Rapid tests for the detection of Prymnesium parvum and its toxins

Linda Medlin, Gundula Ellers, Kerstin Toebe, & Katja Kerkmann
Bremerhaven, Germany

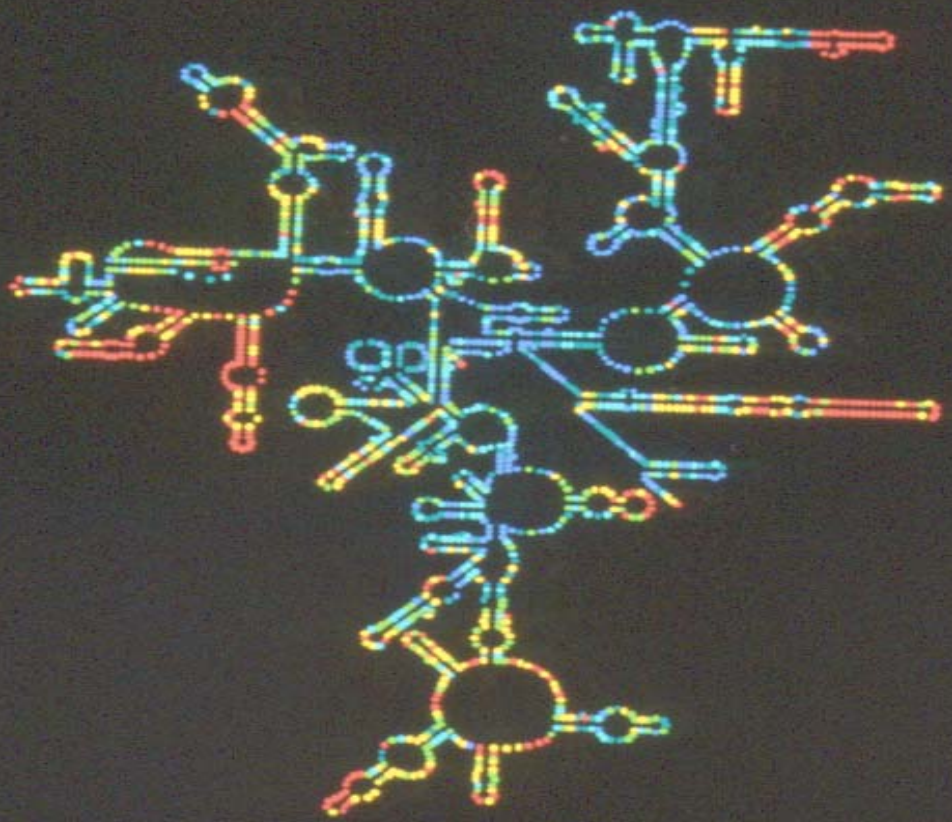
Why rRNA probes?

* universally found

* high target numbers per cell

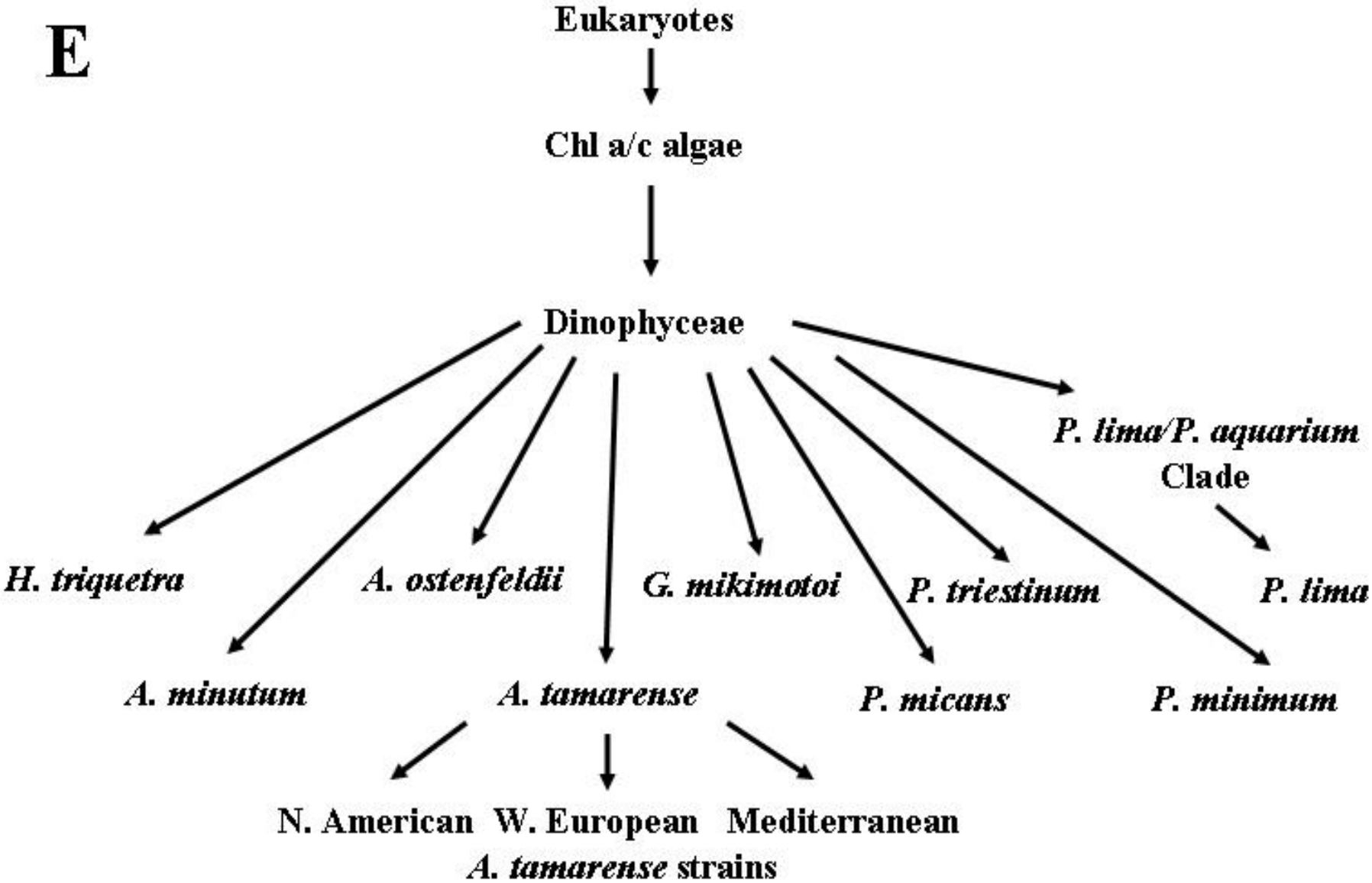
* variable and conserved regions (can make nested probes for quantification)

Variability Map of Eukaryotic Small Ribosomal Subunit RNA



Make hierarchical rRNA probes

E


Eukaryotes
Chl a/c algae
Dinophyceae
P. lima/P. aquarium
Clade
H. triquetra
A. ostenfeldii
G. mikimotoi
P. triestinum
P. lima
A. minutum
A. tamarense
P. micans
P. minimum
N. American
W. European
Mediterranean
A. tamarense strains

P. pungens

How to design and test a probe

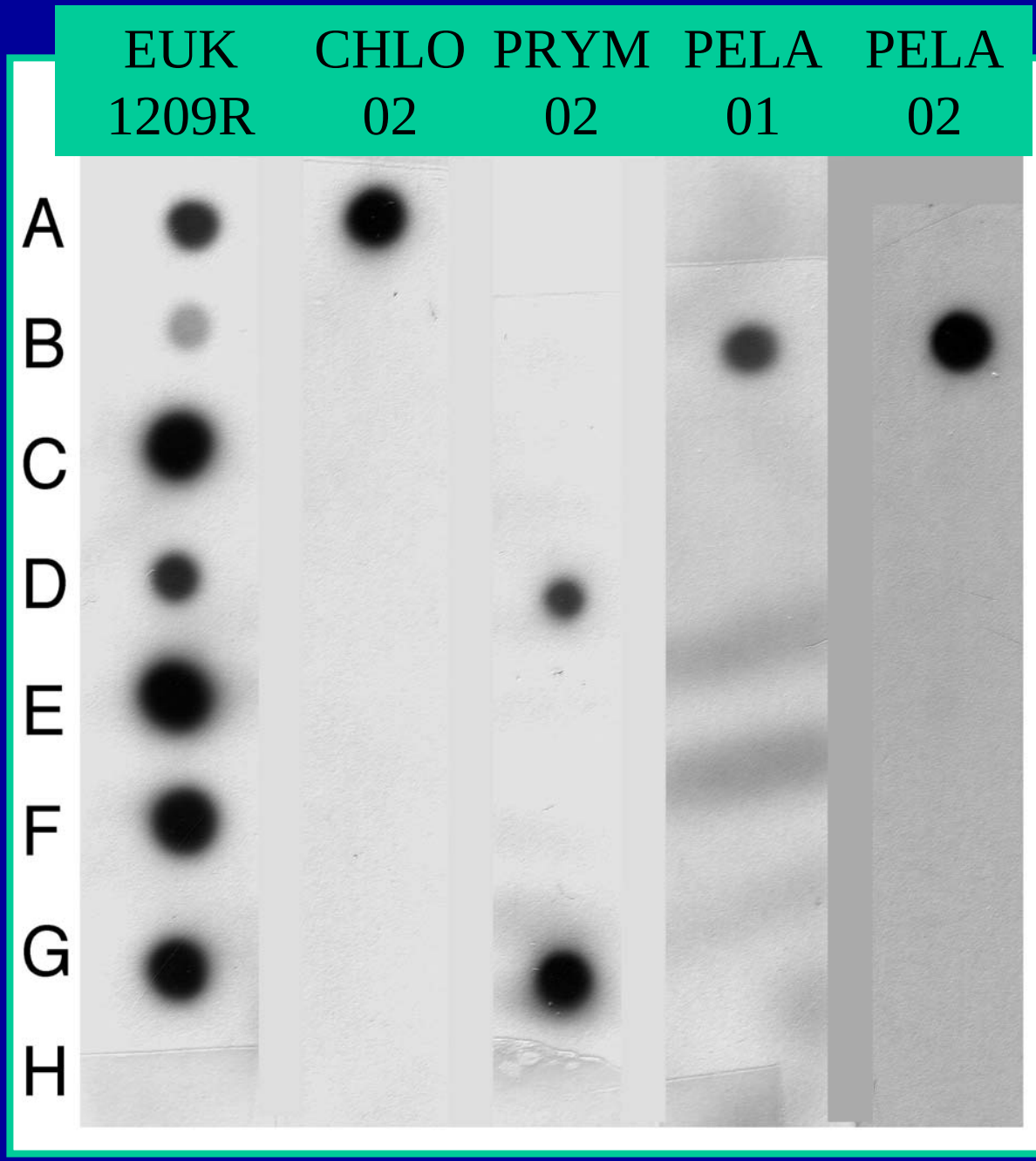
* Amass data bases from rRNA sequences

* 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


EUK 1209R
CHLO 02
PRYM 02
PELA 01
PELA 02
A
B
C
D
E
F
G
H

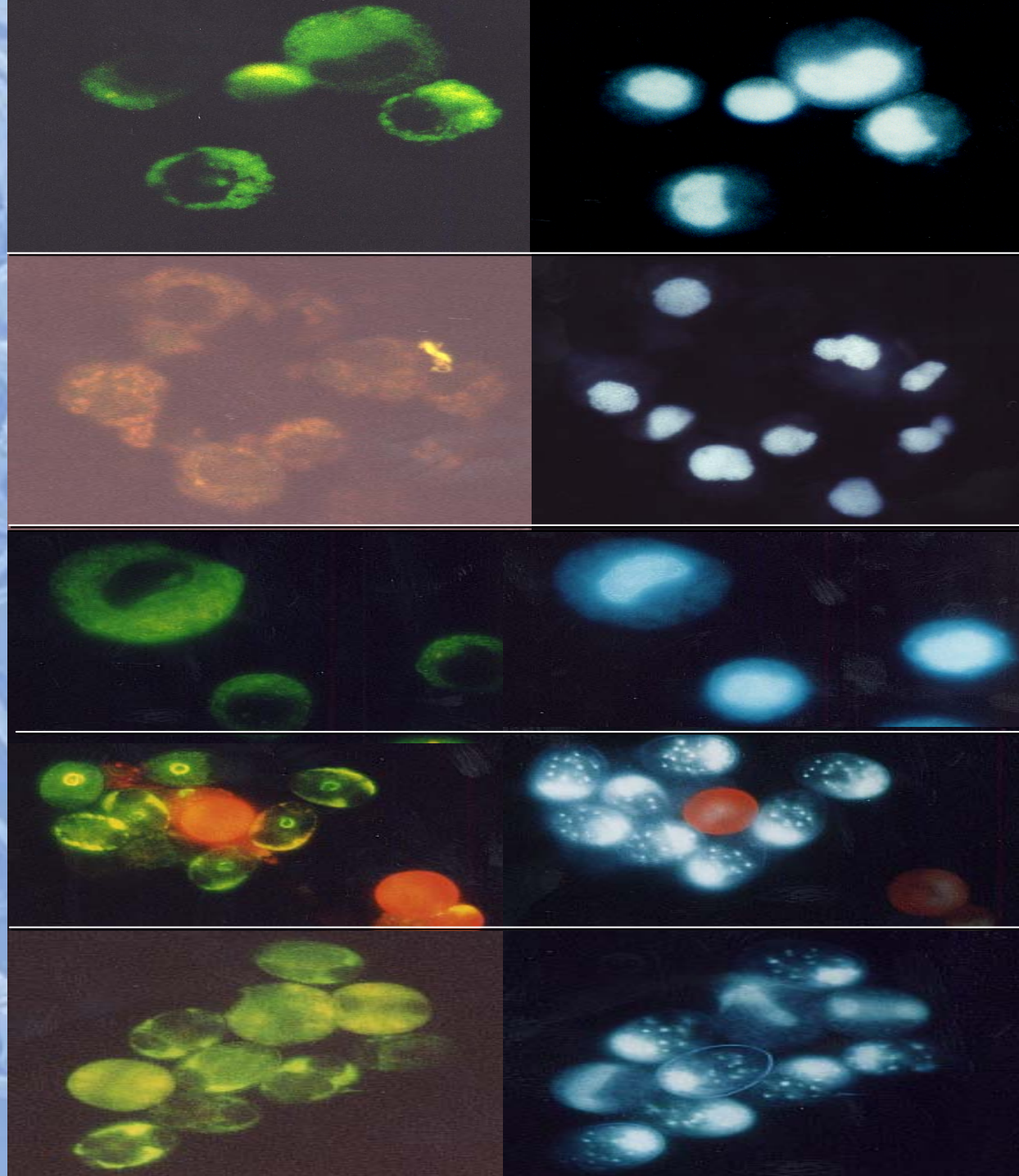

A.C.H.

