Rapid tests for the detection of Prymnesium parvum and its toxins

Linda Medlin, Gundula Ellers, Kerstin Toebe, & Katja Kerkmann
Bremerhaven, Germany
Why rRNA probes?

* universally found
* high target numbers per cell
* variable and conserved regions (can make nested probes for quantification)
Variability Map of Eukaryotic Small Ribosomal Subunit RNA

Make hierarchical rRNA probes
How to design and test a probe

* Amass data bases from rRNA sequences

* use ARB program to design probe

* check probe for possible matches in RDP and Genbank

* test specificity in dot blot (DIG-labelled probe) and in situ (FITC or CY3 labelled probe) tests

* final check with flow cytometer
Change from PFA to ETOH Saline

Change from SDS to Nonidet P-40

Without Dimethyl Formamide

With Dimethyl Formamide
Double Staining of Cells to differentiate cells hierarchically

The cells were hybridised with the universal eukaryotic probe (labelled blue) and the genus specific probe for *Prymnesium PrymGl01A* (labelled green). Mixture of *E.huxleyi* and *Prymnesium parvum*
Analysis of *Alexandrium* strains from European waters using hierarchical probes

**Alex. tamarense** (English Channel)

**Alex. cf. tamarense** (Italy)
Phylogenetic tree of *Alexandrium* (18S rRNA)
Identification of *A. tamarense* in Lab Cultures and Field Samples by Species- & Strain-specific Probes

J. Brenner, unpubl.

**no probe**

**ATAM01**  
(species specific)

**ATNA02**  
(strain specific)

*A. tamarense* (WE strain)  
Field sample (Orkney Islands)

J. Brenner, unpubl. results
Harmful Algal Blooms

Noctiluca

Karenia brevis
Alexandrium tamarense  

A. ostenfeldii

[Images of micrographs showing the microorganisms with labeled V.p.]
Chrysochromulina kappa
Chrysochromulina hirta
Cruciplacolithus neohelis
Coccolithus sp. CCMP 300
Pleurochrysis carterae
Emiliania huxleyi
Prymnesium parvum
Prymnesium patelliferum
Prymnesium calathiferum
Chrysochromulina polylepis
Chrysochromulina kappa
Chrysochromulina hirta
Chrysochromulina campanulifera
Chrysochromulina strombilis
Chrysochromulina simplex
Chrysochromulina leadbeateri
Chrysochromulina cf. ericina
Chrysochromulina throndsenii
Chrysochromulina throndsenii
Chrysochromulina sp. P16
Chrysochromulina sp. TH2
Phaeocystis antarctica
Phaeocystis pouchetii
Phaeocystis globosa
Pavlova sp. CCMP1416
Pavlova gyrans
Pavlova sp. CCMP 625
Pavlova salina
Application & detection methods for rRNA probes

- DNA dot blots
- \textit{In situ} hybridization / Fluorescence Microscopy
- \textit{In situ} hybridization / Flow Cytometry liquid & solid
- DNA microchips
Euk 1209

PrymGenus Probe
18S rRNA

Prym Species Probe
28S rRNA
Conclusions

• Specific probes could be made for different groups of phytoplankton (from higher group down to species and strain level)
• More than a dozen probes for toxic algae are available or under development
• It is possible to use the probes with lab cultures and with field samples
• The probes could be used with different kinds of techniques (dot blots, fluorescence microscopy, flow cytometry, DNA chips, etc.)
Platforms for the detection of toxic algae

Linda Medlin, G. Eller, K. Toebe, R. Groben, M. Lange, & K. Kerkmann
Bremerhaven, Germany
1. ChemScanRDI, a laser based system to quickly analyse FISH-experiments

2. Electrochemical detection of toxic algae via a handheld device

3. Development of DNA-microarrays for monitoring phytoplankton composition
FISH detection of toxic algae via the ChemScan, solid phase cytometer
ChemScanRDI combines fluorescent cell labelling with laser scanning

3. Automated analysis of fluorescent cells by ChemScan RDI (Chemunex Inc.; Maisons-Alfort, France)
All Data:  Total Objects Seen:  107
Electrochemical detection of toxic Algae via a handheld device
A Handheld Device for the Detection of Harmful Algae

- *Alexandrium tamarense*
- *Alexandrium ostenfeldii*

Diagram of the device:

- Helper Electrode
- Reference Electrode
- Work Electrode
- Immobilized Oligo
- Enzyme coupled to an antibody
- Enzyme catalyzed transfer of electrons
- Disposable Sensorchip
Electrochemical Detection of rRNA from *Alexandrium* Species

