The identification of *Chrysochromulina* and *Prymnesium* species (Haptophyta, Prymnesiophyceae) using fluorescent or chemiluminescent oligonucleotide probes: a means for improving studies on toxic algae

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Chrysochromulina and *Prymnesium* are important bloom-forming organisms in marine and brackish waters, respectively. Both genera include toxic species, which are primarily implicated in fish kills. Previous analyses of small subunit (SSU) rDNA sequences from *Chrysochromulina* and *Prymnesium* spp. indicate that *Chrysochromulina* is paraphyletic. *C. polylepis*, which produced a spectacular, harmful bloom in 1988, is more closely related to toxic *Prymnesium* species than to most other *Chrysochromulina* species based on rDNA sequence comparisons. Signatures were identified in the SSU rRNA gene specific for a clade that comprised primarily toxic taxa (*C. polylepis*, *P. parvum*, *P. patelliferum* and *P. calathiferum*) and that recognized *C. polylepis* alone. Oligonucleotide probes complementary to these regions were designed, and their specificity tested using dot-blot hybridization on PCR products of the SSU rRNA gene from 28 strains of *Chrysochromulina* and *Prymnesium*. Whole-cell hybridizations were performed with FLUOS- as well as Cy3-labelled probes on cultured species from both genera, and were detected with both epifluorescence microscopy and flow cytometry. The probes afforded easy identification of clonal isolates of *C. polylepis* and a cluster of closely related species including *C. polylepis* and *Prymnesium* spp. The feasibility of using these probes for species identification and studies of population dynamics in the field is discussed.

Key words: *Chrysochromulina polylepis*, Haptophyta, oligonucleotide probes, small subunit ribosomal DNA, toxic phytoplankton.

Introduction

Chrysochromulina Lackey and *Prymnesium* Massart *ex* Conrad are both important bloom-forming microalgae in marine and brackish systems (Jordan & Green, 1994). The genus *Chrysochromulina* includes some 50 species, most of which are marine (Jordan & Green, 1994). Many are common components of the plankton and make up a significant percentage of the biomass of nanoplanktonic cells (Thomsen *et al.*, 1994). Most *Prymnesium* records are from brackish waters and ponds. Both genera include toxic species: *C. polylepis*, *C. leadbeateri*, *P. parvum* and *P. patelliferum* are responsible for fish kills in many parts of the world. A few other species have been shown to be toxic to the bryozoan, *Electra pilosa* (L.), when the microalgae were in stationary phase (see Moestrup & Thomsen, 1995 for review). Both *Chrysochromulina* and *Prymnesium* recurrently produce blooms in marine and brackish systems (Edvardsen & Paasche, 1997). Hypotheses involving unusual climatic conditions, competition considerations and deleterious effects on predators have been proposed to explain the formation of monospecific blooms of these particular species. Strains isolated from noxious blooms have been studied in the laboratory to understand better the mechanisms of toxin production and mode of action. Although some strains of *C. polylepis*, *P. parvum* and *P. patelliferum* have consistently proven toxic, the dramatic toxic effects seen in nature have been difficult to reproduce in culture (Edvardsen & Paasche, 1997). Clearly, we are still unable to understand how and why a single species may suddenly bloom and cause ecosystem disturbances, such as dramatic toxic events in a water mass.

In order to understand such toxic events, the species behaviour in its environment, or at least in a mesocosm where parameters can be controlled, should ideally be studied. Such monitoring, which requires high-frequency sampling with good spatial resolution is hampered by our inability to identify many *Chrysochromulina* and *Prymnesium* and enumerate these cells rapidly. Electron microscopy, which is required to identify cells of *Chrysochromulina* and *Prymnesium* to the species level (Thomsen *et al.*, 1994; Hajdu *et al.*, 1996; Larsen *et al.*, 1993), is not appropriate for such studies. Molecular probes offer the possibility to identify and quantify cells in the field

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without recourse to electron microscopy and should be the tool of choice to advance our knowledge of species that are difficult to monitor *in situ* (Giovanonni *et al*., 1996; Vrieling *et al*., 1996; Scholin *et al*., 1997).

Phylogenetic studies of the *Chrysochromulina*/*Prymnesium* group revealed two different clades (Medlin *et al*., 1997): in the trees *Prymnesium* species are most closely related to *C. polylepis* and form a distinct lineage within *Chrysochromulina*, whereas most other *Chrysochromulina* spp. analysed belong to a sister clade. The species, *C. polylepis*, and the clade to which it belongs (clade 1, Fig. 1) were chosen as targets for oligonucleotide probes because the species included have been involved in dramatic and/or recurrent fish kills (Moestrup, 1994; Edvardsen & Paasche, 1997). For both *Prymnesium* and *Chrysochromulina*, the toxic potential of most of the other described species remains unknown. Some of them, such as *C. leadbeateri*, have been reported during noxious bloom events (Aune *et al*., 1992), although strains isolated from toxic algal blooms appeared to be non-toxic (Meldahl *et al*., 1994). As our knowledge of the taxonomy and ecology of both genera increases, more probes may be designed to identify other species.