Change from PFA to ETOH Saline

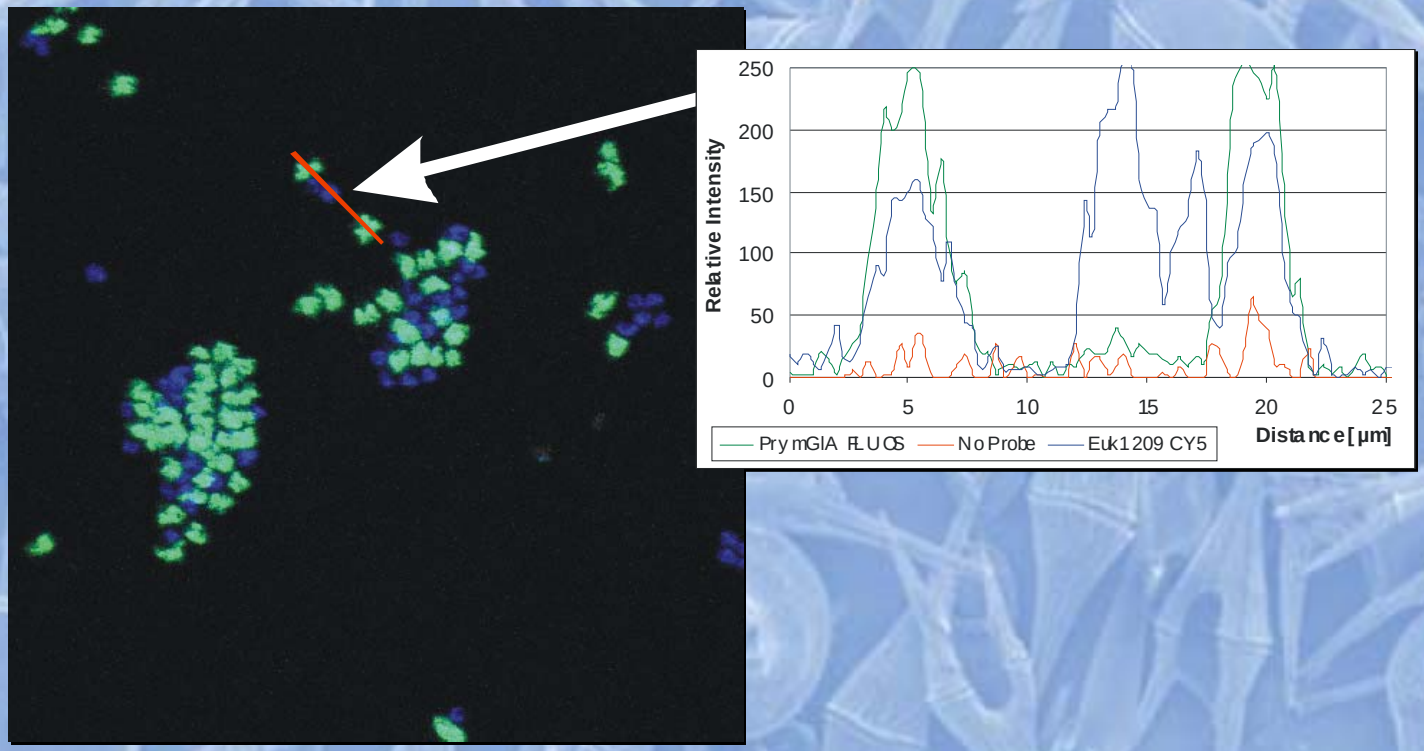
Change from SDS to Nonidet P-40

Without Dimethyl Formamide

With Dimethyl Formamide

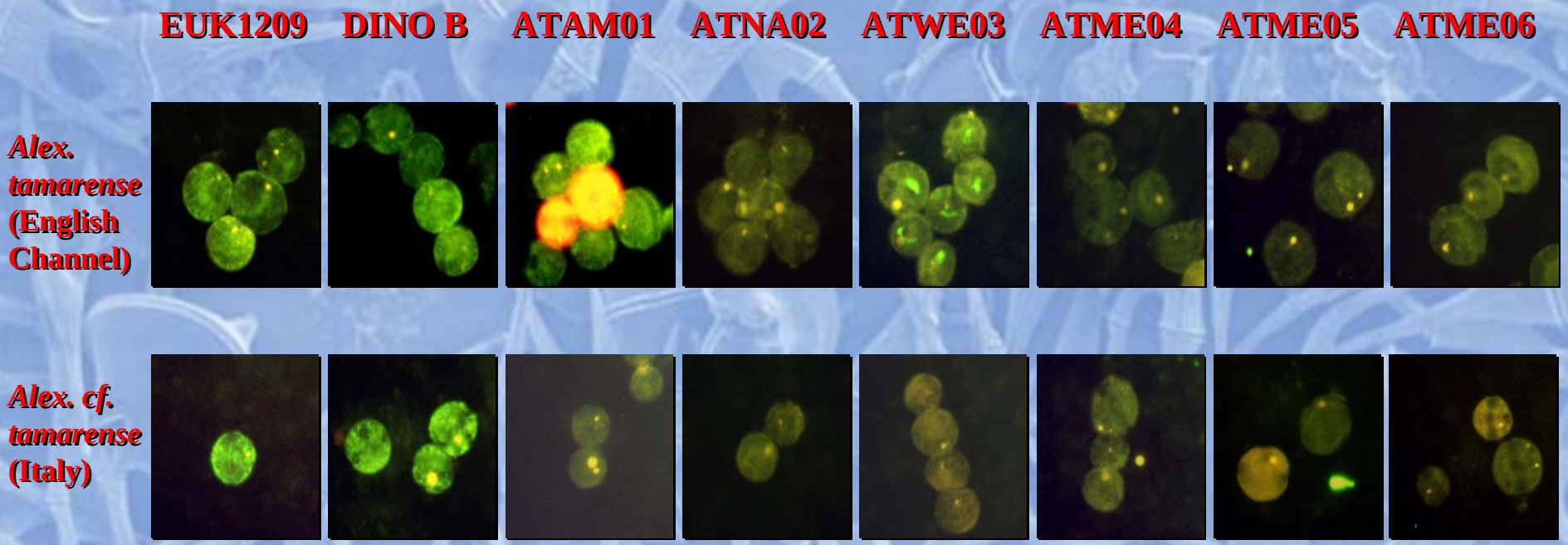


Double Staining of Cells to differentiate cells hierarchically

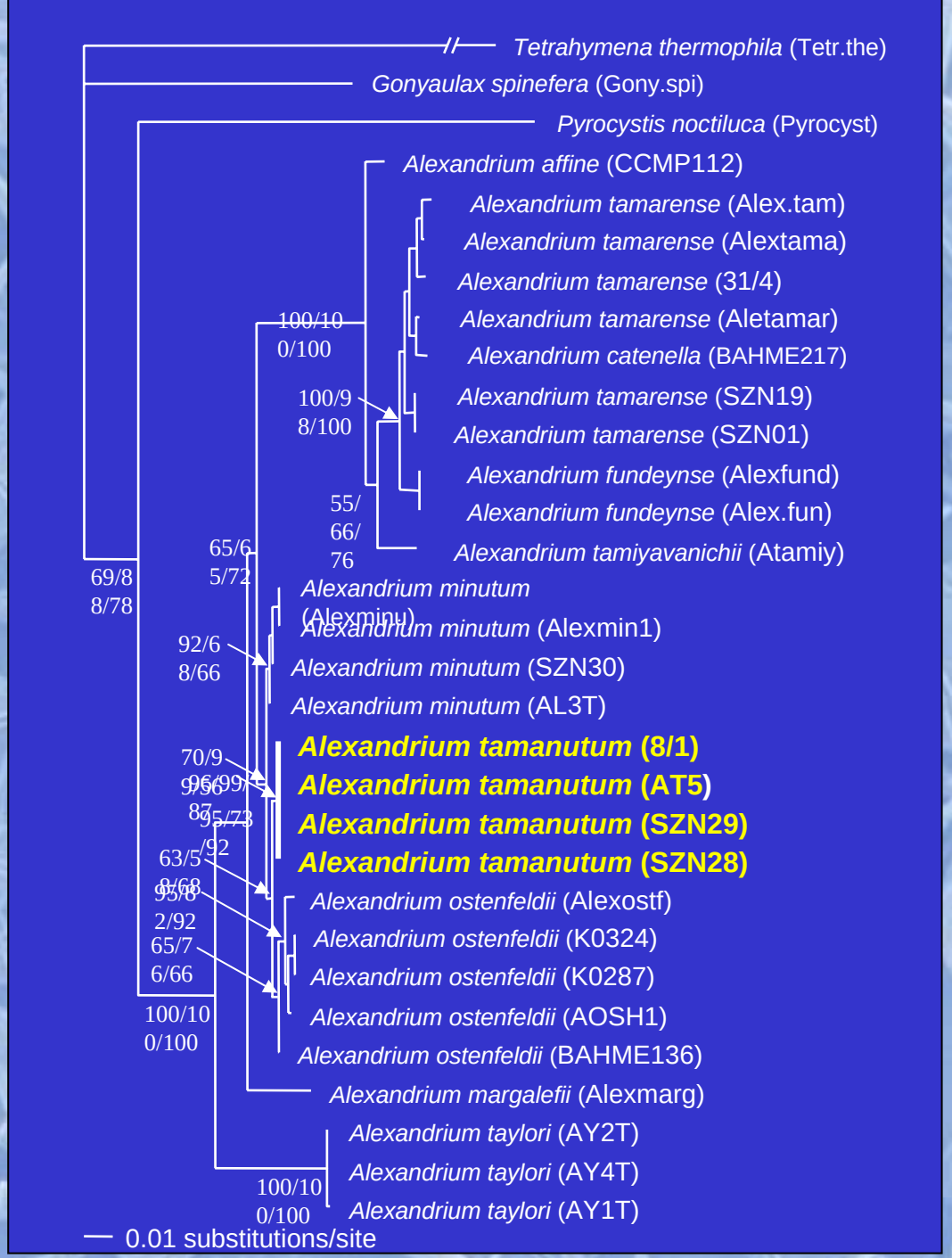


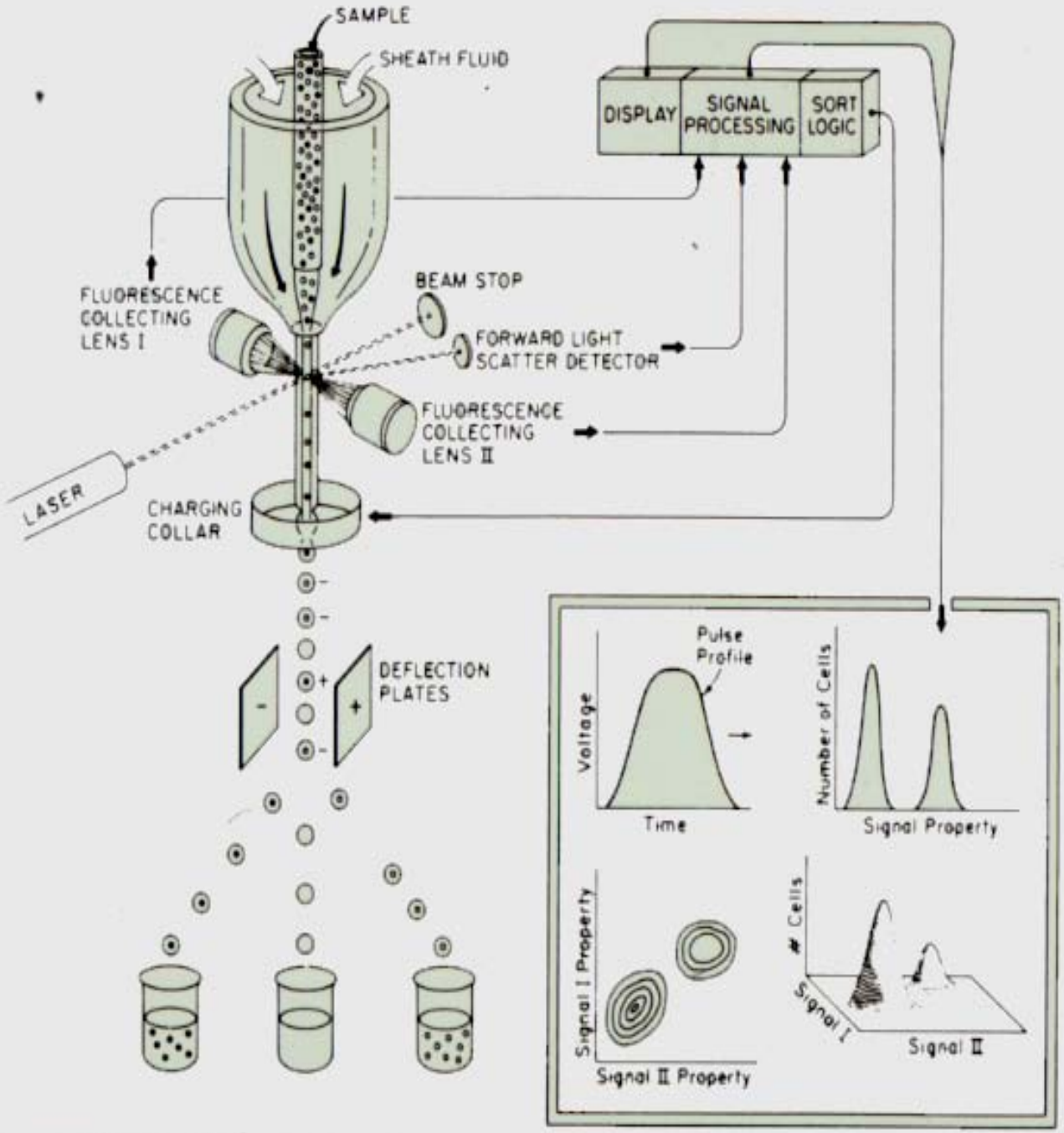
The cells were hybridised with the universal eukaryotic probe (labelled blue) and the genus specific probe for *Prymnesium* PrymGl01A (labelled green). Mixture of E.huxleyi and Prymnesium parvum

Analysis of *Alexandrium* strains from European waters using hierarchical probes



Phylogenetic tree of *Alexandrium* (18S rRNA)



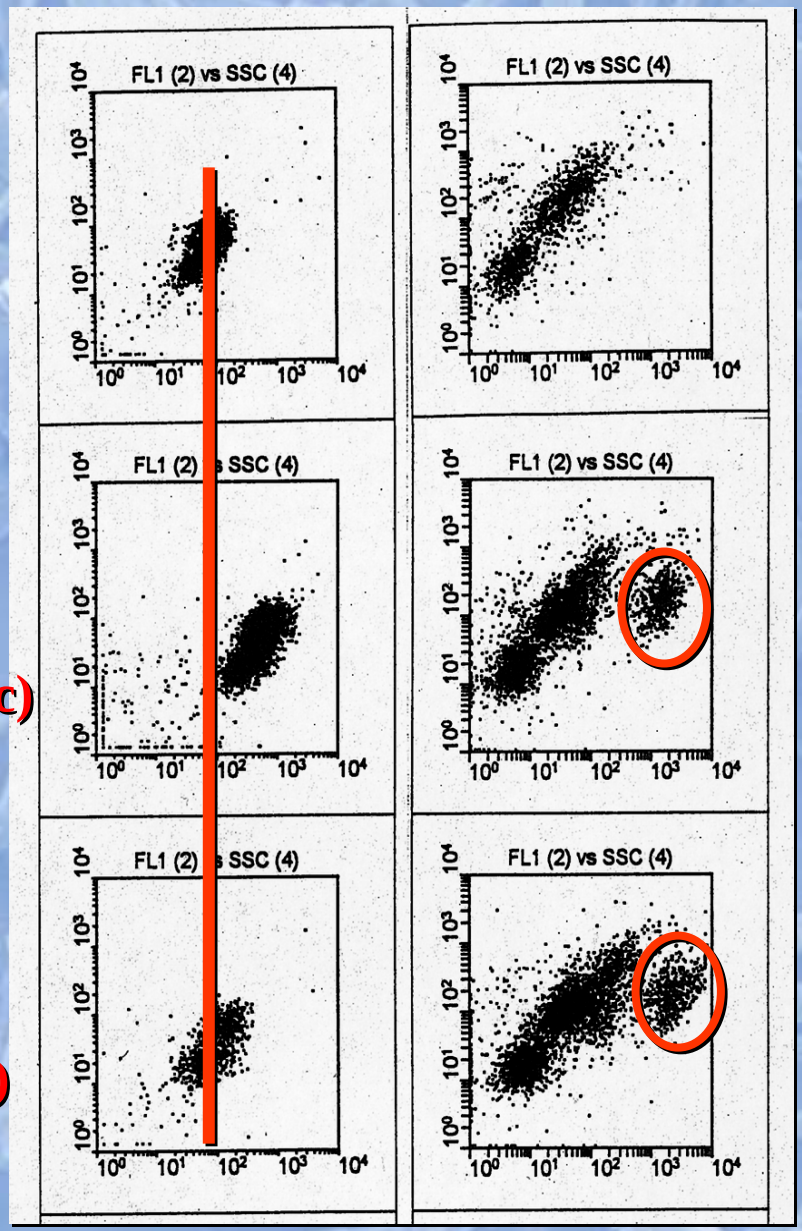

SAMPLE
SHEATH FLUID
DISPLAY
SIGNAL PROCESSING
SORT LOGIC
FLUORESCENCE COLLECTING LENS I
BEAM STOP
FORWARD LIGHT SCATTER DETECTOR
FLUORESCENCE COLLECTING LENS II
LASER
CHARGING COLLAR
DEFLECTION PLATES
Pulse Profile
Voltage
Time
Number of Cells
Signal Property
Signal I Property
Signal II Property
Cells
Signal I
Signal II