- ~ 500 ng rRNA have been hybridized to probes
- The probes were directed against ribosomal RNA of *A. tamarense*, respectively *A. ostenfeldii*
Concentration Series of Decreasing Amounts of Target

A. Decreasing amounts of A. tamarense rRNA hybridized to a A. tamarense probe

B. Decreasing amounts of a 70 bp Oligonucleotide hybridized to a A. ostenfeldii probe
Comparison of cell counts with electrode readings

Data 1

\[ y = 40,919 + 0,032024x \quad R = 0,91791 \]
C. Detection of rRNA in natural samples from the Orkneys islands (column sets 1-4) as compared to rRNA from Prorocentrum mexicanum (column sets 5&6) and hybridized with Alexandrium tamarense probe.
Sensors have been treated with Avidin (Avidin) and Avidin/Probe (DNA)

The coated Sensors were then stored at 4°C over the indicated times

Freshly prepared sensors have been prepared before each hybridization
as positive controls for the experimental conditions (Frisch)

To control the stability of the coated sensors, a hybridization was carried out with a 70 bp oligonucleotide (PC)

For the negative control (NC) a hybridization was carried out without target-DNA
Alexandrium ostenfeldii K0324
RNA per Cell in Log-, Lag- and Stationary Phase

RNA/Cell (pg)
Development of DNA-microarrays for monitoring phytoplankton composition
Scheme of a DNA-Chip Experiment

**Glass-Slide**

Imobilized DNA (Oligonucleotides, PCR-Fragments, cDNA)

Fluorescently labeled ssDNA
Scheme of a DNA-Chip Experiment

Fluorescently labeled ssDNA

Imobilized DNA (Oligonucleotides, PCR-Fragmentes, cDNA)

Glass-Slide
Low Density Chips

- ~ 625 Spots per cm²
- Spot diameter: ~ 200 µm
- Spotting with needles (A) or piezotechnology (B)
Monitoring Phytoplankton composition with DNA-Chip Technology

Phytoplankton samples

Isolation of genomic DNA from the sample

Amplification of the 18S rDNA

Hybridization of the 18S PCR-products with a DNA-Chip that contains probes initially designed for FISH
### Probes and Targets used for preliminary Chip-Experiments

<table>
<thead>
<tr>
<th>Class</th>
<th>Probe</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dinophyceae</td>
<td>DINO B</td>
<td><em>Alexandrium tamarense</em></td>
</tr>
<tr>
<td></td>
<td>DINO E12</td>
<td><em>Prorocentrum minimum</em></td>
</tr>
<tr>
<td>Prymnesiophyceae</td>
<td>PRYM01</td>
<td><em>Prymnesium patelliferum</em></td>
</tr>
<tr>
<td></td>
<td>PRYM02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PRYM03</td>
<td></td>
</tr>
<tr>
<td>Chlorophyceae</td>
<td>CHLO01</td>
<td><em>Dunaniella salina</em></td>
</tr>
<tr>
<td></td>
<td>CHLO02</td>
<td><em>Pyramimonas obovata</em></td>
</tr>
<tr>
<td>Pelagophyceae</td>
<td>PELA01</td>
<td><em>Coccos pelagophyte</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Pulvinaria spec.</em></td>
</tr>
<tr>
<td>Bolidophyceae</td>
<td>BOLI01</td>
<td><em>Clone. No. 151 PICODIV</em></td>
</tr>
<tr>
<td></td>
<td>BOLI02</td>
<td></td>
</tr>
</tbody>
</table>
Localization of the Class-level probes in the 18S-Sequence

18S-DNA ~ 1800 bp
Preliminary results of a DNA-Chip with Class-level Probes

1. *Dunaliella salina*
2. *Prymnesium patelliferum*
3. *Coccoid pelagophyte*
4. *Alexandrium tamarense*
Identification of Phytoplankton on Class-level in a Mix of Laboratory Strains

Genomic DNA

18S-PCR

Hybridisation to DNA-Chip

A1- PC
A2- Euk 328
A3- Euk 1209
A4- Chlo 01
A5- Chlo 02
A6- Hetero 01
B1- Boli 01
B2- Boli 02
B3- Prym 01
B4- Prym 02
B5- Prym 03
B6- Pela 01
C1- Pela 02
C2- Dino B
C3- Dino E-12
C4- NS 03
C5- NS 04
C6- Pras 04
D1- Bathy 01
D2- Micro 01
D3- Ostreo 01
D4- Crypto B
D5- NC
D6- PC
- *Prymnesium parvum*
- *Alexandrium ostenfeldii*