The present study concerns the specificity of the probes we have developed, and the use of several techniques (dot-blots on extracted nucleic acids, whole-cell hybridization combined with epifluorescence microscopy or flow cytometry) for the identification of isolates. Potentially these techniques could be used for studying population dynamics of *Chrysochromulina* and other toxic species.

Material and methods

Cultures of Chrysochromulina *and* Prymnesium *strains*

Cultures of *Chrysochromulina* and *Prymnesium* used in this study are shown in Table 1. Batch cultures were grown in 30 ml borosilicate tubes or Erlenmeyer flasks with sea water diluted to 30‰ salinity and enriched with vitamins, chelated trace metals and nitrate, as in IMR 1/2 medium (Eppley *et al*., 1967) and with 10^{-8} M selenite (Dahl *et al*., 1989). Cultures were grown at 15 °C under white fluorescent light with a quantum flux of 100 μmol photons m^{-2} s^{-1} and a 12:12 h light:dark cycle.

DNA extraction and amplification

Cultures were harvested during exponential phase by filtration onto nitrocellulose filters (Nuclepore, 3 μm pore size) or by centrifugation (20 min, 7000 g, 4 °C, RC-SB Sorvall centrifuge). DNA was extracted using a 3% CTAB (hexadecyltrimethylammonium bromide) extraction procedure (Doyle & Doyle, 1990). Total nucleic acid preparations were used as templates for the amplification of the nuclear gene coding for the SSU rRNA molecule using polymerase chain reactions (PCR, Saiki *et al*., 1988). Reaction mixtures of 100 μl contained 100 ng of DNA in 1X PCR buffer as in Medlin *et al*. (1988) and 30 nM of the primers designed by Medlin *et al*. (1988) for amplifying eukaryotic SSU rDNA (excluding the polylinkers). Amplification products were used to test probe specificity.

Table 1. Origin of the algal strains used in this study

Species	Strain code	Culture collection
Prymnesium patelliferum Green, Hibbert *et* Pienaar	K-O252	SCCAP
	PCC 527	PCC
	RH pat 93	UIB
P. parvum N. Carter	K-0081	SCCAP
	RC 10 parv 93	UIB
	PCC 94	PCC
Chrysochromulina polylepis Manton *et* Parke	B11	UIO
	S	UIO
	A	UIO
	K	UIO
	CCMP 287	CCMP
	PCC 200	PCC
C. hirta Manton	1Y	UIO
	PN2	UIO
C. kappa Parke *et* Manton	EN3	UIO
C. campanulifera Manton *et* Leadbeater	J10	UIO
C. cf. *strobilus* Parke *et* Manton	R18	UIO
C. simplex Estep, Davis, Hargraves *et* Sieburth *emend.* Birkhead *et* Pienaar	Jomf B	UIO
C. cf. *simplex*	PCC 384A	PCC
C. leadbeateri Estep, Davis, Hargraves *et* Sieburth	ERIK	UIO
C. ericina Parke *et* Manton	Q17	UIO
C. throndsenii Eikrem	K11	UIO
	L12	UIO
Chrysochromulina sp. 1	TH2	UIO
C. scutellum Eikrem *et* Moestrup	G7	UIO
Chrysochromulina sp. 2	S19	UIO
C. acantha Leadbeater *et* Manton	T20	UIO
Chrysochromulina sp. 3	P16	UIO
C. brevifilum Parke *et* Manton	PCC 143	PCC
C. chiton Parke *et* Manton	PCC 146	PCC
C. cf. *minor* Parke *et* Manton	PCC M115	PCC

PCC = Plymouth Culture Collection, Plymouth, U.K.; CCMP = Culture Center for Marine Phytoplankton, Bigelow Laboratory, U.S.A.; UIO = University of Oslo, Marine Botany, Norway; UIB = University of Bergen, High Technology Center, Department of Biology, Bergen, Norway; SCCAP = Scandinavian Culture Collection for Protozoa, Copenhagen, Denmark.

Probe design

The tree presented in Medlin *et al*. (1997) and redrawn in Fig. 1 served as a basis for our probe design. The *Chrysochromulina*/*Prymnesium* SSU rDNA sequences were aligned with those for more than 250 other published and unpublished algal species and loaded into the ARB program (W. Ludwig, Technical University of Munich, Germany) and a search was performed for regions of 15 to 22 nucleotides in length that were unique for the following taxa/clades among the *Prymnesium*/*Chrysochromulina* clade: (1) the species *C. polylepis* (probe CPOLY01), (2) a clade (clade 1 in Fig. 1) containing the toxic haptophytes