Identification of *A. tamarense* in Lab Cultures and Field Samples by Species- & Strain-specific Probes

no probe

ATAM01
(species specific)

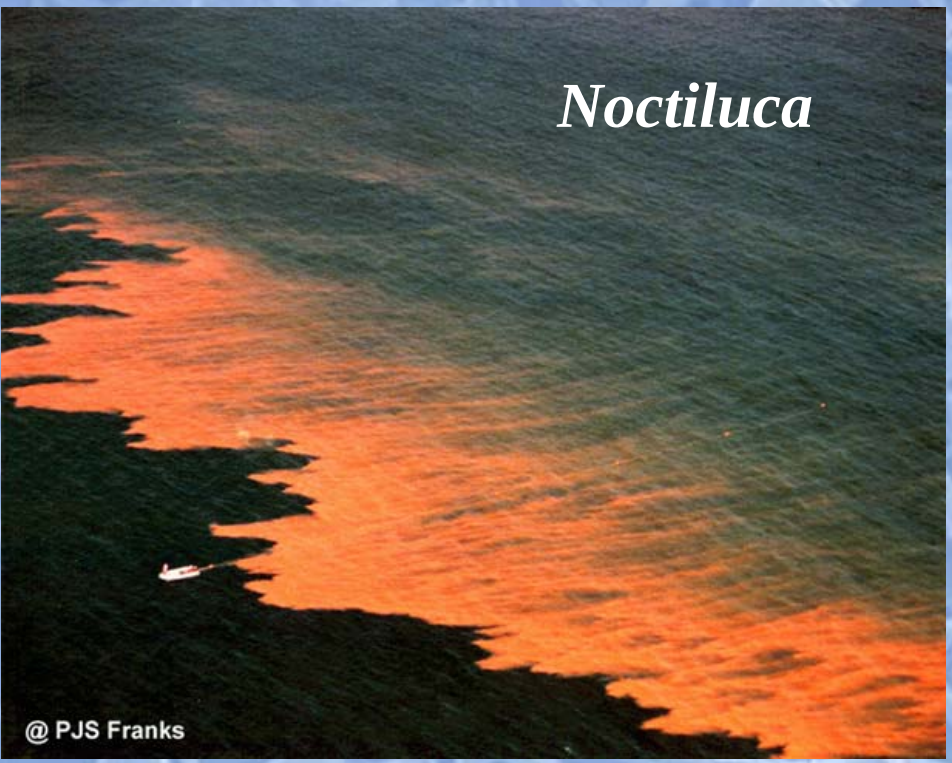
ATNA02
(strain specific)



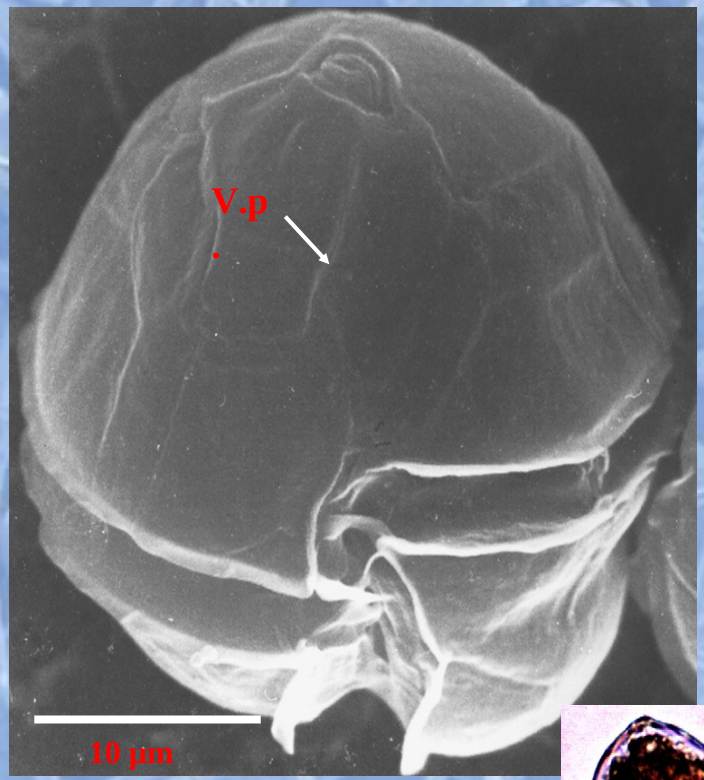
A. tamarense (WE strain) Field sample (Orkney Islands)

J. Brenner, unpubl. results

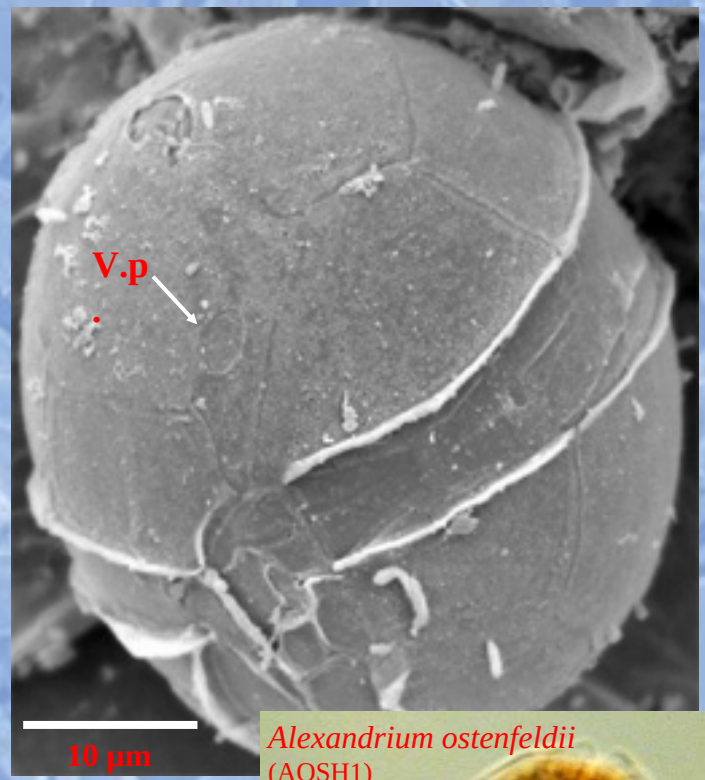
Harmful Algal Blooms

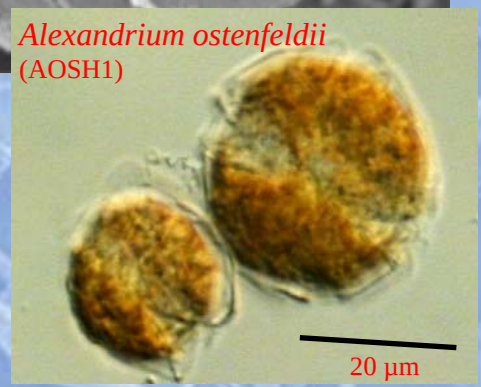


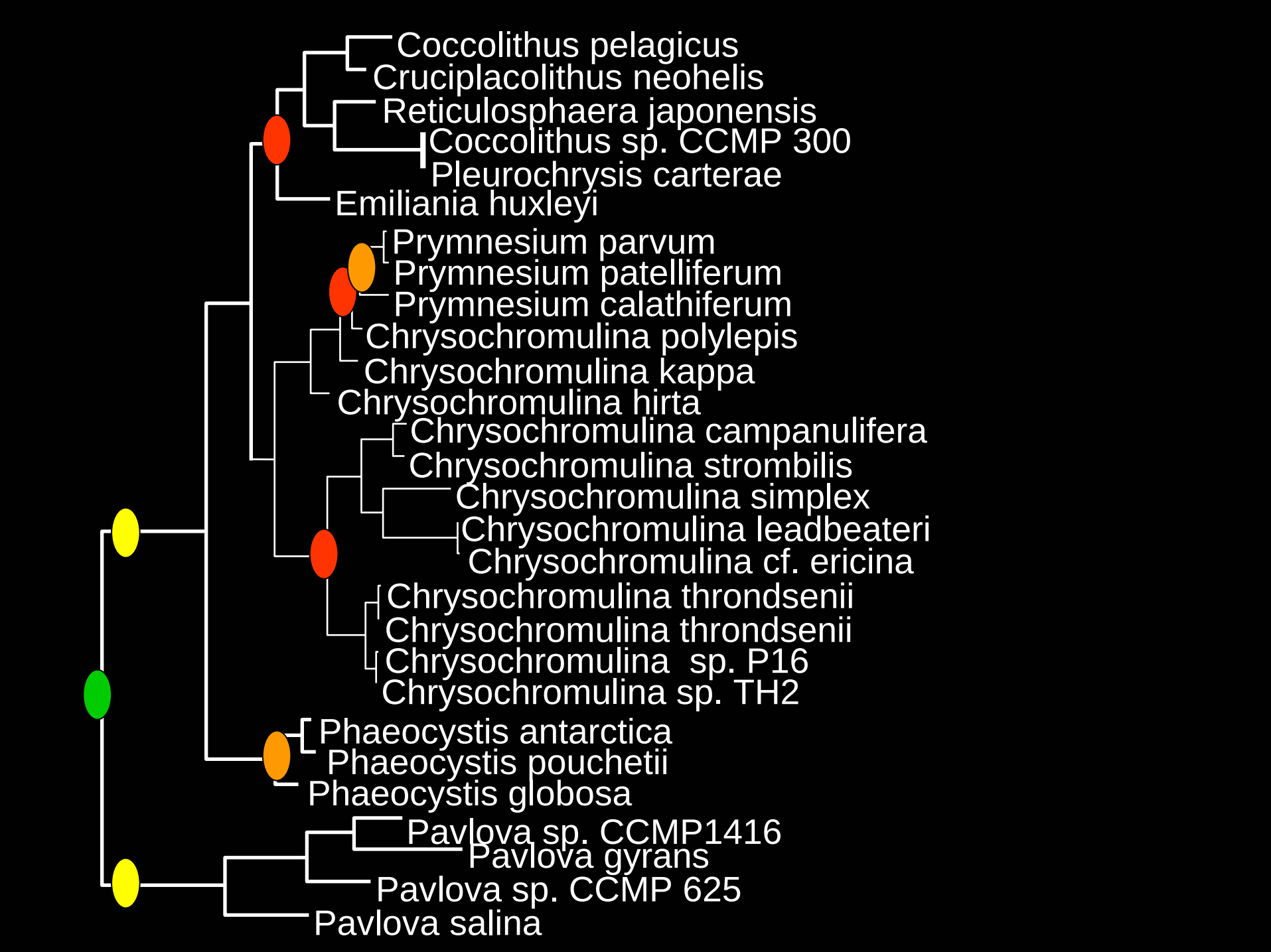
Alexandrium tamarense

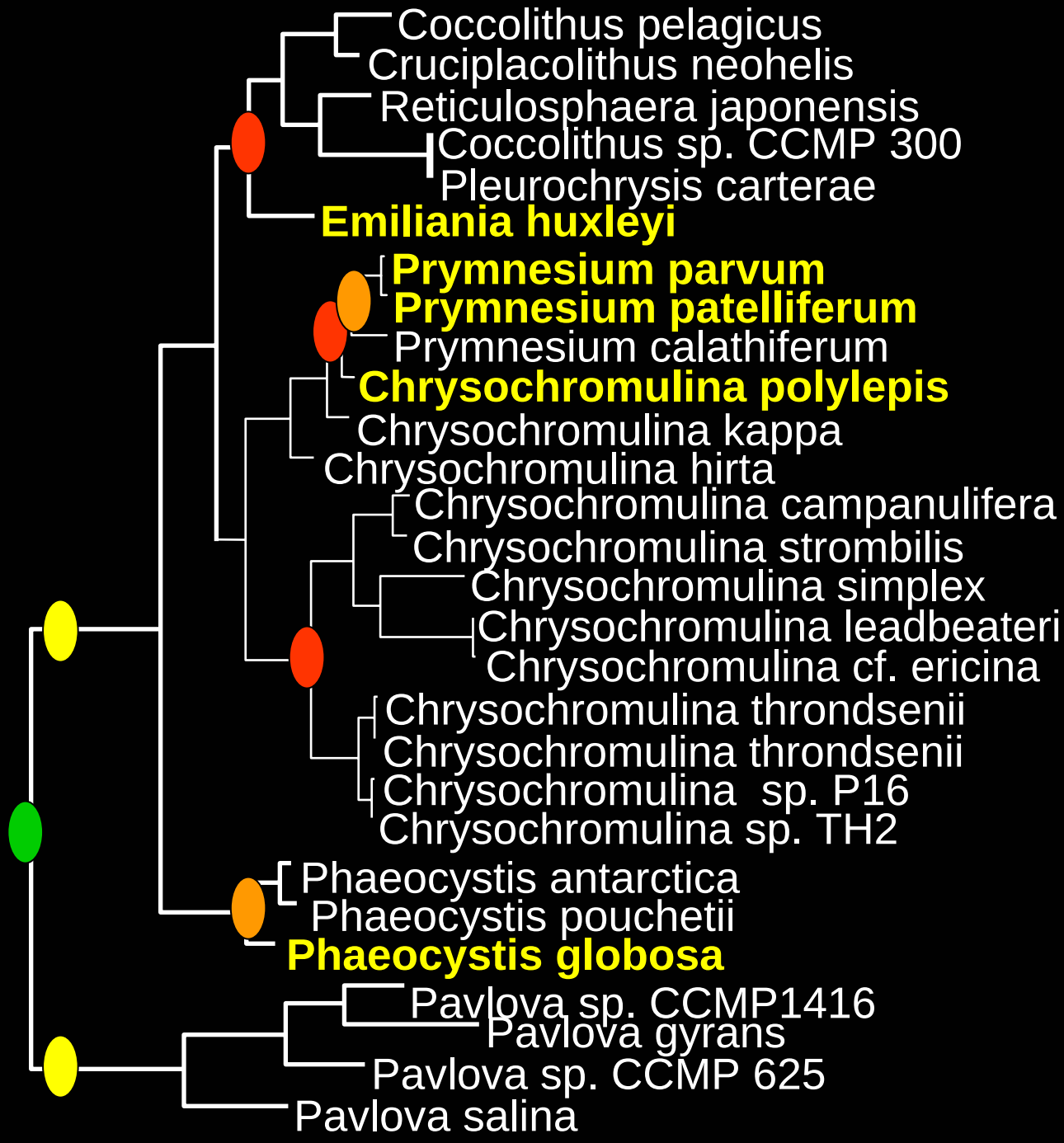


A.ostenfeldii






Coccolithus pelagicus
Cruciplacolithus neohelis
Reticulosphaera japonensis
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


Coccolithus pelagicus
Cruciplacolithus neohelis
Reticulosphaera japonensis
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