**Threshold for a positive signal:**

signal/noise ratio $\geq 2$

<table>
<thead>
<tr>
<th>A1</th>
<th>PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2</td>
<td>Euk 328</td>
</tr>
<tr>
<td>A3</td>
<td>Euk 1209</td>
</tr>
<tr>
<td>A4</td>
<td>Chlo 01</td>
</tr>
<tr>
<td>A5</td>
<td>Chlo 02</td>
</tr>
<tr>
<td>A6</td>
<td>Hetero 01</td>
</tr>
<tr>
<td>B1</td>
<td>Boli 01</td>
</tr>
<tr>
<td>B2</td>
<td>Boli 02</td>
</tr>
<tr>
<td>B3</td>
<td>Prym 01</td>
</tr>
<tr>
<td>B4</td>
<td>Prym 02</td>
</tr>
<tr>
<td>B5</td>
<td>Prym 03</td>
</tr>
<tr>
<td>B6</td>
<td>Pela 01</td>
</tr>
<tr>
<td>C1</td>
<td>Pela 02</td>
</tr>
<tr>
<td>C2</td>
<td>Dino B</td>
</tr>
<tr>
<td>C3</td>
<td>Dino E-12</td>
</tr>
<tr>
<td>C4</td>
<td>NS 03</td>
</tr>
<tr>
<td>C5</td>
<td>NS 04</td>
</tr>
<tr>
<td>C6</td>
<td>Pras 04</td>
</tr>
<tr>
<td>D1</td>
<td>Bathy 01</td>
</tr>
<tr>
<td>D2</td>
<td>Micro 01</td>
</tr>
<tr>
<td>D3</td>
<td>Ostreo 01</td>
</tr>
<tr>
<td>D4</td>
<td>Crypto B</td>
</tr>
<tr>
<td>D5</td>
<td>NC</td>
</tr>
<tr>
<td>D6</td>
<td>PC</td>
</tr>
</tbody>
</table>
Summary

- The ChemScanRDI is a laser-based system that reduces the time required for FISH due to an automatic analysis, visual recovery of cells with positive signals.

- It is possible to detect toxic *Alexandrium* species via a handheld device.

- DNA-Chip technology provides the possibility to analyse numerous hybridizations in parallel.
Research Needs

- Monitoring of toxic phytoplankton populations is an important scientific issue.

- Efficient monitoring requires quick and reliable techniques.

- Currently the identification of species is done mainly by light or electron microscopy.

- New tools are needed to be developed which cut down the time necessary for toxic phytoplankton classification.

- Methods that involve oligonucleotide probes have the potential to fulfill these needs.
People involved in the projects

Dr. Gundula Eller
Dr. Kerstin Toebe  FISH/ ChemScanRDI

Susanne Huljic  Handheld device/ A. ostenfeldii

Dr. Katja Kerkmann  DNA-Chip Technology

Dr. Martin Lange  Handheld device/ A. tamarense

Dr. Rene Groben  Probe  Hybridisation optimisation

Dr. Linda Medlin  Principal investigator
Rapid Tests for the Detection of Haemolytic Compounds
Absorbance spectrum of lysed erythrocytes and algal extract.

- Lysed erythrocytes peak at 414 nm.
- Algal extract peaks at 540 and 575 nm.

Absorbance is measured in units along the vertical axis, with wavelengths from 350 to 700 nm on the horizontal axis.
y = 0.9829x - 0.0024
R² = 0.9994

y = 0.1051x - 0.0007
R² = 0.9994

0.0 0.2 0.4 0.6 0.8 1.0

abs. units

414 nm:

540 nm:
Prymnesium parvum RL10

The graph shows the percentage of lysis (% lysis) over the number of cells *10^3 / well for Prymnesium parvum RL10. The data is presented for both 24 h and 48 h time points. The percentage of lysis decreases with increasing cell numbers, and there is a noticeable difference between the two time points.
Alexandrium tamarense CCMP115

![Graph showing % lysis of Alexandrium tamarense CCMP115 over different concentrations of cells and time points (24 h and 48 h). The x-axis represents the number of cells *10^2/well, and the y-axis represents % lysis.](image-url)
Allelochemical effects
ELA Tests provide rapid means for detecting haemolytic compounds but these must be coupled with species tests for 100% Reliability