-Biotin. 500 ng of Psoralen-biotin-labelled and non-labelled 18S-DNA of *Prymnesium patelliferum* were separated on a 1.5% agarose gel.

The labelled 18S-PCR-fragment was hybridised to a DNA-chip. In addition a hybridisation was carried out with a 18S-PCR-fragment that had been biotinylated by a biotinylated PCR-primer. A comparison of the signal-intensities of both hybridisations shows that the absolute signal intensities after the labelling with Psoralen-Biotin are in average 3.5 x higher than the absolute signal-intensities measured after the hybridisation of the PCR-labelled target-DNA. But the same time the background intensity was a lot higher then in experiments in which PCR-labelled DNA was used as a target. Therefore the signal to noise-ratios are better if the target-DNA is labelled by PCR. Nevertheless it was possible to observe a clear signal for Euk 1209 for the Psoralen-biotin-labelled target-DNA which was hardly detectable if the target-DNA was labelled with a biotinylated primer. The signal to noise ratio generated in the experiment with Psorlen-Biotin-labelled target-DNA of Euk 1209 was almost as high as the signal to noise-ratio of Prym 02 that gave a good signal in previous results. It was only 1.7 x less then the signal to noise-ratio of Prym 02. In contrast to this the signal to noise-ratio for Euk 1209 was 21 x less than the signal to noise-ratio of Prym02 if the DNA was labelled with a biotinylated PCR-Primer (Fig. 5). This means that the signal-intensity of Euk 1209 is approximately 10 x stronger in relation to Prym 02, if the DNA is fragmented, which happens during the process of the Psoralen-labelling-procedure.

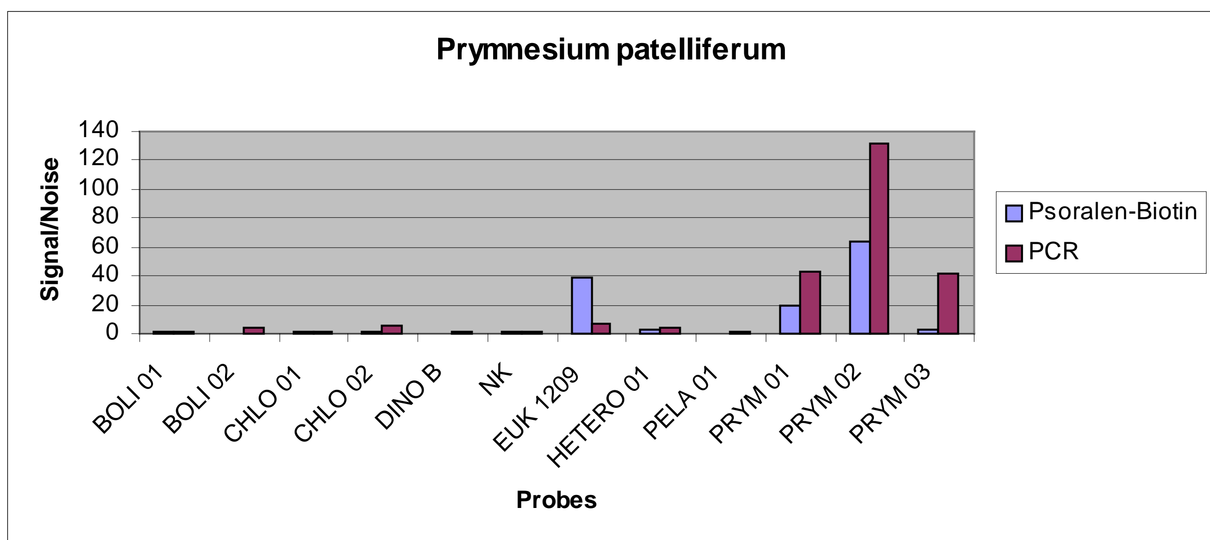

Fig. 5. : Quantitative analysis of a hybridisation in which Prymnesium patelliferum was either labelled with Psoralen-Biotin or with a biotinylated Primer.

5. Determination of the average Hybridisation-signal per ng Target-DNA

The results of the experiments that have been described in this report have been done with DNA-chips that have been spotted in collaboration with the University of Bremen. To ensure that the results are reproducible, the signal-intensity gained per ng DNA following a hybridisation, has been estimated for Prym 02 and Boli 02 for all the hybridisations that have been carried out so far. The signal intensities obtained per ng target-DNA are highly similar over the experiments, independent how much DNA was hybridised (Fig. 6). This indicates that the spotting of the DNA-chips is highly reproducible. In addition to that the results also show that the average signal-intensity for the different probes differs a lot. Most likely this is due to the fact that the chosen probes have different optimal hybridisation conditions. Probes that ought to be designed in the future should be designed under the aspect that they theoretically have very similar hybridisation conditions.