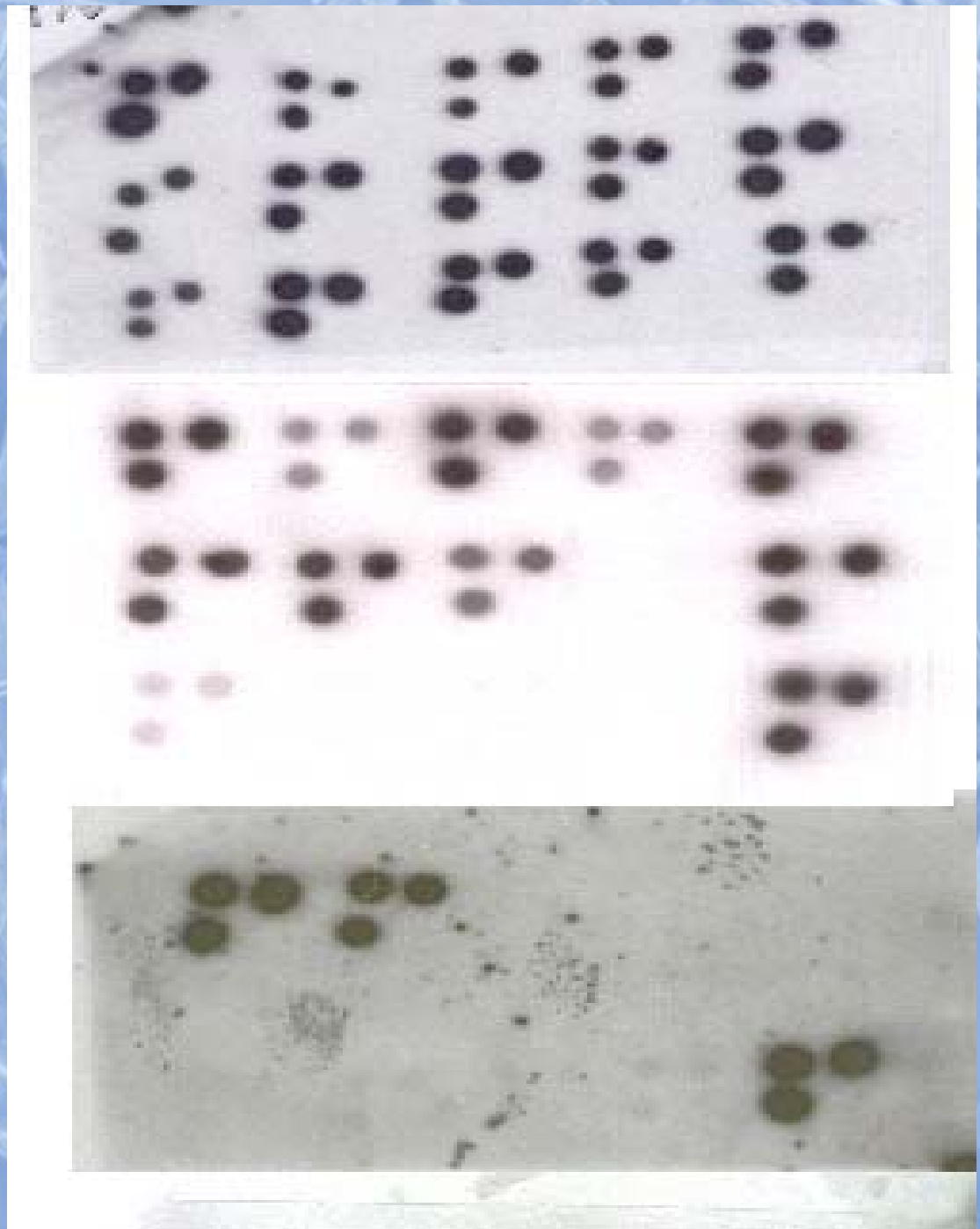
Application & detection methods for rRNA probes

- **DNA dot blots**
- ***In situ* hybridization / Fluorescence Microscopy**
- ***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, Gernmany

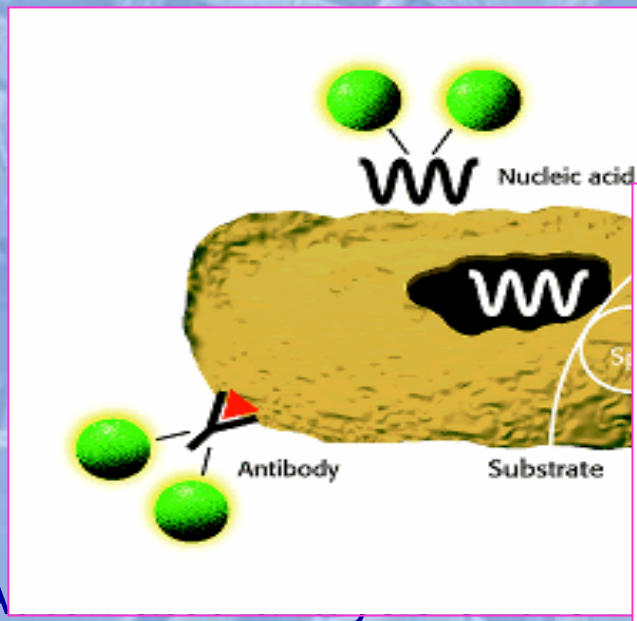
1. Chem*ScanRDI*, a laser based system to quickly analyse FISH-experiments

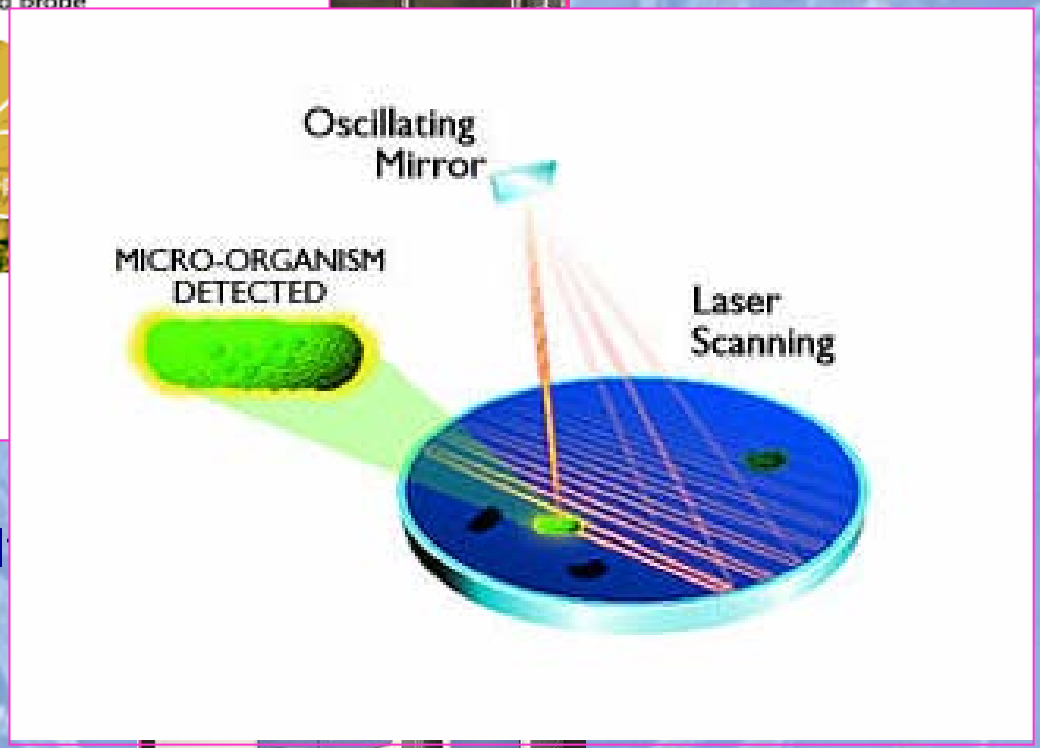
2. Electrochemical detection of toxic algae via a handhold device

3. Development of DNA-microarrays for monitoring phytoplankton composition

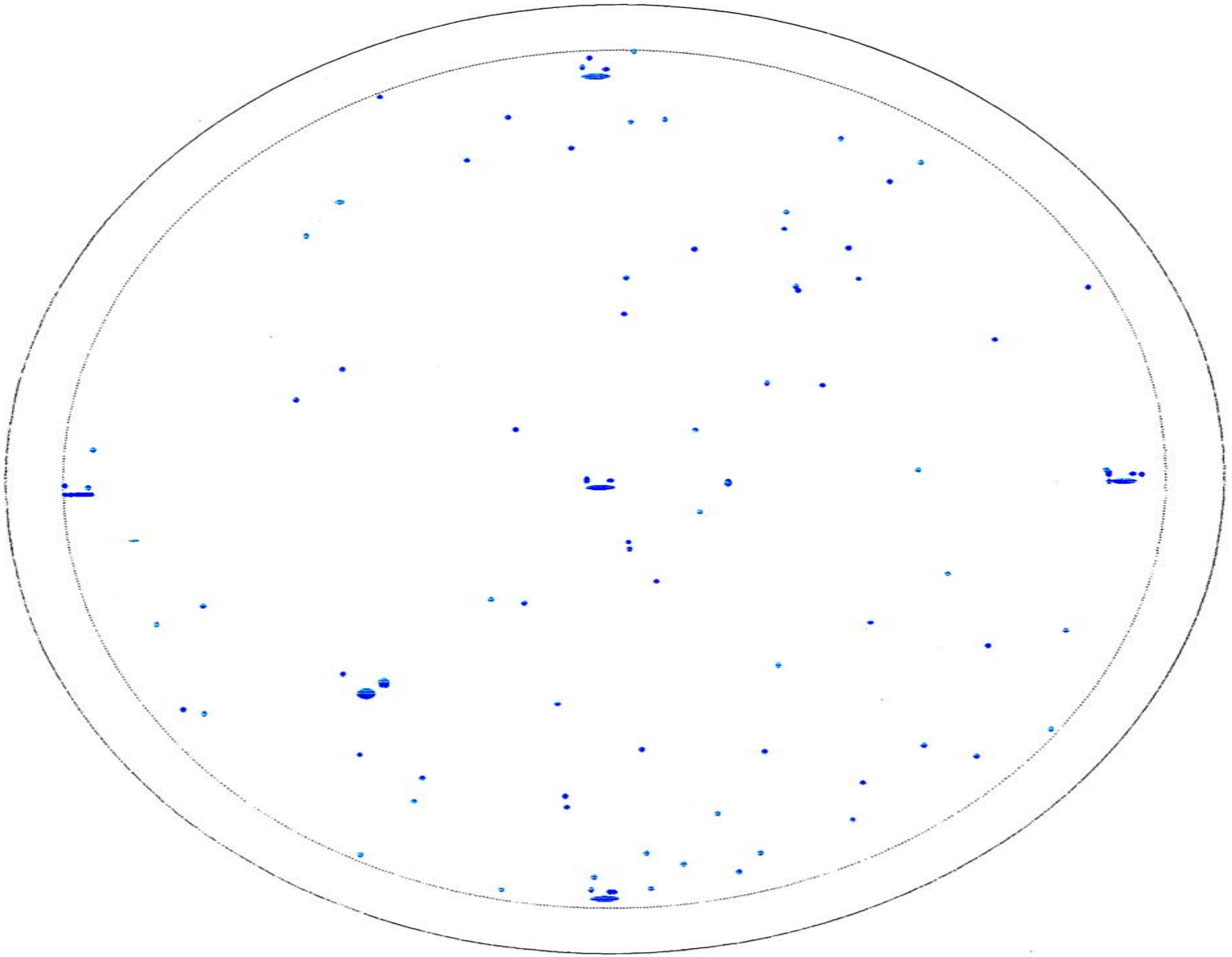
FISH detection of toxic algae via the Chem*Scan, solid phase cytometer*

ChemScanRDI combines fluorescent cell labelling with laser scanning





3. A
cells by Chem*Scan RDI*
(Chemunex Inc.; Maisons-Al

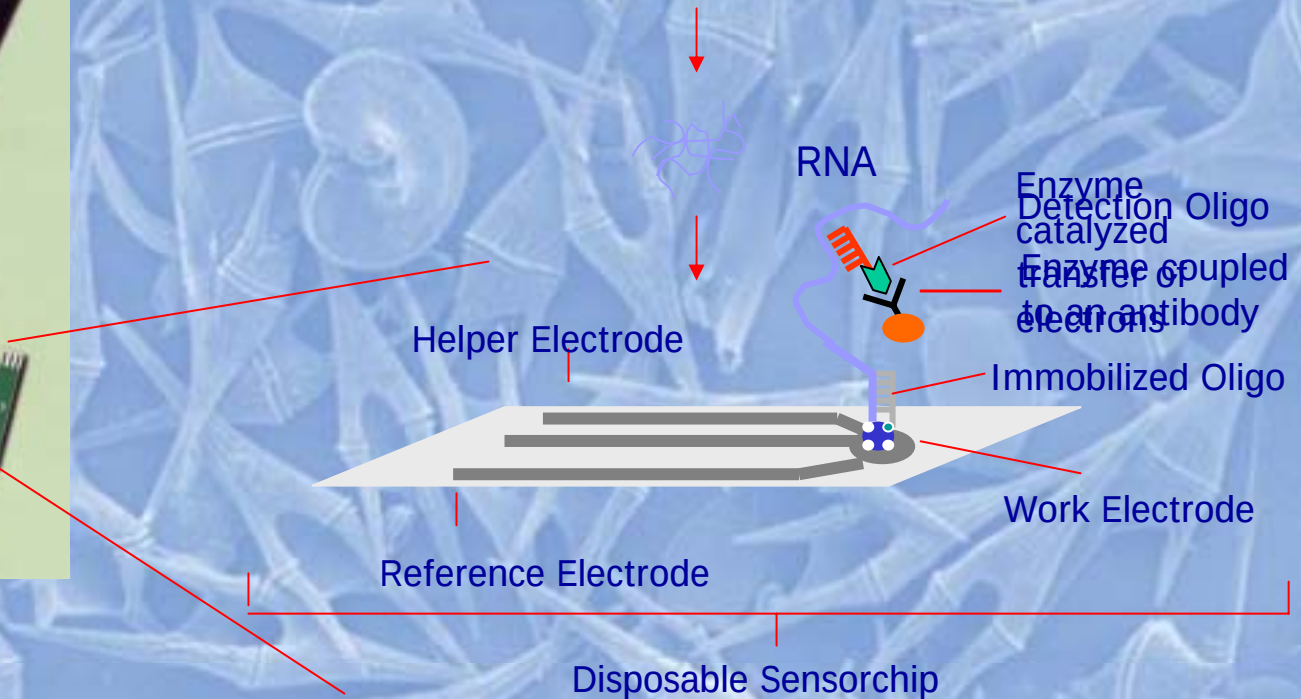


All Data: Total Objects Seen: 107

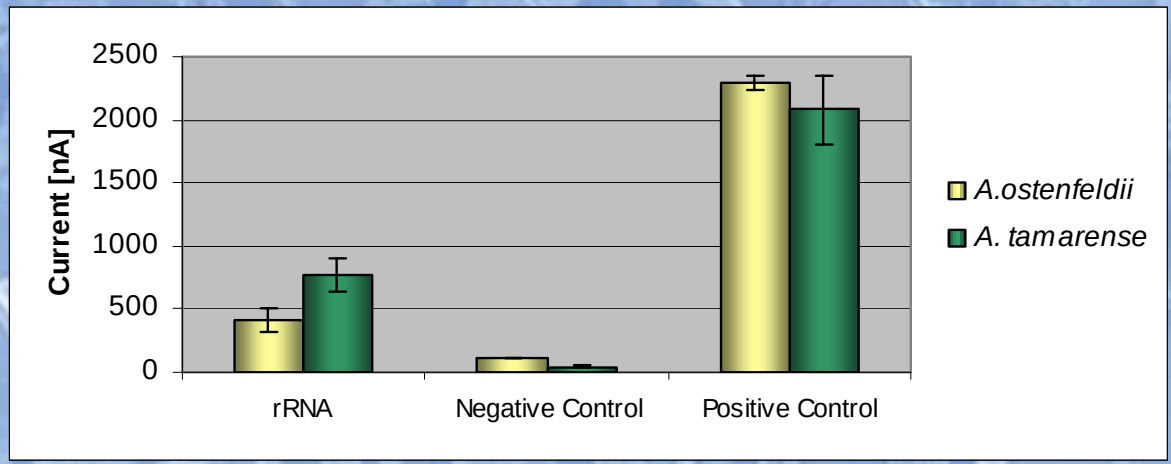
Electrochemical detection of toxic Algae via a handheld device