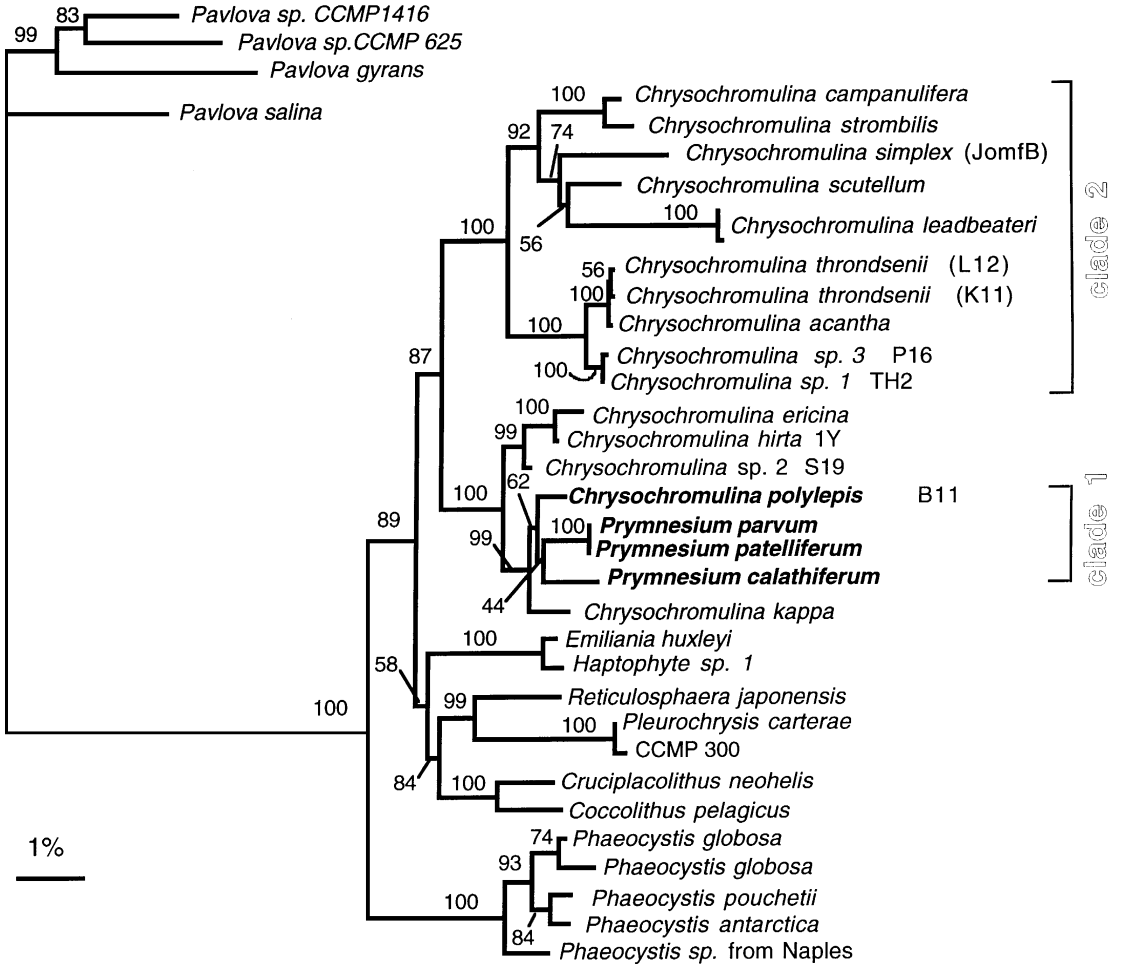


Fig. 1. Molecular phylogenetic tree inferred from the SSU rRNA gene from the *Prymnesium/Chrysochromulina* group using the neighbour-joining method (redrawn from Medlin *et al.*, 1997). Strain origins for *Chrysochromulina* spp. and *Prymnesium* spp. are as in Table 1. Numbers above the nodes represent percentages of 100 bootstrap repetitions (Felsenstein, 1985).

C. polylepis, *P. parvum*, *P. patelliferum* and *P. calathiferum* (probe CLADE1) but excluding *C. kappa*, (3) a second clade of *Chrysochromulina* species (clade 2 in Fig. 1, probe CLADE2, which served as a control).

Dot-blot hybridization

Amplified SSU rRNA genes from *Chrysochromulina* and *Prymnesium* species were denatured by heating at 95 °C for 10 min. Samples were immediately chilled in an NaCl-ice bath for 10 min before blotting onto positively-charged nylon membranes (Boehringer Mannheim, Mannheim, Germany). The oligonucleotide probes that we designed, as well as the probe PRYM01 (Prymnesiophyceae specific, Lange *et al.*, 1996), were labelled with digoxigenin (DIG)-dUTP using the Boehringer Mannheim DIG Oligonucleotide 3′-End Labelling Kit. The membranes were hybridized with 1 pmol l^{-1} of DIG-labelled probes at 50 °C for CPOLY01 and 54 °C for CLADE1, CLADE2 and PRYM01 probes. Detection was performed by chemiluminescence (DIG detection Kit with CSPD as a substrate, Boehringer Mannheim). All steps were performed according to the manufacturer's instructions. Membranes were exposed to X-ray films (Amersham, Arlington Heights, IL) for up to 3 h and results were documented by laser copying.