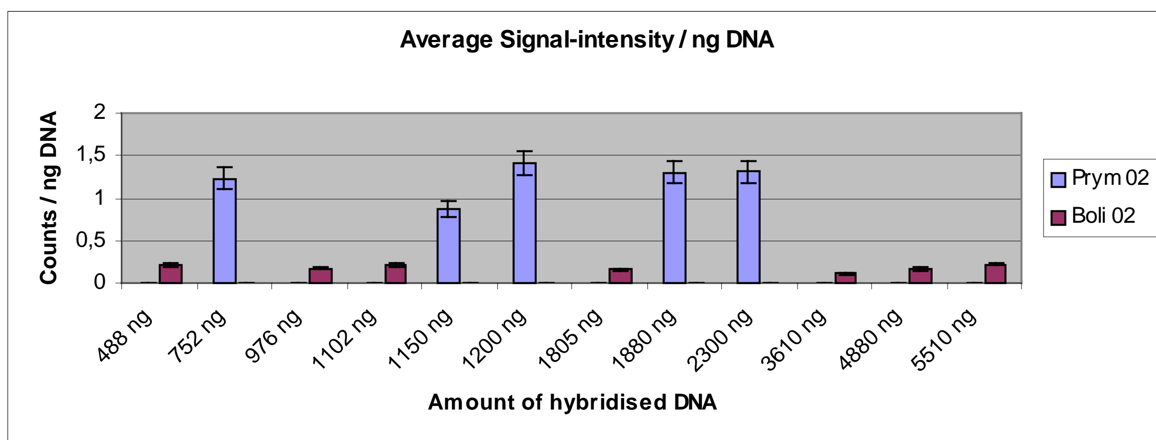

Fig. 6.: The average specific signal-intensity that has been obtained per ng target-DNA was calculated.

7. Probes to be used for the environmental samples.

We have now obtained all probe sequences from new clades characterised in the PICODIV Project hat we plan to use on the microarrary for the analysis of the environmental samples. These include: EUK 1209, EUK 328, EUK 1528, CHLO 01,

CHLO 02, PRYM 01, PRYM 02, PRYM 03, DINO B, DINO E-12, BOLI 01, BOLI 02, HETERO 01, PELA 01, PELA 02, PK, NK, NS 3, NS 4, PRAS 04, BATHY 01, MICRO 01, OSTREO 01, CRYPTO 07, CRYPTO B. These have been sent away for spotting and we should receive the chips in the next few weeks and can begin testing the environmental DNA. We await the DNA samples from our partners.

8. Summary

In the past three months since the last report was written, experiments have been carried out to obtain more experience with the application of DNA-chips for the identification of phytoplankton. First of all PCR-fragments from the 18S-DNA of picoplankton species isolated in the PICODIV-project could be specifically identified with a DNA-chip. Second it was possible to show that a DNA-chip is suited to identify specifically single species in a mix of different target-DNAs. In addition to that, we were able to show that the concentration of the target-DNA corresponds closely to the signal-intensities on the DNA-chip after a hybridisation. The results of the described experiments indicate one very important aspect that should be considered for the probe-design in the future. We made the experience that probes, besides having as similar hybridisation-conditions as possible, should be located in a maximum distance of 1000 bp from the 3'-End of the 18S-Gene. If this is not possible it should be considered to fragment the DNA prior to a hybridisation, since a fragmented target-DNA let to clear signals that could not be observed if the target-DNA was not fragmented. Our chips will soon be ready to complete the last phase of the environmental characterization.

Presentations

Medlin, L., Groben, R. , John , U., Lange, M., and Kerkmann K.. rRNA probes for identification and characterization of marine phytoplankton: their potential application for DNA microchips. Invited speaker, Culture Collection of Algae: Increasing accessiblility and exploring algal biodiversity. Goettingen Sept 2/6 2002

Kerkmann, K., Groben, R. ,Valentin, K. and Medlin, L. Development of a NDA-microarray for the monitoring of phytoplankton diversity Invited speaker, Culture Collection of Algae: Increasing accessibility and exploring algal biodiversity. Goettingen Sept 2/6 2002

Valentin, K. Assessing picoplankton cultures using 18S fingerprinting and partial sequencing. Poster presented at the Deutsche Botanikertagung, Freiburg Sept. 2002-10-08

Valentin K, Mehl H, and Medlin L Molecular analysis of the eukaryotic marine picoplankton at Helgoland (North Sea). Invited talk by KV at the Deutsche Botanikertagung, Freiburg Sept. 2002-10-08