A Handheld Device for the Detection of Harmful Algae

- *Alexandrium tamarense*
- *Alexandrium ostenfeldii*

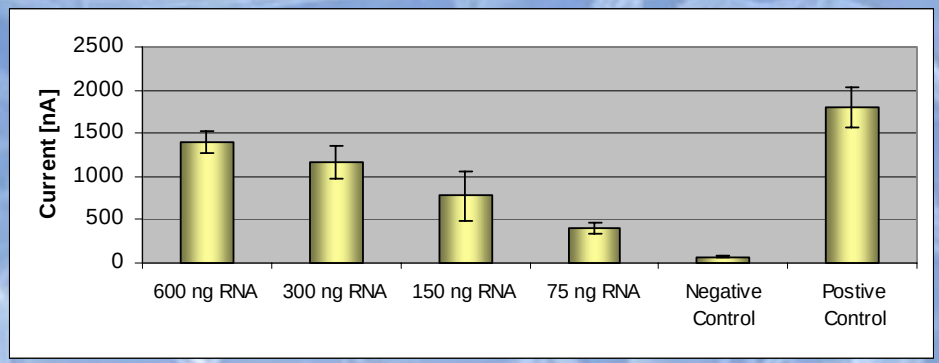


Electrochemical Detection of rRNA from *Alexandrium* Species

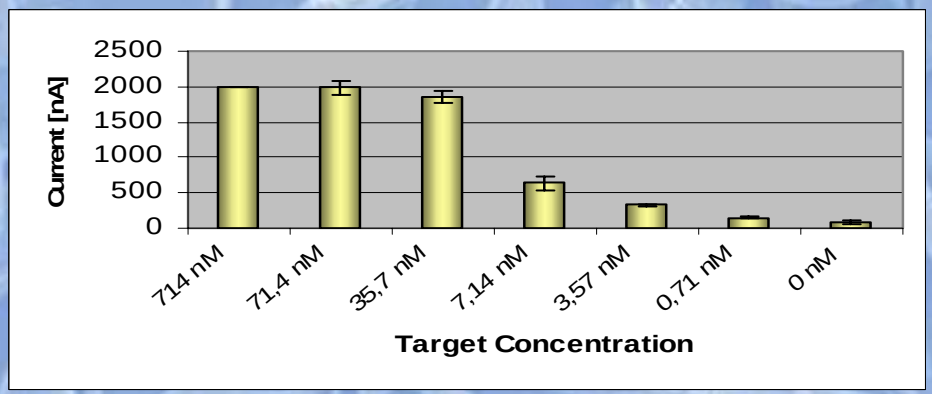


- ~ 500 ng rRNA have been hybridized to probes
- The probes were directed against ribosomal RNA of *A. tamarense,* respectively *A. ostenfeldii*

Concentration Series of Decreasing Amounts of Target

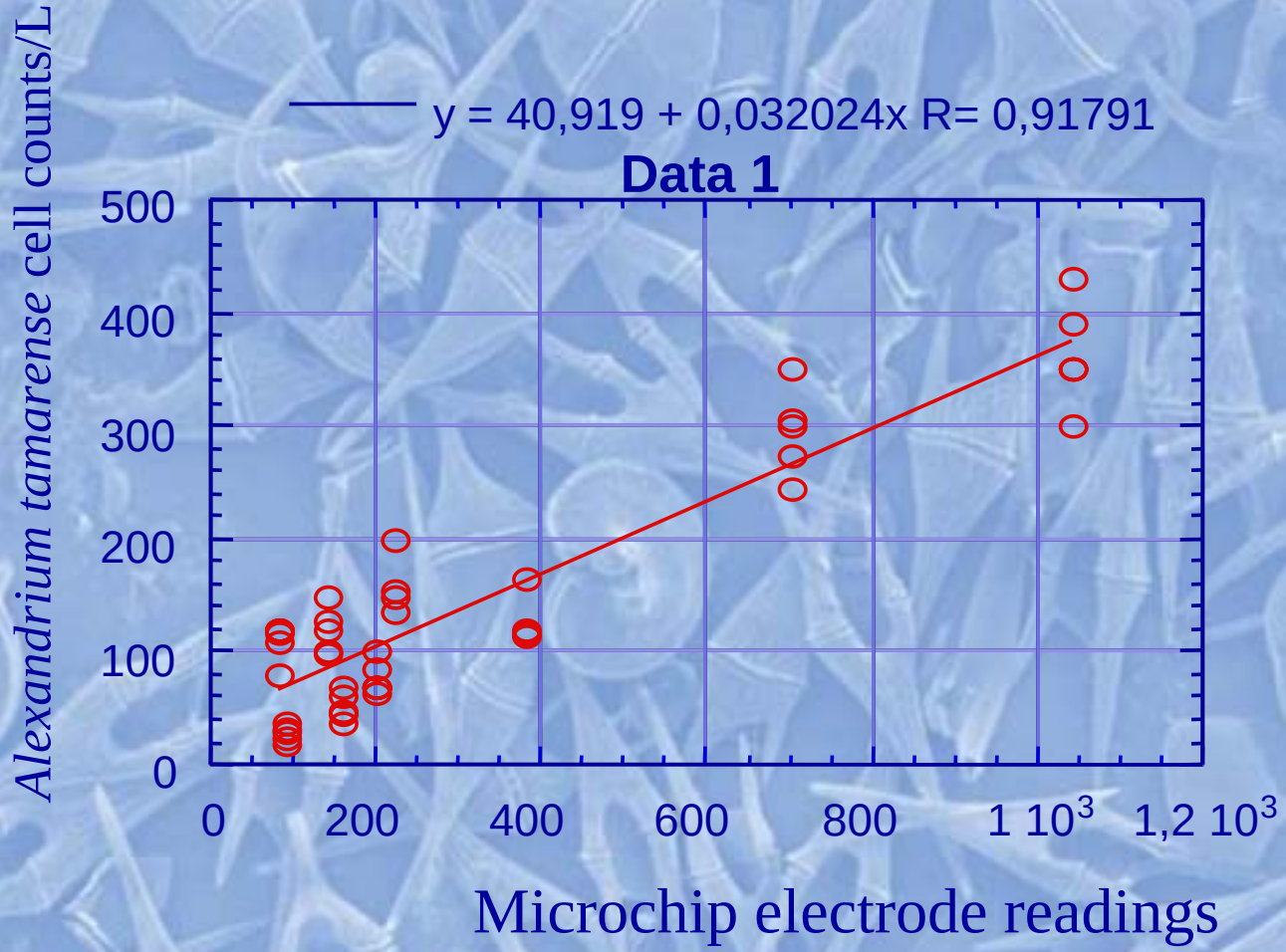


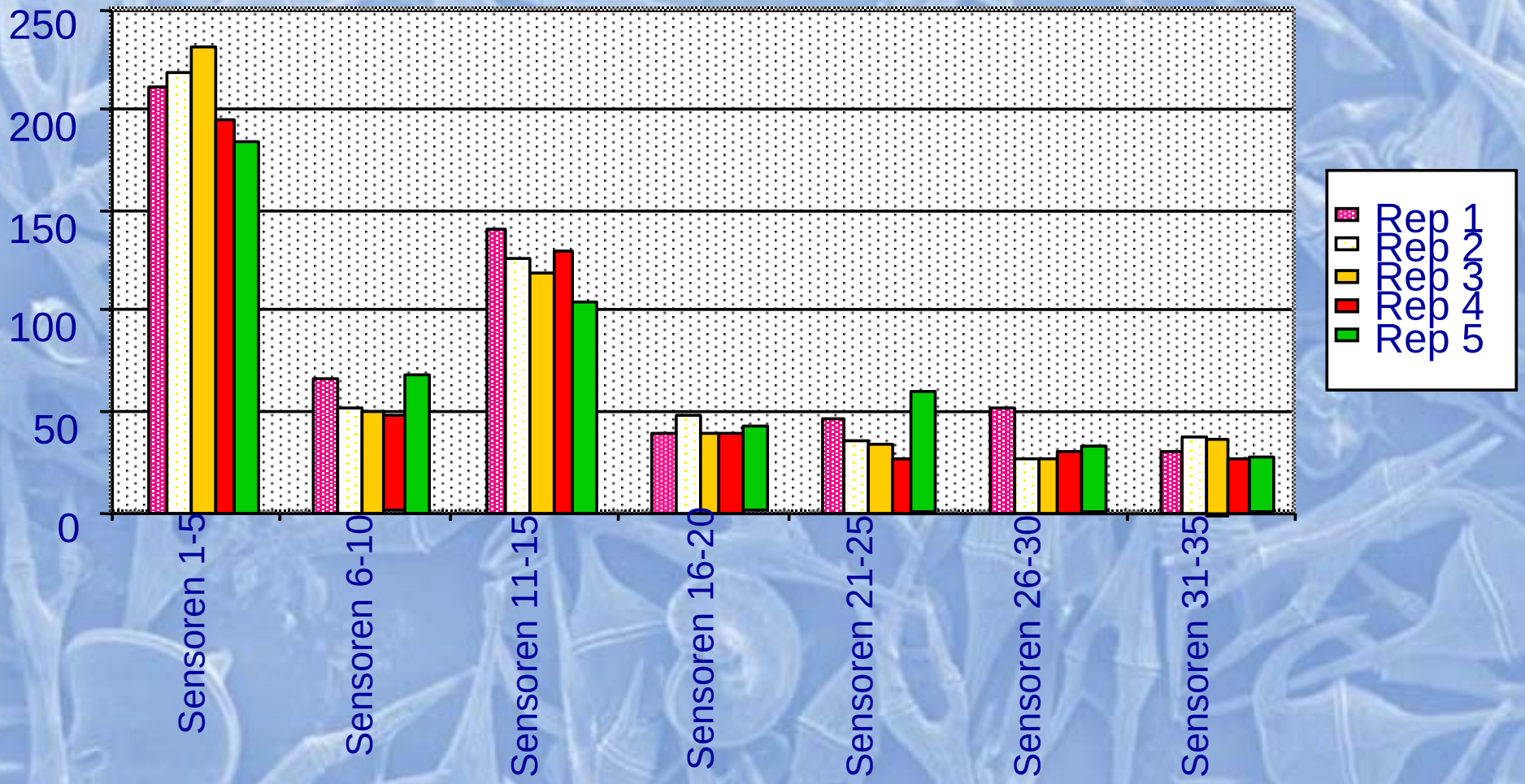
A. Decreasing amounts of A. tamarense rRNA hybridized to a *A. tamarense* probe



B. Decreasing amounts of a 70 bp Oligonucleotide hybridized to a *A.ostenfeldii* probe

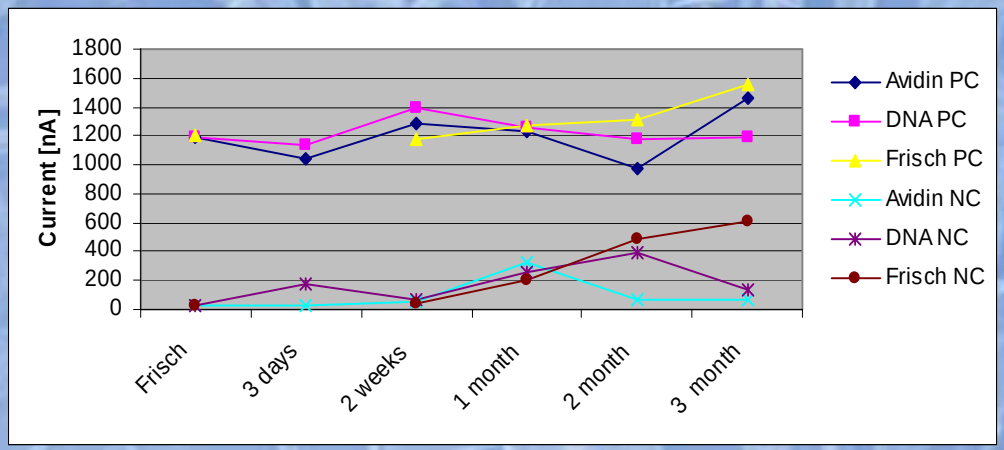
Comparison of cell counts with electrode readings





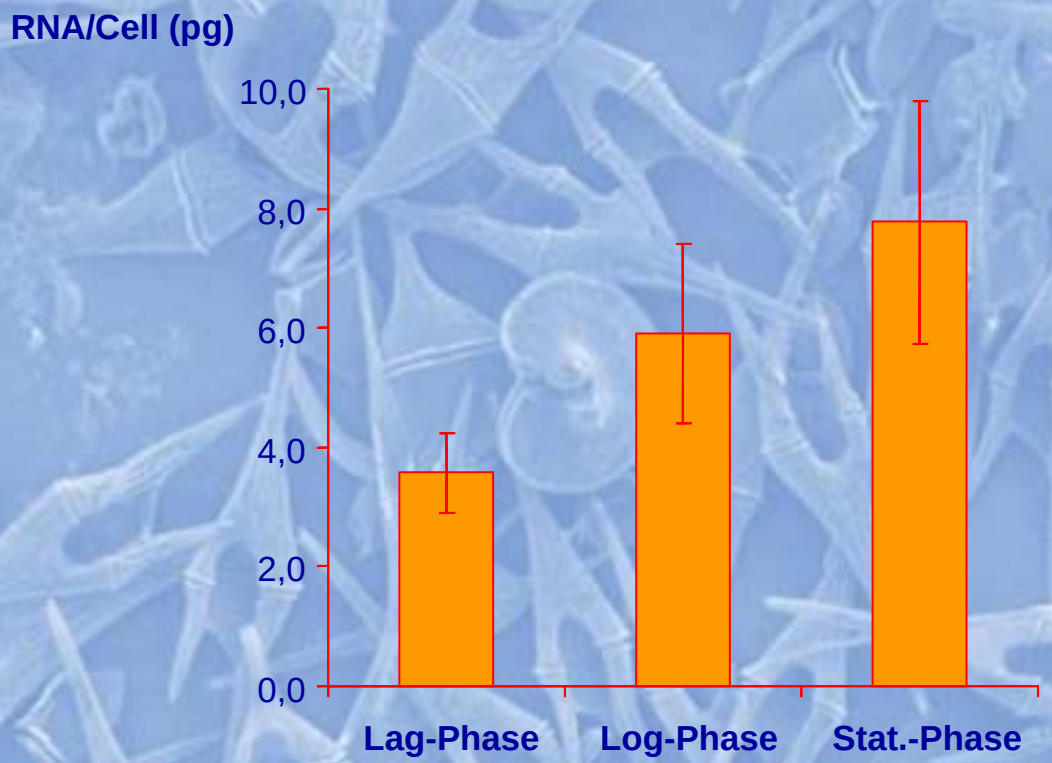
C. Detection of rRNA in natural samples from the Orkneys islands (column sets 1-4) as compared to rRNA from Prorocentrum mexicanum (column sets 5&6) and hybidized with Alexandrium tamarense probe.

