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Automated detection and enumeration for toxic algae by solid-phase cytometry and the introduction of a new probe for *Prymnesium parvum* (Haptophyta: *Prymnesiophyceae*)

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Harmful algal blooms have a severe impact on aquaculture and fishery and can be caused by toxic haptophytes and dinoflagellates. Different toxic species, which are not easy to distinguish from their morphologically similar and non-toxic relatives, occur in both groups. Sequencing of the large subunit ribosomal RNA of different strains and taxonomic relatives allowed the design of a probe specific to the toxic Prymnesium parvum spp. For the rapid detection and enumeration of Prymnesium and Alexandrium cells in cultures and environmental samples, respectively, protocols for fluorescence in situ hybridization were adapted for automated detection by a solid-phase cytometer, the ChemScan. This cytometer enables the automated counting of fluorescently labelled cells on a membrane filter and subsequently a microscopic verification of these results by the user, because the motorized stage of the microscope is driven to each positive signal by the computer software to localize that cell on the filter. With this fast detection method, it was possible to detect, enumerate and verify microalgal cells on a filter, with a detection limit of one cell per membrane filter.

INTRODUCTION

Toxic microalgae have become a severe problem in many coastal areas, and haptophytes and dinoflagellates have been frequently reported as causative agents for harmful algal blooms (HABs). The species *Chrysochromulina polylepis* (Manton and Parke, 1962) and *Prymnesium parvum* (Carter, 1937) and *Prymnesium patelliferum* (Green *et al.*, 1982; Larsen, 1999), members of the family *Prymnesiophyceae*, have been responsible for numerous fish kills, especially in low salinity water (Kaartvedt *et al.*, 1991; Moestrup, 1994; Edvardsen and Paasche, 1998; Johansson and Granéli, 1999) through the production of ichthyotoxins. To date, an oligonucleotide probe for only *C. polylepis* has been developed (Simon *et al.*, 1997).

A further taxon of harmful microalgae responsible for HABs is the genus *Alexandrium*, which comprises many species capable of producing paralytic shellfish poisons. These toxins cause a serious illness that can affect humans and cause severe neurological problems (Hallegraeff, 1995). Different phylogenetic studies have shown that the three closely related, toxigenic morphotypes *A. tamarense*, *A. fundyense* and *A. catenella* are separated into phylogenetic clades according to their geographic origin and are therefore often combined to the *A. tamarense* species complex (John *et al.*, 2003a, 2005). John *et al.* (John *et al.*, 2005) developed oligonucleotide probes targeting the 28S ribosomal RNA (rRNA) to differentiate toxic from non-toxic geographic clades,

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which are morphologically indistinguishable in HABs. Because so many species of the genus *Alexandrium* are toxin producers, and there is an existing variability in toxicity in some *Alexandrium* species, each bloom of any member of this genus should be investigated for possible paralytic shellfish poisoning (PSP) outbreaks (Taylor *et al.*, 2003).

A detection method for monitoring toxic microalgae with a high sample throughput and a low detection limit is desirable because it would allow the detection of increasing cell numbers of toxic species well before the actual bloom is initiated. Consequently, appropriate mitigation measures can be taken in time to prevent economic loss. In contrast to classical electron microscopy methods, the use of fluorescence *in situ* hybridization (FISH) allows the rapid detection of different algal groups by epifluorescence microscopy and even the separation of closely related and morphologically similar species (Lim *et al.*, 1993; Scholin *et al.*, 1994a, 1996; Miller and Scholin, 1996, 2000; Simon *et al.*, 1997, 2000; John *et al.*, 2003b, 2005; Groben *et al.*, 2004). Epifluorescence microscopy and flow cytometry are usually used to detect and count cells after FISH. However, because of their limited sensitivity at lower cell concentrations, neither of these techniques is suited to detect cells at low environmental levels. Presently, solid-phase cytometry (SPC) is the only technique that can detect and enumerate microorganisms at very low concentrations (Lemarchand *et al.*, 2001). This method combines the advantages of flow cytometry and image analysis (Kamentsky, 2001) and enables the rapid measurement of several thousand cells with similar precision to flow cytometry (Darynkiewicz *et al.*, 2001).

The ChemScanRDI system (Chemunex, France) is a recently designed solid-phase cytometer for the detection and enumeration of rare events (Mignon-Godefroy *et al.*, 1997; Reynolds and Fricker, 1999). Prior to scanning, cells are collected by filtration onto a membrane filter and fluorescently labelled. Fluorescent events are detected by photomultipliers and undergo various discrimination steps by the computer software to differentiate positively labelled microorganisms from non-target autofluorescent particles. This allows an accurate enumeration down to a detection limit of one cell per filter. After scanning, cells can be visualized by epifluorescence microscopy. For this purpose, an epifluorescence microscope with a motorized stage is connected to the ChemScan. The filter is transferred to the microscope stage and can be moved to each positive scan point on the filter for microscopic validation (Reynolds and Fricker, 1999).