Whole-cell hybridization

For whole-cell hybridization purposes, all probes were synthesized with an amino linker at the 5′ end and labelled with 5(6) carboxyfluorescein N-hydroxysuccinimide ester (FLUOS) (Boehringer Mannheim) or the Fluorolink Cy3 (Amersham) according to Amann (1995). Two different protocols were used for cell fixation. Modifications of the protocols described by Amann (1995) and Wallner *et al.* (1993) were employed respectively for epifluorescence microscopy and flow cytometry. Cells were spotted onto a gelatin-coated slide (Paul Marienfeld KG, Bad Mergentheim, Germany) and dried at 46 °C for 5 min before dehydration in an ethanol series (50, 80 and 96% aqueous ethanol, v/v). In some specificity studies cultures were mixed in equal proportions and spotted onto slides. For flow cytometry, cells were fixed with 1% paraformaldehyde for 1 h at 4 °C prior to dehydration in ethanol:PBS (70:30, v/v). Cells were further incubated in hybridization buffer (0.9 M NaCl, 20 mM Tris HCl pH 7.2, 0.01% SDS, 10% formamide). Cells were then

rinsed in 0.45 M NaCl, 20 mM Tris HCl, 0.01% SDS and either mounted in Citifluor (Citifluor Ltd, Canterbury, UK) before examination with epifluorescence microscopy or resuspended in PBS pH 9 for flow cytometry. Cells were viewed with an Axioskop 20 epifluorescence microscope (Zeiss, Oberkochen, Germany) or analysed with a FACS Analyzer flow cytometer (Becton Dickinson, San Jose, California) equipped with a mercury arc lamp. Our microscope was equipped with Zeiss filter sets 09 (FLUOS) or 14 (Cy3). Photographs were taken with 200 or 1600 ISO Fuji films following a 15 to 60 s exposure for epifluorescence. For flow cytometry, excitation was at 546 (10) nm and fluorescent signals were collected through a 580 (10) nm band pass filter.

Results and discussion

Probe sequences and localization

Information concerning our taxon-specific oligonucleotides (target species, localization in the rRNA secondary structure, nucleotide sequence) is summarized in Table 2. A signature region for *Chrysochromulina polylepis* was found in helix 26-27-28 of the SSU rRNA molecule (see secondary structure model in Chesnick *et al*., 1997). A probe (CPOLY01) complementary to this region was designed. It had at least 2 mismatches with the corresponding region of the SSU rDNA of all other species sequenced to date. Similarly, a signature region (helix 26-27) was found for a group of species (*C. polylepis*, *Prymnesium parvum*, *P. patelliferum* and *P. calathiferum*) which forms a small clade within *Chrysochromulina*/*Prymnesium*. Because all species in this group are potentially toxic, a probe specific for this clade was designed (CLADE1 probe). This does not imply that toxin production is related to the phylogeny of the species. As a control, we designed a probe that is specific for another clade of *Chrysochromulina* species (CLADE2 probe, helix 9-10). Whereas CLADE1 probe had at least 3 mismatches with all other available rDNA sequences, CLADE2 probe had only 1 mismatch with *C. polylepis*, *P. parvum*, *P. patelliferum* and *Cruciplacolithus neohelis* (Macintyre *et* Abé) Reinhardt SSU rDNA gene sequences. We therefore designed a probe complementary to the target region of CLADE2 for those latter species. Such a competitor should prevent unspecific labelling of CLADE 2 probe to these species' SSU rRNAs (Manz *et al*., 1992; Simon *et al*., 1995).

We are aware that our search for probes was conducted on only a fraction of the species belonging to *Chrysochromulina* and *Prymnesium*, the actual number of which may exceed 100 according to Thomsen *et al*. (1994). Similarly, sequence data for only one strain of *C. polylepis* was used for the design of CPOLY01. Therefore, more species and strains (especially for *C. polylepis*) were used to assess the specificity of the probes by dot-blot hybridization (see below).

Table 2. Probe sequence and localization in a SSU rRNA secondary structure model (Chesnick *et al*., 1997)

Probe name	Sequence (5′ to 3′)	Localization (Helix number)	Number of mismatches*
PRYM01 (Lange *et al*., 1996)	TCGTAAACGGTCCCTACA	24	1
CLADE1	GGACTTCCGCCGATCCCTAGT	26-27	3
CPOLY01	GACTATAGTTTCCCATAAGGT	26-27-28	2
CLADE2	AGTCGGGTCTTCCTGCATGT	9-10	1
CLADE2 competitor	AGTCGGGACTTCCTGCATGT	9-10	0

* Numbers of mismatches in the corresponding region of the SSU rDNA from the closest relatives. Mismatches were positioned as close as possible to the centre of the probe.