Long term stability of treated sensors



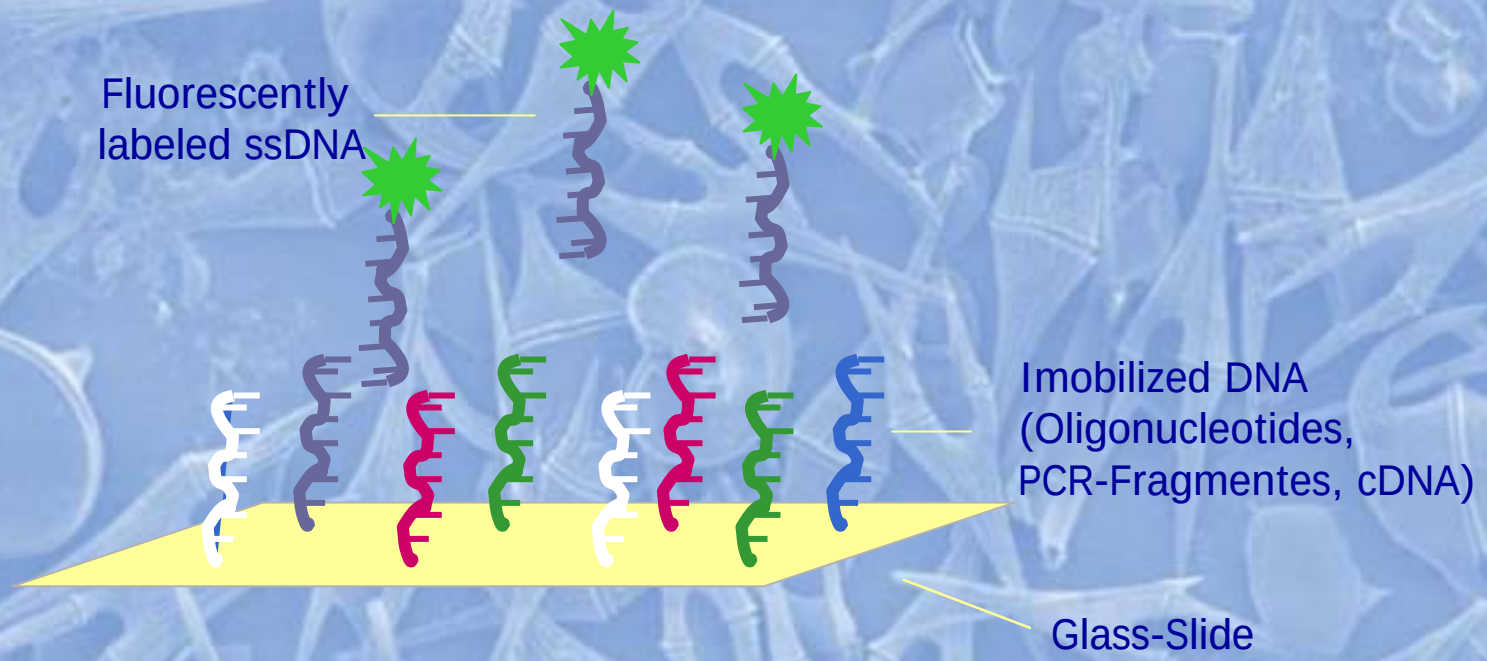
- Sensors have been treated with Avidin (Avidin) and Avidin/Probe (DNA)
- The coated Sensors were then stored at 4°C over the indicated times
- Freshly prepared sensors have been prepared before each hybridization as positve controls for the experimental conditions (Frisch)
- To control the stability 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

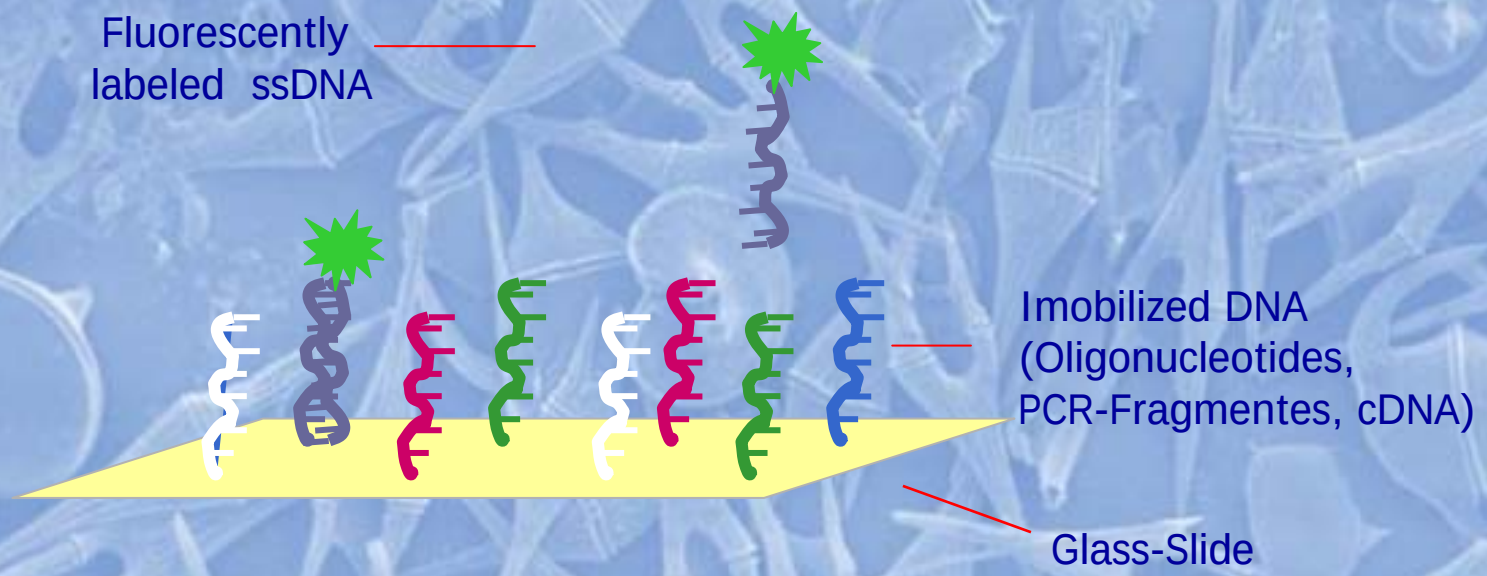


Development of DNA-microarrays for monitoring phytoplankton composition

Scheme of a DNA-Chip Experiment

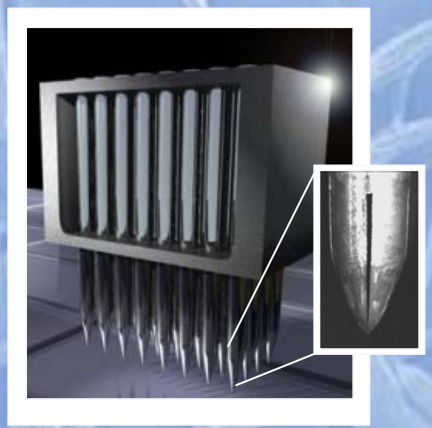


Scheme of a DNA-Chip Experiment

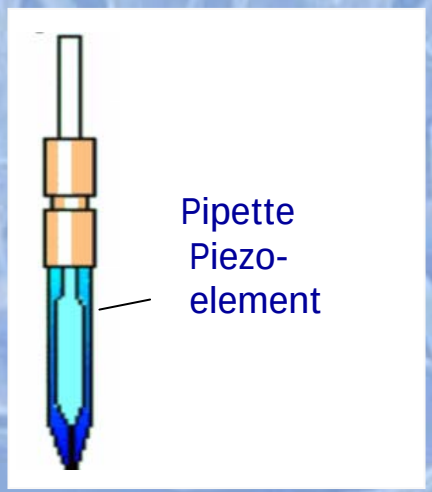


Low Density Chips

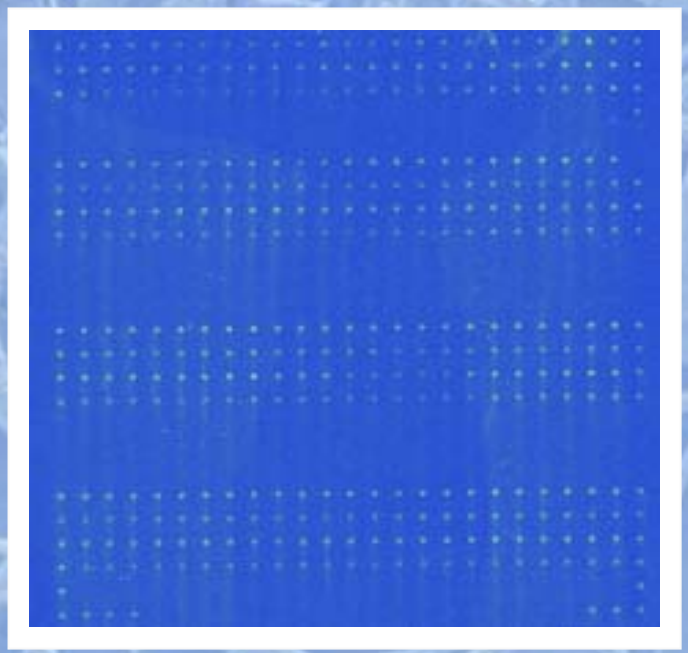
- ~ 625 Spots per cm^2
- Spotdiameter: ~ 200 µm
- Spotting with needles (A) or piezotechnology (B)



A.



B.



500 µm

Monitoring Phytoplankton composition with DNA-Chip Technology

Phytoplankton samples

↓

Isolation of genomic DNA from the sample

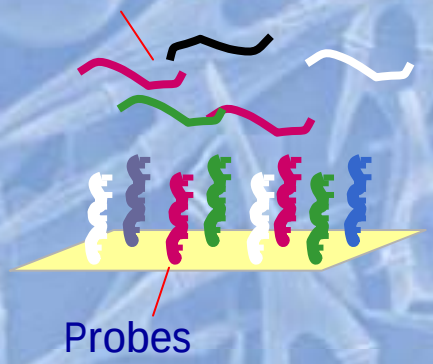
↓

Amplification of the 18S rDNA

↓

Hybridization of the 18S PCR-products with a DNA-Chip that contains probes initially designed for FISH

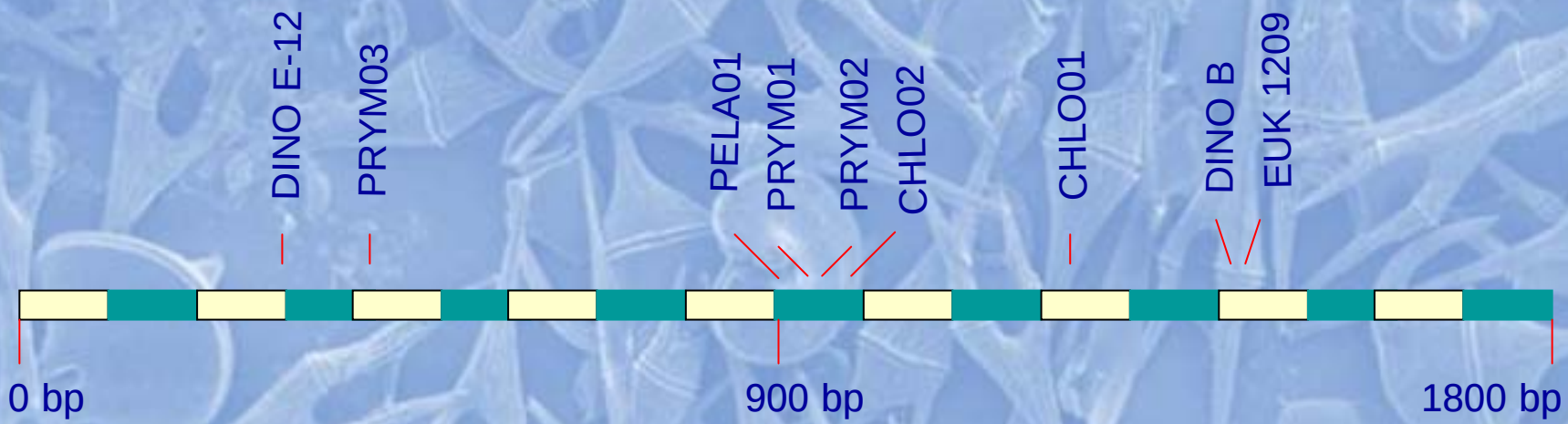
18S- PCR products



Probes and Targets used for preliminary Chip-Experiments

Class	Probe	Species
Dinophyceae	DINO B	▪ *Alexandrium tamarense* ▪ *Prorocentrum minimum*
	DINO E12	
Prymnesiophyceae	PRYM01	▪ *Prymnesium patelliferum*
	PRYM02	
	PRYM03	
Chlorophyceae	CHLO01	▪ *Dunaniella salina* ▪ *Pyramimonas obovata*
	CHLO02	
Pelagophyceae	PELA01	▪ *Coccoid pelagophyte* ▪ *Pulvinaria spec.*
Bolidophyceae	BOLI01	▪ *Clone. No. 151 PICODIV*
	BOLI02	

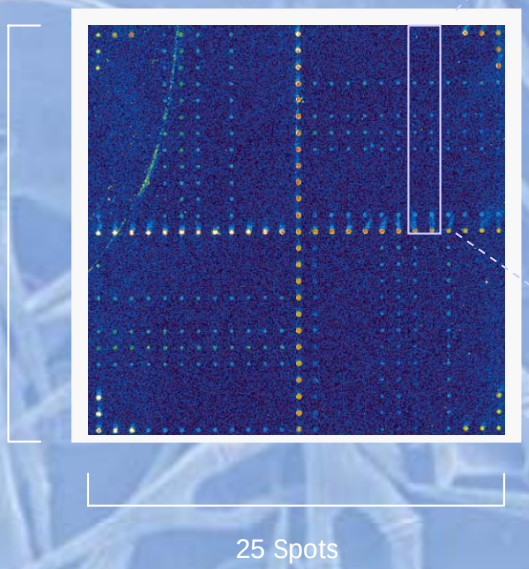
Localization of the Class-level probes in the 18S-Sequence

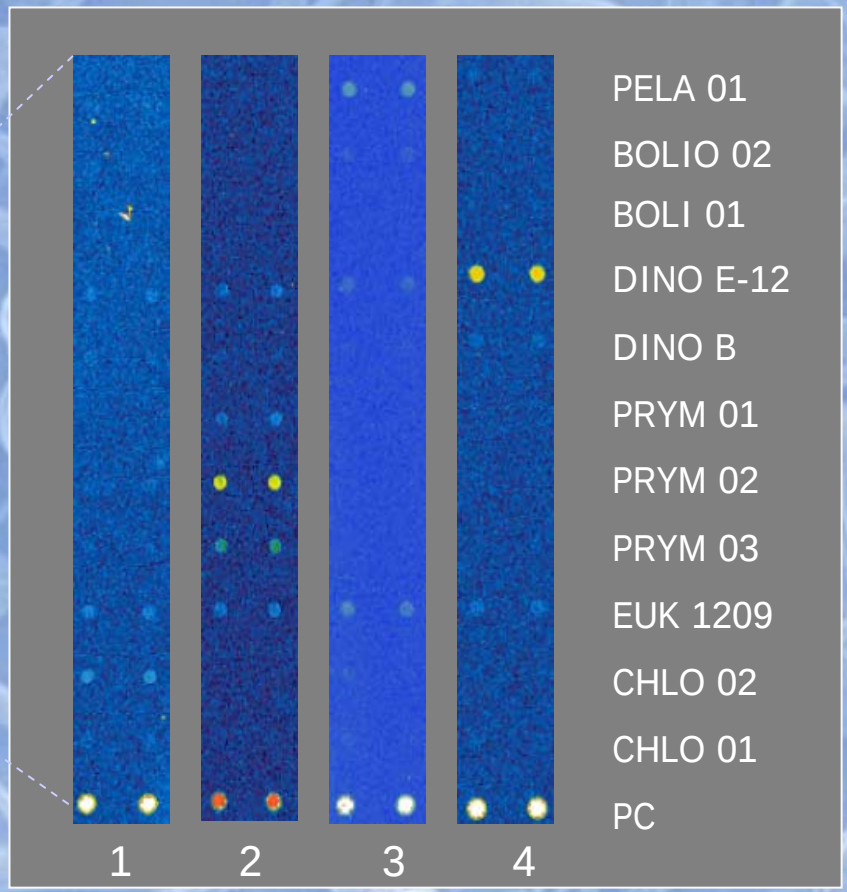


18S-DNA ~ 1800 bp

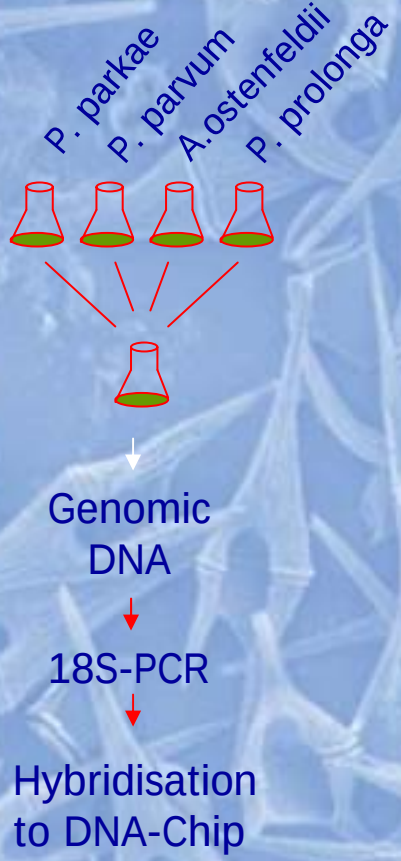
Preliminary results of a DNA-Chip with Class-level Probes

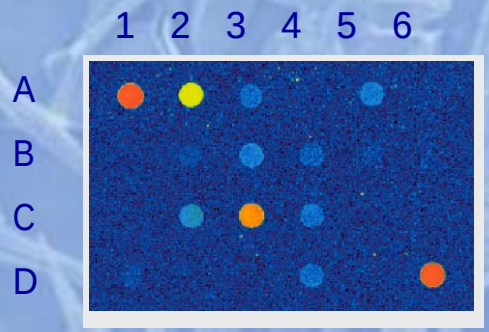
1. *Dunaniella salina*
2. *Prymnesium patelliferum*
3. *Coccoid pelagophyte*
4. *Alexandrium tamarense*