To date, the ChemScan system has been mainly used for the detection and enumeration of bacteria in drinking water (Lemarchand *et al.*, 2001), visualized by fluorescing viability tests (Jones *et al.*, 1999; Reynolds and Fricker, 1999; Roubin *et al.*, 2002) and by monoclonal antibodies (Rushton *et al.*, 2000; Pougnard *et al.*, 2002; Aurell *et al.*, 2004. Baudart *et al.* (Baudart *et al.*, 2002) applied FISH in combination with a direct viable count tests to detect bacteria in freshwater and drinking water with the ChemScan. West *et al.* (West *et al.*, 2006) applied the ChemScan system to detect and quantify *P. parvum* cells in environmental samples with monoclonal antibodies. However, SPC in combination with FISH and microalgae has never been attempted and would be useful for the monitoring of harmful algae. The aim of this work was to develop an oligonucleotide probe targeting the rRNA of the toxic species *P. parvum* and to establish a FISH protocol suitable for the semi-automated detection and enumeration of microalgae with a solid-phase cytometer, the ChemScan.

METHOD

Culture conditions

Pure cultures of different microalgae (Table I) were grown in enriched seawater media K (Keller *et al.*, 1987), IMR/2 (Eppley *et al.*, 1967) or Drebes (Drebes, 1966), respectively. Cultures were grown with a light/dark photocycle of 12/12 h at 15°C and a photon flux density of 100 μmol m^{-2} s^{-1}.

DNA extraction

DNA extractions from microalgae Table 1 were made from 1 L of culture in logarithmic growth phase and were harvested with filtration through a 3-μm Isopore membrane filter (Millipore) and afterwards washed once with sterile seawater. Cells were washed from the filters with 3 mL prewarmed (65°C) 3% hexadecyl-trimethyl-ammonium bromide (CTAB) into 15-mL reaction tubes (Sarstedt, Germany), and DNA was extracted as described by Doyle and Doyle (Doyle and Doyle, 1990). Cells were lysed in 3% CTAB buffer at 60°C for 1 h. DNA was purified by subsequent extraction with phenol/chloroform/isoamylalcohol (PCI) and chloroform/isoamylalcohol (CI) and precipitated with isopropanol. The DNA pellet was washed with ethanol and resuspended in PCR-grade water. RNA and proteins were removed by Rnase and Proteinase K treatment, proteins were extracted again with PCI and CI, and DNA was precipitated with 100% ethanol. Finally, DNA was resuspended in pH-stabilized PCR-grade water and concentration estimated photometrically at 260 nm.

PCR amplification

The small subunit rRNA genes of the selected algae were amplified using the universal eukaryotic primers 1F (5′-AAC CTG GTT GAT CCT GCC AGT A-3′) and 1528R (5′-GAT CCT TCT GCA GGT TCA CCT AC-3′) as described by Medlin *et al.* (Medlin *et al.*, 1988) without the polylinkers. Each 100-µL PCR reaction contained 10 µL of 10× reaction buffer [100 mM Tris (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$ and 0.01% gelatine; Applied Biosystems, USA,] 0.1 mM of each dNTP, 0.1 µM of each primer and 2.5–5 units of Ampli-Taq DNA polymerase (Applied Biosystems, USA). PCR reactions were prepared including the Taq polymerase and cooled on ice until placed in an Eppendorf Mastercycler Gradient (Eppendorf, Germany), with the block preheated to 94°C. After the initial denaturation (94°C, 5 min), 30 cycles of 94°C for 2 min, annealing at 56°C for 2 min and elongation at 72°C for 4 min were carried out, followed by a final extension at 72°C for 10 min and cooling to 4°C. The variable D1/D2 region of the large subunit rRNA of eukaryotes was amplified using the forward primer DIR-F and the reverse primer D2CR (Scholin *et al.*, 1994a) with initial denaturation (94°C, 1 min) followed by 30 cycles of 94°C for 1 min, annealing at 50°C for 2 min, and elongation at 72°C for 3 and 5 min and a final extension at 72°C for 7 min. Amplification products were cooled to 4°C and checked for appropriate length and purity by agarose gel electrophoresis.

Sequencing

PCR products for the D1/D2 region were purified using the QIAQuick PCR purification or the MiniElute Kit (Qiagen, Germany) following the manufacturer's instructions. Sequencing reactions were performed using the Big Dye Terminator Ready Reaction Mix (BigDye v. 3.0, Applied Biosystems), following the manufacturer's instructions and using the same primer sets as in the initial PCR. Approximately 10 ng of templates was added to each reaction mix and the annealing temperature set to 50°C. Amplification products were filled up to a volume of 20 µL with PCR-grade water, purified using Sephadex G-50 columns (Amersham Biosciences, Germany) into MultiScreen plates (Millipore) and desalted by gel filtration according to the manufacturer's instructions. Sequences were determined with a capillary sequencer (ABI Prism 3100 Genetic Analyzer, Applied Biosystems). All sequence outputs were checked manually and consensus sequences calculated using the DNAStar 4.05 software package SeqMan II (Lasergene).