Probe specificity: dot-blot hybridization

To check the specificity of our probes, we chose 19 species from the more than 60 described taxa of *Prymnesium*/*Chrysochromulina*. These 19 species are represented by 28 strains including some whose SSU rRNA gene has been sequenced, and others, such as *C. chiton*, *C.* cf. *minor*, and multiple strains of *C. polylepis*, and *Prymnesium* spp., whose SSU rRNA gene has not been sequenced. A total of 6 *Chrysochromulina polylepis* strains was used in this study, including two forms isolated from a noxious bloom in Norwegian coastal waters (Edvardsen & Paasche, 1992; Edvardsen & Vaulot, 1996), as well as an isolate from the English Channel (PCC 200). *P. parvum* and *P. patelliferum* were represented by a total of 6 strains. Other *Chrysochromulina* species tested comprised strains that were closely related to *C. polylepis* (e.g. *C. kappa*) in terms of SSU rDNA phylogeny, as well as more distantly related species (e.g. *C. leadbeateri*, *C. throndsenii*) (Fig. 1). Hybridization conditions were attained under which the probes labelled only their targets (Fig. 2). PRYM01 was used as our positive control and labelled all strains, except for *C. leadbeateri* (examination of its SSU rDNA sequence revealed a single mismatch with the PRYM01 probe). Probes CPOLY01, CLADE1 and CLADE2 labelled only their intended targets and proved highly specific for the taxonomic groupings on which they were designed. CPOLY01 labelled all *C. polylepis* strains including the toxic Norwegian strains (Edvardsen, 1993) and the British isolate PCC200 whose toxicity has not yet been tested. Electron microscopy revealed that the latter isolate differs slightly from the Norwegian clones with respect to scale morphology, as well as to SSU rDNA sequence (B. Edvardsen and L. Medlin, unpublished data).

Preliminary information concerning the phylogenetic affinities of those species for which sequence was not available during the 'probe-design' process could be obtained using dot-blot hybridization. *C. chiton* gave a positive signal with the probe CLADE1 and no signal with

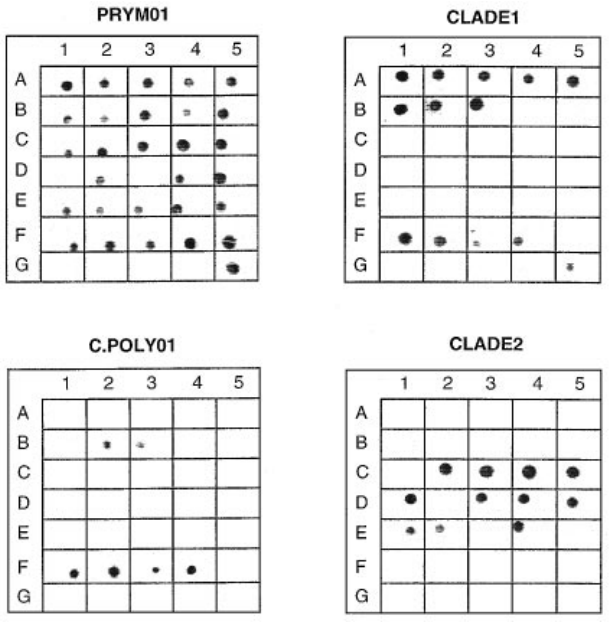


Fig. 2. Dot-blot hybridization of filter bound DNA (amplified SSU rRNA gene) with the digoxigenin-labelled oligonucleotide probes PRYM01, CLADE1, CPOLY01 and CLADE2. The grid shows the following members of the *Chrysochromulina/Prymnesium* group. A-1, -2, -3; *Prymnesium patelliferum* (K-0252, PCC 527, RH pat 93); A-4, -5, B-1: *P. parvum* (K-0081, RC 10 parv 93 and PCC 94); B-2, -3, F-1, -2, -3, -4: *Chrysochromulina polylepis* (clones S, B11, CCMP 287, PCC 200, A and K, respectively); B-4: *C. kappa*; B-5, C-1: *C. hirta* (clones 1Y and PN2-14); C-2: *C. campanulifera*; C-3, -5: *C.* cf. *strobilus*; C-4: *C.* cf. *simplex*, D-1, -3: *C. leadbeateri*; D-2: *C. ericina*; D-4: *C. throndsenii*; D-5, *Chrysochromulina* sp. 1 (TH2); E-1: *C. scutellum*; E-2; *C. acantha*; E-3: *Chrysochromulina* sp. 2 (S19); E-4: *Chrysochromulina* sp. 3 (P16); E-5: *C.* cf. *minor*; F-5: *C. brevifilum*; G-5: *C. chiton*. G-1, -2, -3, -4 are empty.

both probes CLADE2 and CPOLY01. *Chrysochromulina* cf. *minor* did not hybridize with any of the probes and should be targeted for sequencing. Although dot-blot hybridizations do not give the same amount of information as sequence data, they may be used for preliminary screening of isolates or even for community structure studies as proposed by Giovanonni *et al.* (1996).