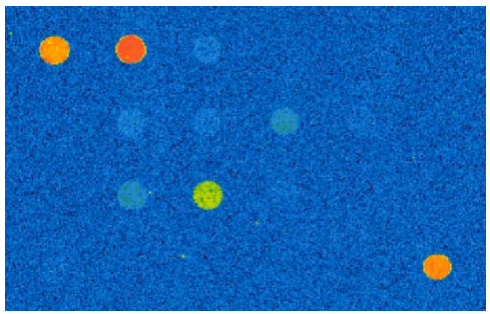


Identification of Phytoplankton on Class-level in a Mix of Laboratory Strains



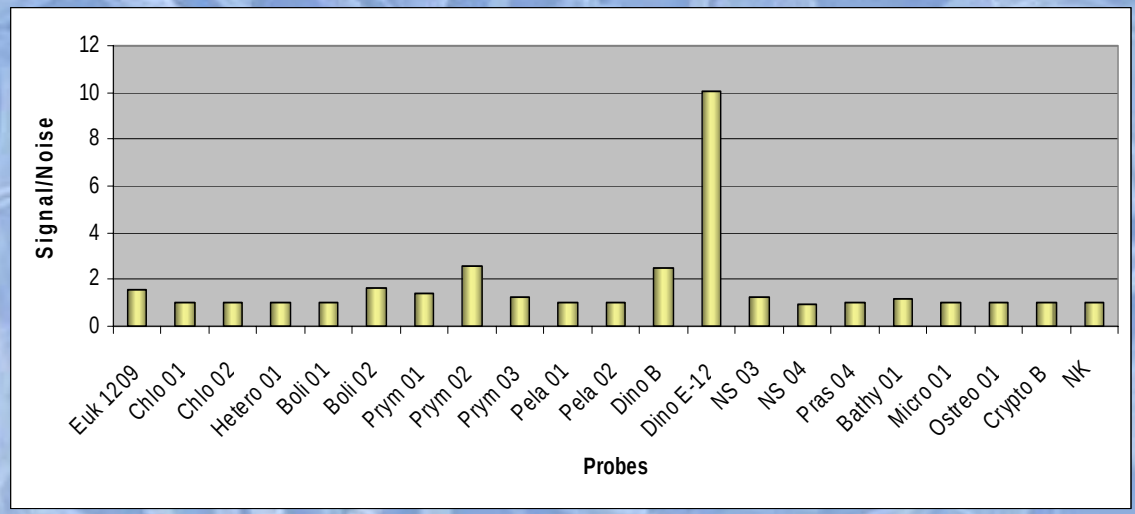


A1- PC	C1- Pela 02
A2- Euk 328	C2- Dino B
A3- Euk 1209	C3- Dino E-12
A4- Chlo 01	C4- NS 03
A5- Chlo 02	C5- NS 04
A6- Hetero 01	C6- Pras 04
B1- Boli 01	D1- Bathy 01
B2- Boli 02	D2- Micro 01
B3- Prym 01	D3- Ostreo 01
B4- Prym 02	D4- Crypto B
B5- Prym 03	D5- NC
B6- Pela 01	D6- PC



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

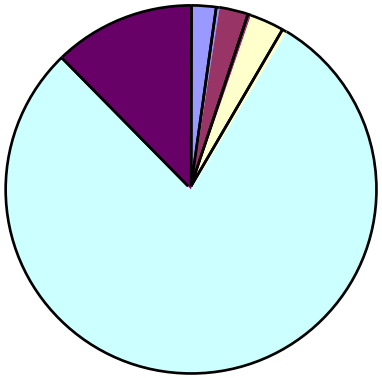
- *Prymnesium parvum*
- *Alexandrium ostenfeldii*
- Threshold for a positive signal: signal/noise ratio ≥ 2

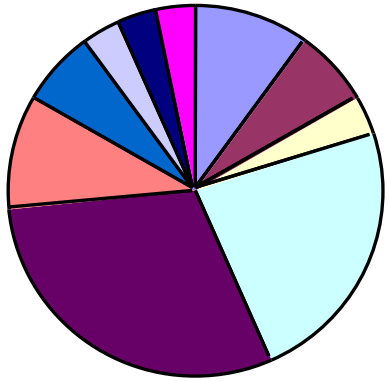


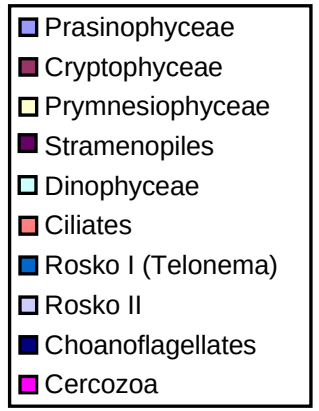
DNA CHIP

Clone librairy

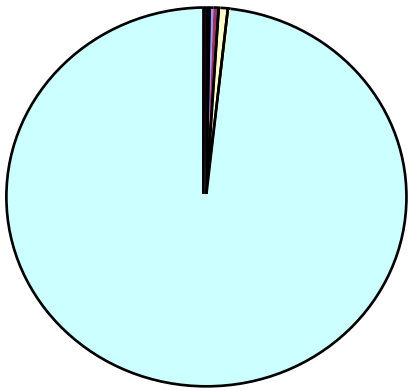
Dec 2000

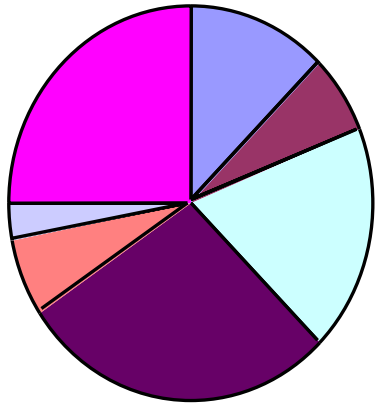


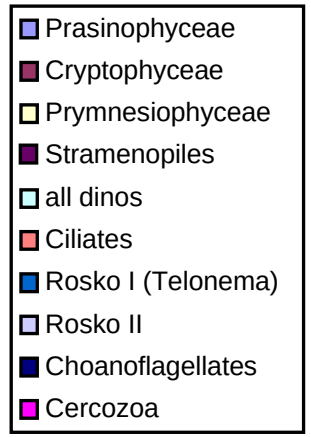




April 2001







Summary

- The ChemScanRDI is a laserbased 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 hybridzations in parallel

Research Needs

- Monitoring of toxic phytoplankton populations is an important scientific issue
- Efficient monitoring requieres 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/ ChemS*canRDI*

Susanne Huljic Handheld device/ *A.ostenfeldii*

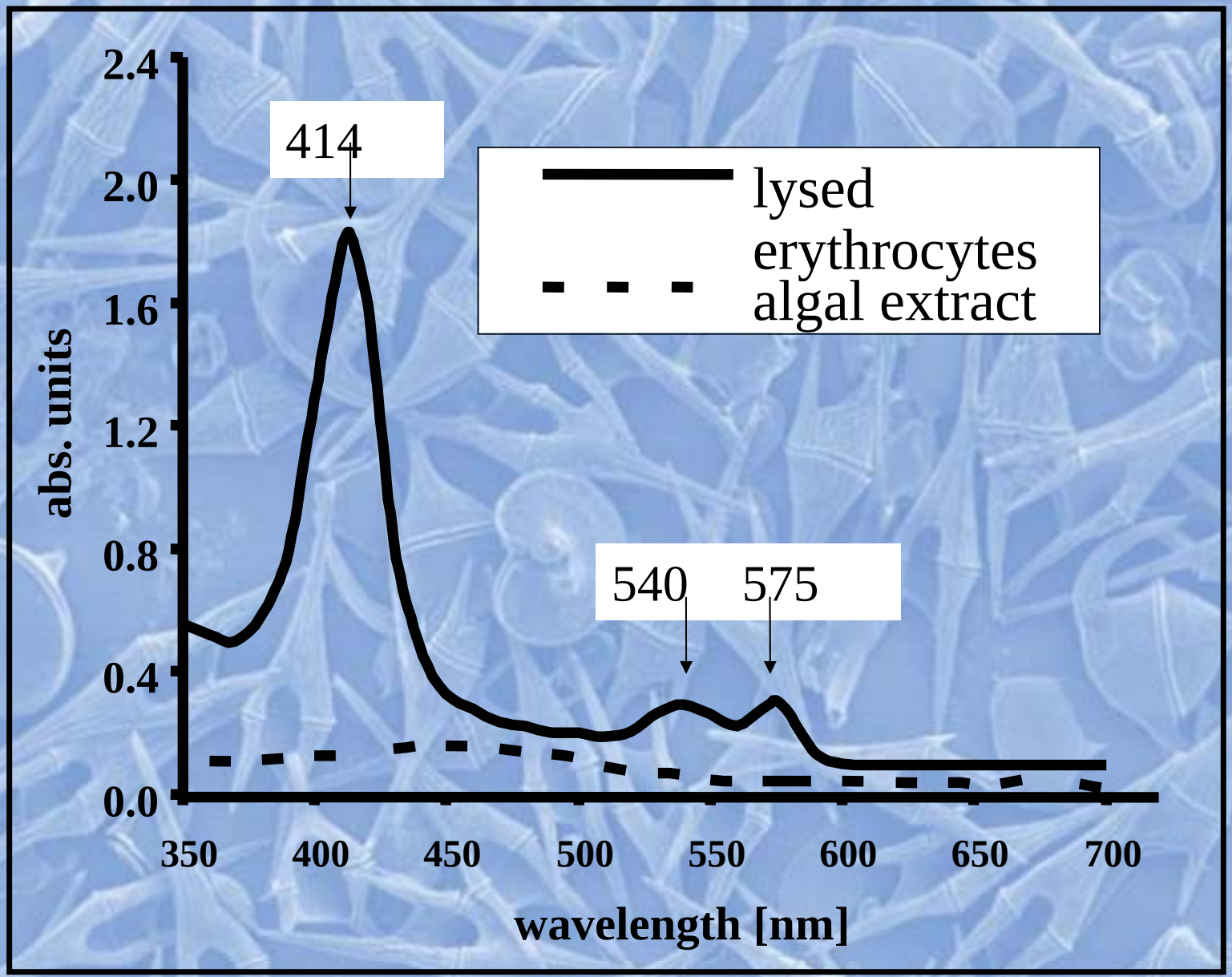
Dr. Katja Kerkmann DNA-Chip Technology

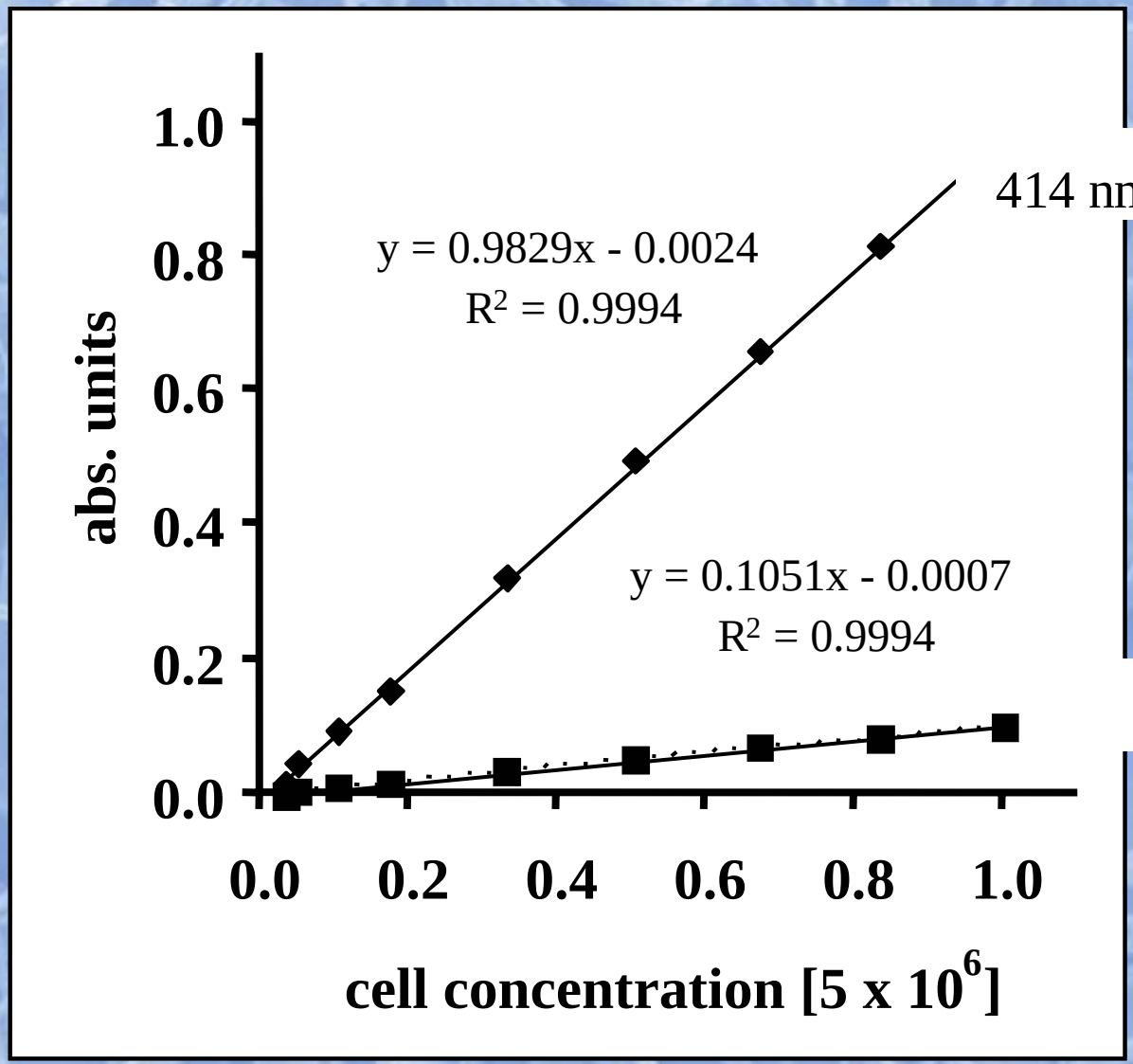
Dr. Martin Lange Handheld device/ *A. tamarense*

Dr, Rene Groben Probe Hybridisation optimisation

Dr. Linda Medlin Principal investigator

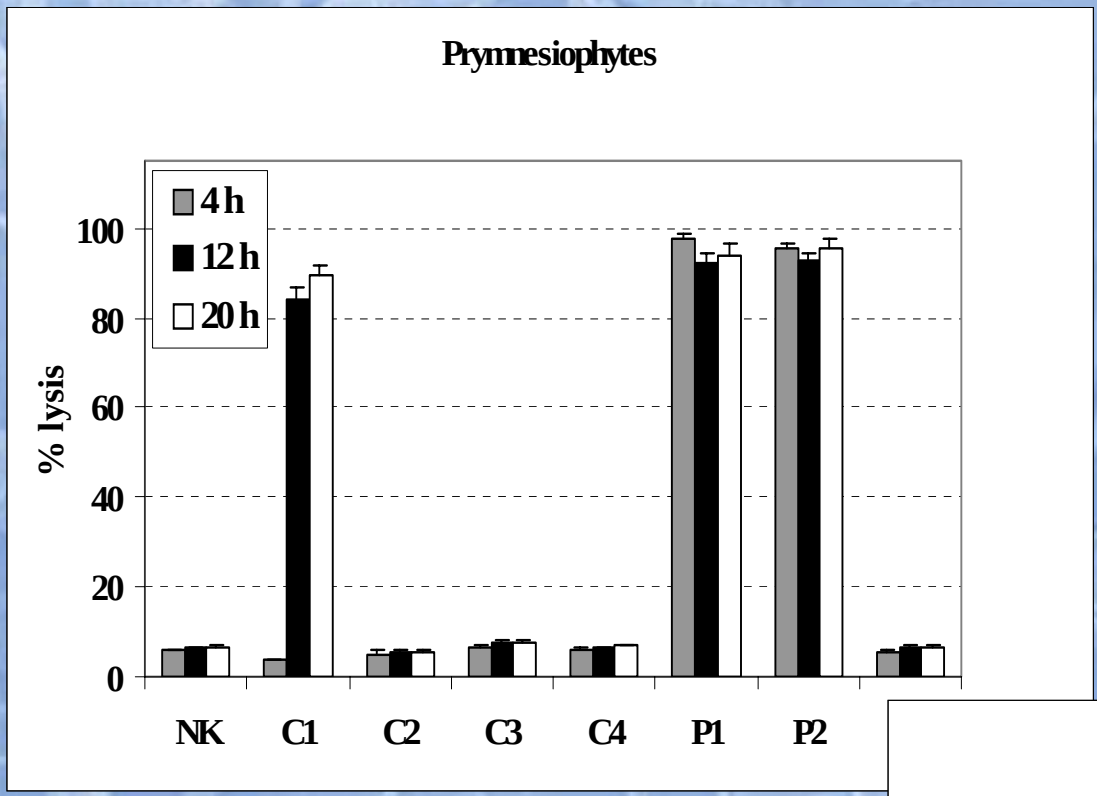
Rapid Tests for the Detection of Haemolytic Compounds

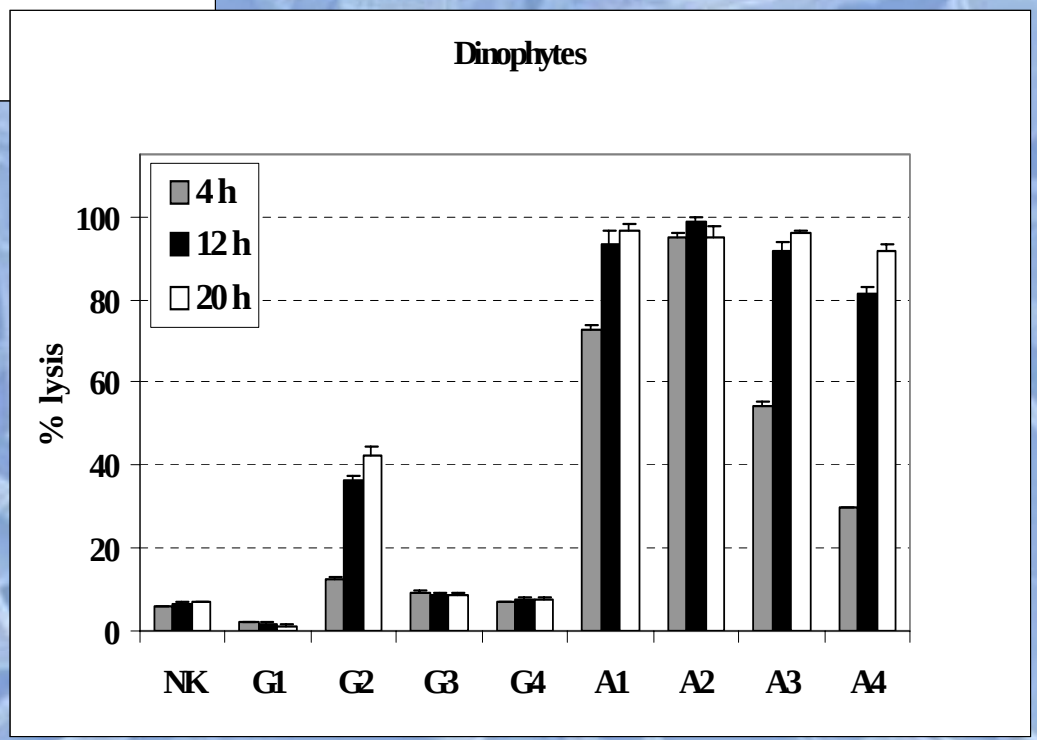

2.4
2.0
1.6
1.2
0.8
0.4
0.0
abs. units
414
540
575
lysed erythrocytes
algal extract
350
400
450
500
550
600
650
700
wavelength [nm]

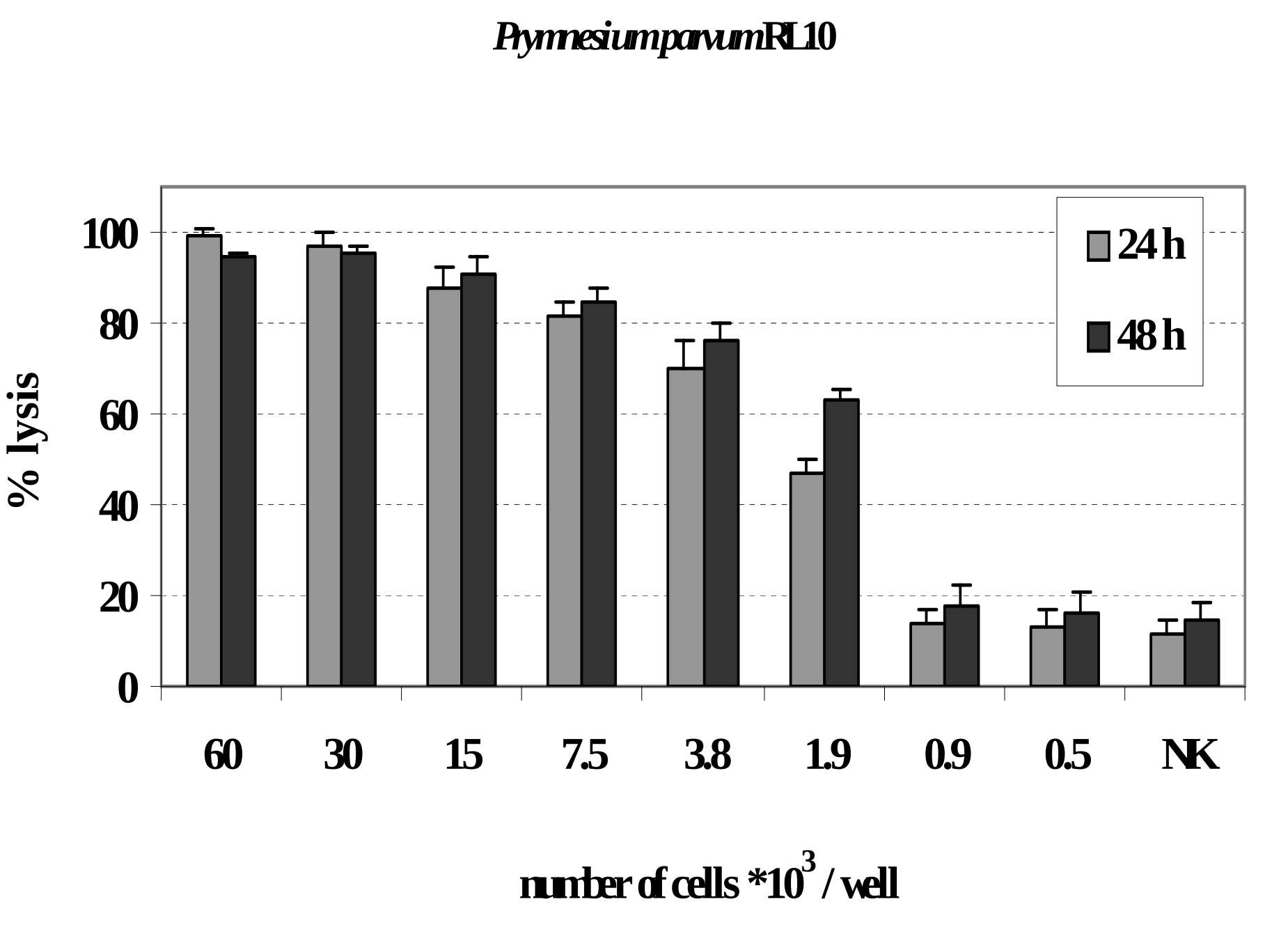

1.0
0.8
0.6
0.4
0.2
0.0
abs. units
y = 0.9829x - 0.0024
R2 = 0.9994
y = 0.1051x - 0.0007
R2 = 0.9994
0.0
0.2
0.4
0.6
0.8
1.0
cell concentration [5 x 10^6]

414 nm:

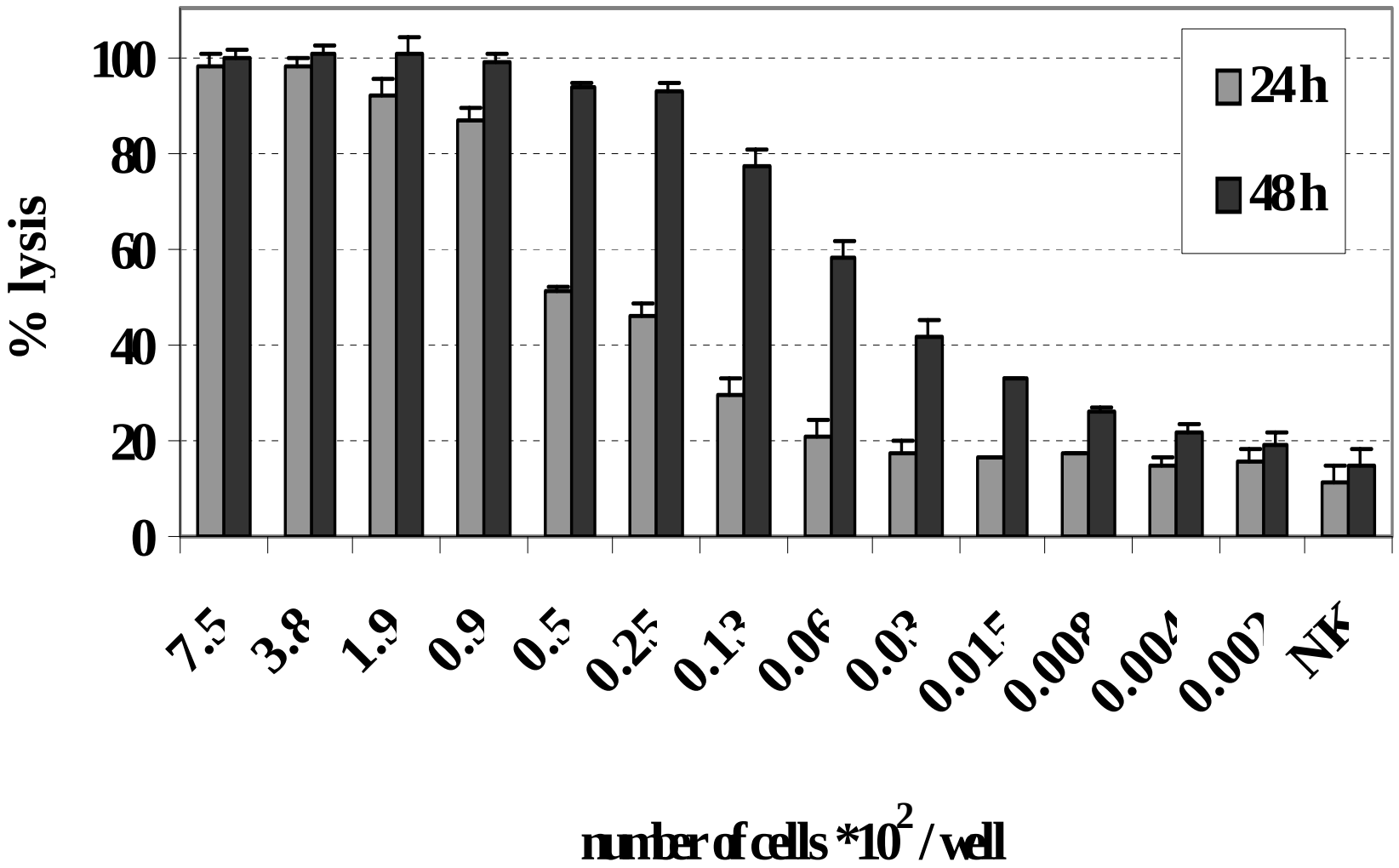
540 nm:


Prymnesiophytes
4 h
12 h
20 h
% lysis
100
80
60
40
20
0
NK
C1
C2
C3
C4
P1
P2

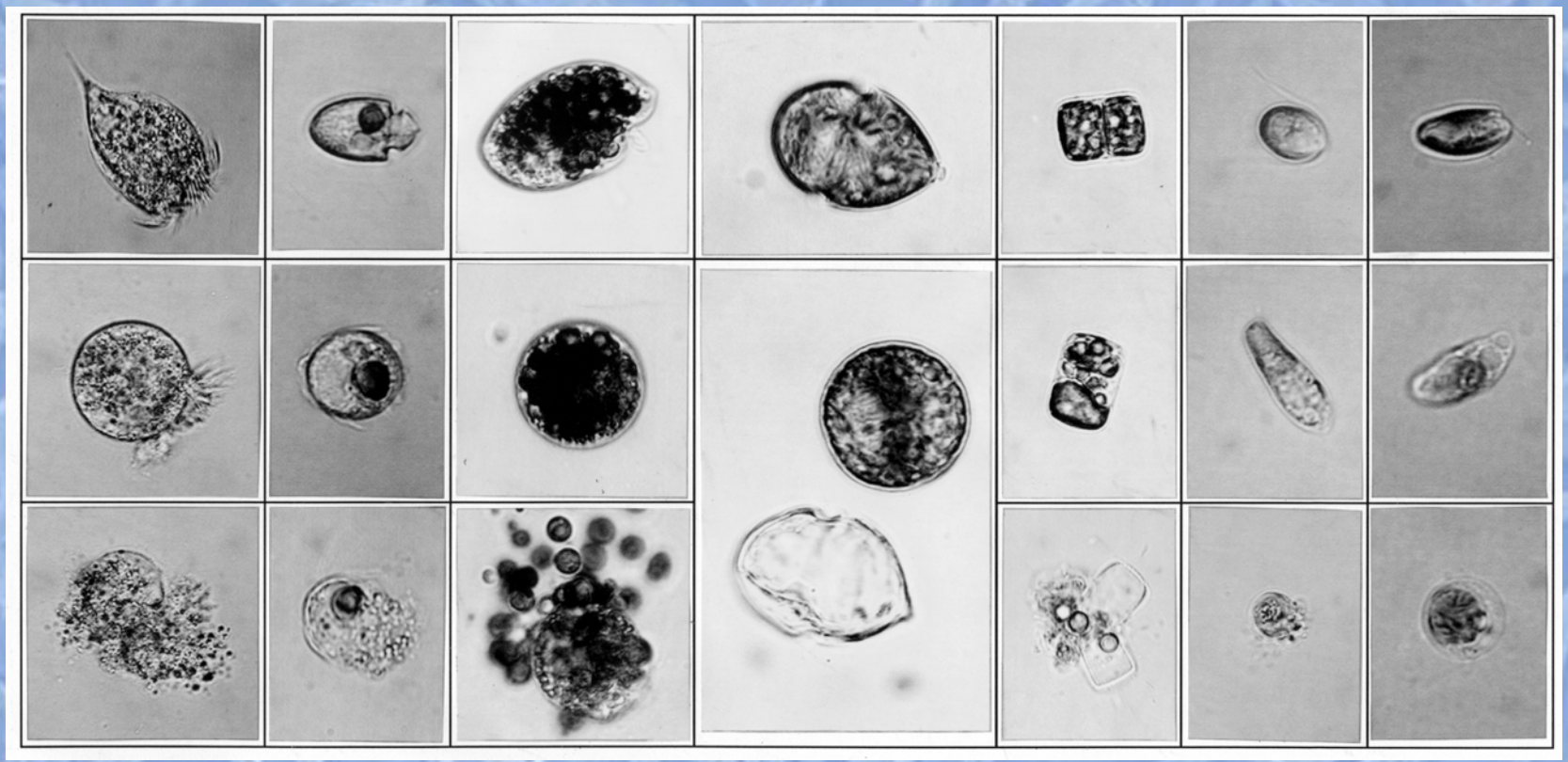

Dinophytes
4 h
12 h
20 h
% lysis
100
80
60
40
20
0
NK
G1
G2
G3
G4
A1
A2
A3
A4


Prymnesium parvum RL10
100
80
60
40
20
0
% lysis
24 h
48 h
60
30
15
7.5
3.8
1.9
0.9
0.5
NK
number of cells *10^3 / well

Alexandrium tamarense CCMP115



Allelochemical effects



ELA Tests provide rapid means for detecting haemolytic compounds but these must be coupled with species tests for 100% Reliability