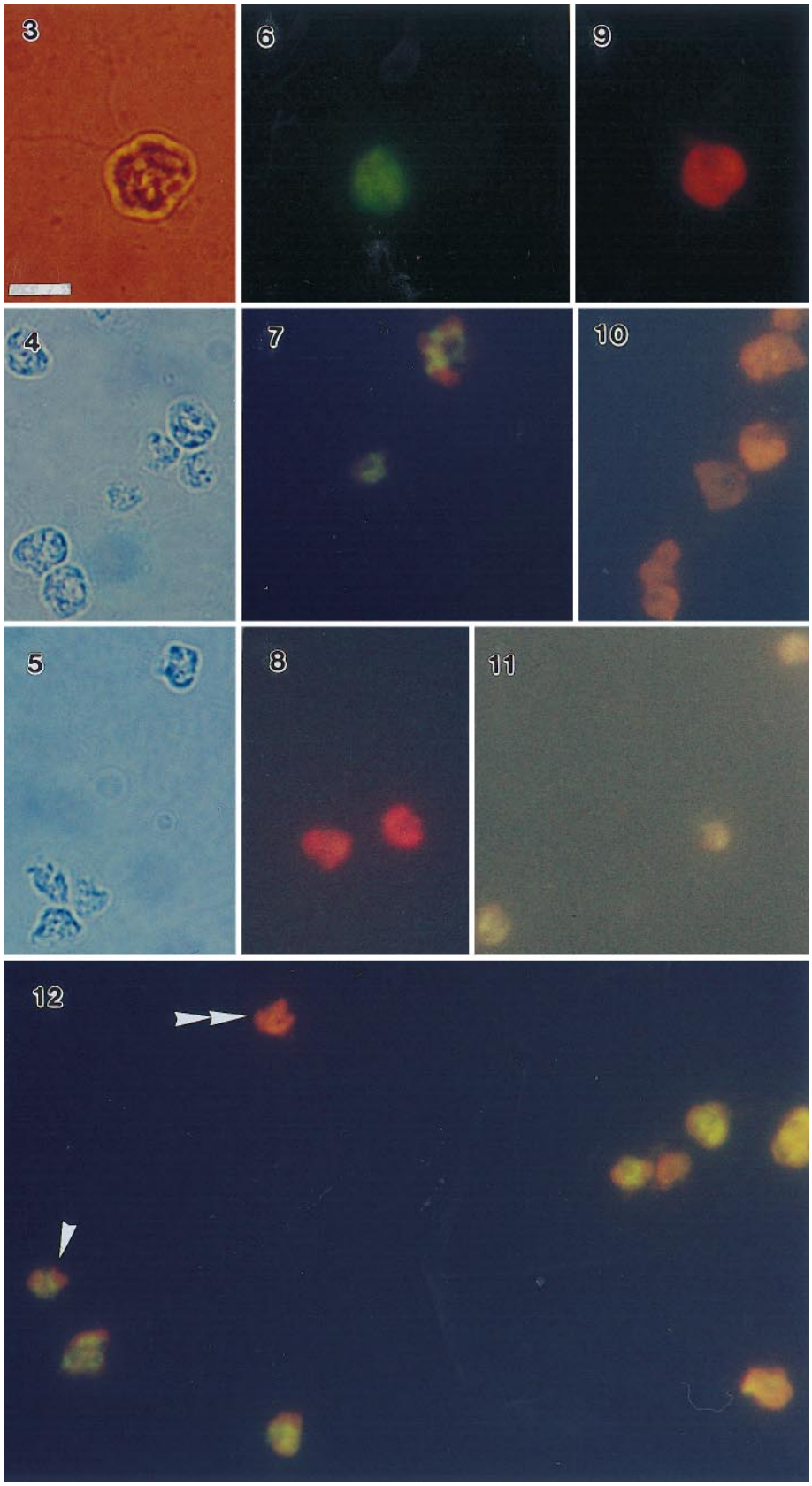
Whole-cell hybridization

Given the high specificity of the probes as shown from the dot-blot analysis, our tests for whole-cell hybridization were conducted only on a reduced number of species. *P. parvum*, *C. polylepis* and *C. throndsenii* were chosen for hybridization experiments (Figs 3–5). FLUOS-labelled CLADE1 probes applied to *P. parvum* and *C. polylepis* gave a bright green signal (Figs 6 and 7, respectively). CLADE1 probe did not label *C. throndsenii* (Fig. 8). Conversely, CLADE2 probe did not label *P. parvum* and *C. polylepis* (Figs 9, 10) but conferred a bright signal to *C. throndsenii* (Fig. 11). *C. polylepis* was also clearly detected using the CLADE1 probe, when mixed with *C. throndsenii* (Fig. 12). Similarly, the CPOLY01 probe was highly specific and afforded detection of *C. polylepis* to the exclusion of *P. parvum* and *C. throndsenii* (results not shown). Thus, our probes have been tested successfully in mixed populations, albeit limited in species number.

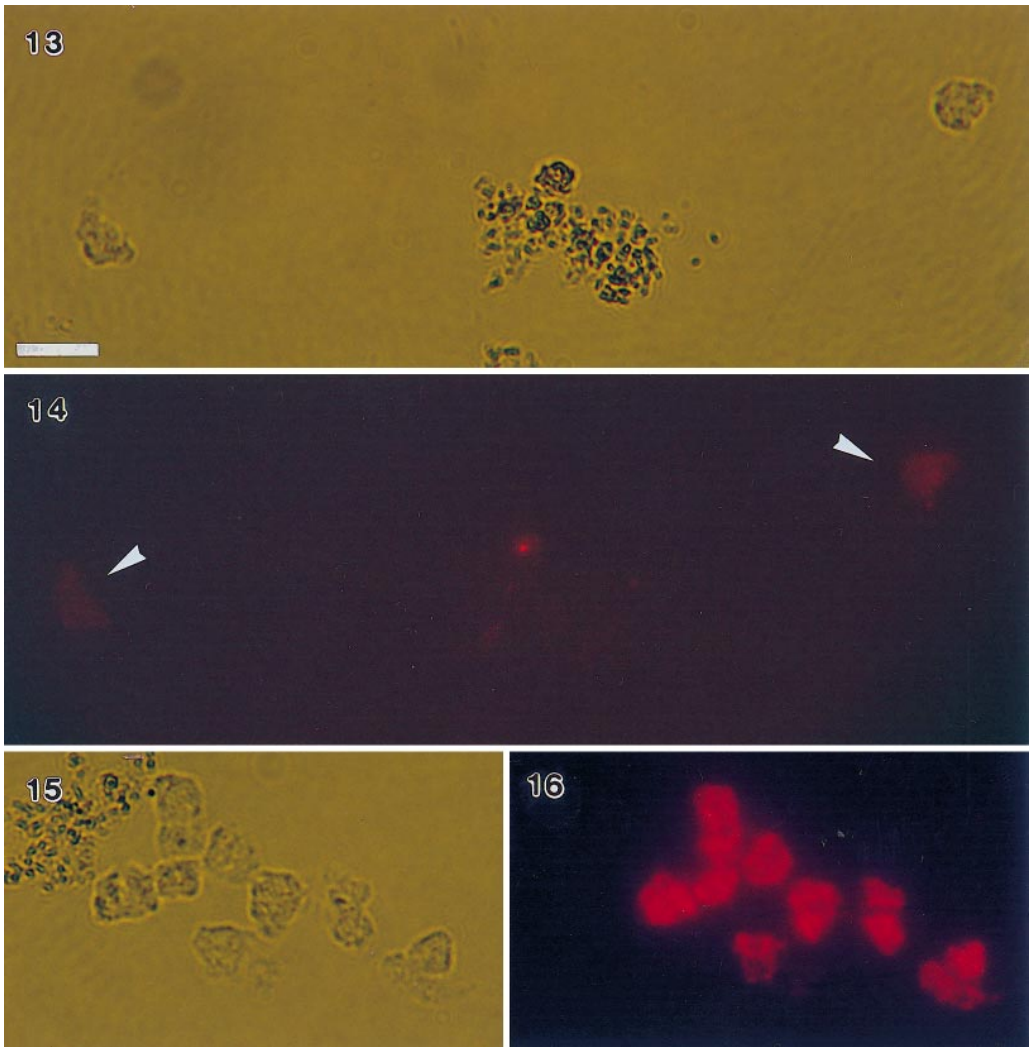
Monitoring of natural samples for selected species requires the examination of large numbers of cells. For such purposes, a stronger signal is likely to be needed in order to identify target (toxic) species rapidly. Cy3, an orange fluorochrome with an elevated signal strength, can be used as an alternative to FLUOS for whole-cell hybridization when stronger signals are required (Alfreider *et al.*, 1996) (Figs 13–16). As expected, a very bright signal was observed for exponentially growing cells hybridized with Cy3-labelled CLADE1 probe (Fig. 16). Autofluorescence was low (Fig. 14). For cells in stationary phase, the autofluorescence was higher than for those in exponential growth phase and would probably interfere with cell detection and identification if the target cells were in this physiological state (data not shown).

Cy3-labelled probes were further tested for whole-cell hybridization in combination with flow cytometry. Our aim was to evaluate how probes would respond using tools that facilitate rapid identification and quantification of cells. Four strains were chosen for these tests: *C. polylepis* (PCC 200), *P. parvum* (RC 10 parv 93), *C. acantha* (T20) and *C. leadbeateri* (ERIK). The intensity of the probe-conferred fluorescence was always highest when probe specificity matched the phylogeny of the cells (Fig. 17). Ratios of fluorescence of hybridized cells to that of control cells (no probe added in the hybridization buffer) were 2 to 10 times higher for probe/target cell combinations than for probe/non-target combinations (Table 3). Highest ratios were obtained for *C. polylepis*.

Rapid identification of *C. polylepis* isolates can be obtained utilizing the CPOLY01 probe in conjunction with either flow cytometry or epifluorescence microscopy (no TEM). The use of the probes we designed for natural sample examination has not yet been tested. Fluorescent oligonucleotide probes for larger organisms such as *Pseudo-nitzschia* species have already been used for the study of seawater samples by epifluorescence microscopy (Scholin *et al.*, 1996, 1997; Rhodes *et al.*, 1997). For the examination of natural assemblages of nanoplanktonic protists with oligonucleotide probes, a quantitative method has also been developed (Lim *et al.*, 1996). With the availability of our probes and these methods it should be possible to gather data on the abundance and distribution of *Chrysochromulina* and *Prymnesium* species. The use of flow cytometry combined with 'probe technology' already appears to be a powerful tool for the rapid analysis of microbial communities in activated sludges used in waste water treatments (Wallner *et al.*, 1995). The analysis of natural samples will require improvement in sample processing (centrifugation and resuspension steps may cause cell loss) and an increase in the signal to noise ratio. The use of indirect labelling (Knauber *et al.*, 1996) or of multiple probes (Lee *et al.*, 1994) might help us to obtain higher specific fluorescent signals.



Figs 3–12. Hybridization of the rRNA-based probes CLADE1 and CLADE2 labelled with FLUOS to different *Chrysochromulina/Prymnesium*. Cells were visualized by light (Figs 3–5) or epifluorescence microscopy (Figs 6–12). Scale bar represents 10 μm and applies to all figures. Positive label of the FLUOS probe appears green, autofluorescence of the cells is red. Figs 3–5. *Prymnesium parvum, Chrysochromulina polylepis* and *C. throndsenii*, respectively, after fixation and incubation in hybridization buffer. Figs 6–8. *Prymnesium parvum, Chrysochromulina polylepis* and *C. throndsenii*, respectively, after incubation in hybridization buffer with the probe CLADE1. Figs 9–11. *Prymnesium parvum, Chrysochromulina polylepis* and *C. throndsenii*, respectively, after incubation in hybridization buffer with the probe CLADE2. Fig. 12. Mixture of both *C. polylepis* (simple arrowhead) and *C. throndsenii* (double arrowhead) hybridized with the probe CLADE1.



Figs 13–16. Hybridization of the rRNA-based probe CLADE1 labelled with Cy3 to *Chrysochromulina polylepis*. Scale bar in Fig. 13 represents 10 μm and applies to all figures. Figs 13, 14. Bright field photomicrograph (Fig. 13) and the corresponding field viewed by epifluorescence microscopy (Fig. 14) for a preparation of cells incubated in hybridization buffer without probe, showing autofluorescence of the cells (arrowheads). Positive label of the Cy3 probe is bright red (Fig. 16) in comparison to the paler red autofluorescence of the cells (Fig. 14). Figs 15, 16. Cells hybridized with CLADE1 labelled with Cy3 visualized by light microscopy (Fig. 15) and showing strong fluorescence with epifluorescence microscopy (Fig. 16).

Conclusions

All probes we designed are highly specific for their target taxa as proven by dot-blot hybridization. Monitoring studies could be readily conducted in the environment using those probes according to the protocols developed by Giovanonni *et al*. (1996) for hybridization with nucleic acids extracted from natural samples. However, the study of bloom dynamics requires a relatively high temporal and spatial resolution. Most blooms occur in relatively limited areas (ponds or lakes for *Prymnesium*, coastal water masses for *Chrysochromulina*) and develop in one or two months (Edvardsen & Paasche, 1997). Whole-cell hybridization would probably afford the most precise study of such events and would be, in that respect, more appropriate than other indirect or more time-consuming methods. With this method, information concerning the identity of individual cells and actual cell counts may be obtained daily from chosen sampling sites. This information may be combined with other biological and physical parameters to understand how and why a species may suddenly bloom in a discrete water mass. Our results are the necessary first steps towards such studies of species in their environment.

Table 3. Ratio of fluorescence intensities of cells hybridized with the different probes (CLADE1, CLADE2, CPOLY01) to the intensity of the autofluorescence of the cells (control: no probe in the hybridization buffer)

Species	CLADE1/ control	CLADE2/ control	CPOLY01/ control
Chrysochromulina polylepis	18.5	1.9	14.4
Prymnesium parvum	10.8	3	4.3
Chrysochromulina acantha	4.5	9.7	nd
Chrysochromulina leadbeateri	3.2	7.1	1.3

Ratios were calculated from mean values for a whole cell population (> 4000 cells) analysed by flow cytometry. nd = not determined.

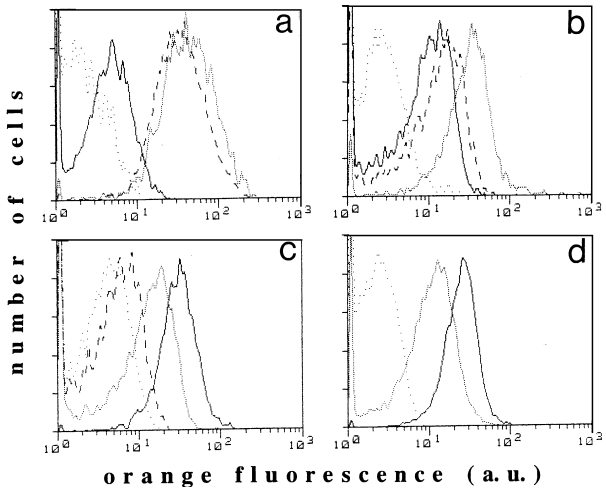


Fig. 17. Histograms (number of cells *vs* orange fluorescence) obtained from the analysis of cell populations of (a) *Chrysochromulina polylepis*, (b) *Prymnesium parvum* (c) *C. leadbeateri* and (d) *C. acantha* by flow cytometry. Number of cells were standardized for clarity (Y axis). A. u. represents arbitrary units (X axis). Cells were fixed, permeabilized and incubated either without probes (autofluorescence of cells, ...) or with CY3-labelled CLADE2 (——), CLADE1 (……) and CPOLY01 (-----). When cells are positively labelled with a probe there is an increase in the intensity of the fluorescense signal as detected by the flow cytometer. With maximum separation of labelled and non-labelled/non-specific labelled populations, the histograms have minimum overlap. For example, in panel A, C. polyeis is labelled equally well by both CLADE1 and CPOLY01 probes with little overlap with CLADE2 probe. The remaining panels show some overlap between target (histogram with the highest fluorescence values) and non-specific labelling.

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