Probe design

All probes were designed using the ARB software package (Ludwig *et al.*, 2004) and its sub-function 'probe design'. One probe for the species *P. parvum* was designed from the 28S rDNA database that included 230 sequences of eukaryotic organisms. The probe sequences suggested by the probe design tool were first compared with all sequences in the dataset, using the 'probe match' function implemented in ARB. The probe sequences with the highest number of mismatches to non-target sequences and a location of the mismatches in the centre of the probe sequence were further checked in GenBank by a BLAST sequence similarity search (http://www.ncbi.nlm.nih.gov/blast). Two additional probes targeting the 18S rDNA were used with the species-level probe for hierarchical hybridization experiments: a probe recognizing all Prymnesiophytes (Prym03, 5′-GTC AGG ATT CGG GCA ATT-3′, G. Eller *et al.*, unpublished data) and the genus-level probe PrymGl01A for *Prymnesium* (5′-TGC TCG CCA ACG AGG TGT-3′, G. Eller *et al.*, unpublished data). Both probes were hybridized for 2 h at 50°C and with 20 formamide in the hybridization buffer, or at 37°C with 35% formamide in the hybridization buffer for TAS-FISH, respectively.

Probe labelling and dot-blot hybridization

The oligonucleotide probes (MWG-Biotech, Germany) were labelled with digoxigenine (DIG) using a DIG Oligonucleotide Tailing Kit (Roche, Germany) according to the manufacturer's instructions. Labelled probes were mixed with dot-blot hybridization buffer to a final probe concentration of ~0.1 pmol mL^{-1}. The specificity of the probe was tested with PCR amplified 18S or 28S rDNA genes in dot-blot hybridizations as described in John *et al.* (John *et al.*, 2003b). In brief, PCR products were denatured and spotted onto positively charged nylon membranes (Roche, Germany), air-dried and cross-linked to the membrane with UV light. Membranes were pre-hybridized in hybridization buffer (5× SSC, 0.1% *N*-laurylsarcosine, 0.02% SDS and 1% blocking solution) without probe for 2–4 h at hybridization temperature, before the buffer was exchanged to probe–buffer mix and then hybridized overnight. Detection was carried out using the DIG detection kit and CSPD (Roche, Germany), following the manufacturer's instructions. Signals were detected by exposure to X-ray films from 15 min to 4 h.

FISH on membrane filters for microscopy

The protocol used for FISH with algal cells filtered onto polycarbonate membranes (Millipore) was used as described in Groben and Medlin (Groben and Medlin, 2005), modified from Miller and Scholin (Miller and

Table I: *Nucleotide accession numbers of Prymnesium parvum f. parvum and P. parvum f. patelliferum strains and taxonomic relatives used in this study*

Organisms	Strain code	Origin	Culture collection	Nucleotide accession numbers
P. parvum f. patelliferum	PLY527	Plymouth, England	PML	AJ876801
P. parvum f. parvum	K-0081	Flade Sø, Denmark	SCCAP	AJ876802
P. parvum f. parvum	RL10parv93	Bergen, Norway	Aud Larsen	AJ876803
P. parvum f. parvum	RL9parv93	Bergen	Aud Larsen	AJ876804
P. parvum f. patelliferum	RS2pat94	Bergen	Aud Larsen	AJ876794
P. parvum f. patelliferum	RLpat93	Bergen	Aud Larsen	AJ876795
P. parvum f. patelliferum	RHpat93	Bergen	Aud Larsen	AJ876796
P. parvum f. patelliferum	K-0252	Wilson Promontory, Norman Bay, Victoria Australia	SCCAP	AJ876797
P. nemamethecum	South Africa	St James False Bay, South Africa	S.A.	AJ876798
Chrysochromulina throndsenii	K11	Off Arendal, S Norway	UIO	AJ876799
Chrysochromulina campanulifera	J10	Skagerrak	UIO	AJ876800

Scholin, 1996, 2000) and Lim *et al.* (Lim *et al.*, 1996). For fixation, 10 mL of culture was filtered, covered with saline ethanol [25 mL of 100% ethanol, 2 mL of deionized water, 3 mL of 25× SET (3.75 M NaCl, 25 mM EDTA, 0.5 M Tris–HCl and pH 7.8)] and fixed for 1 h at room temperature. The fixative was filtered, precipitates were resolved by applying hybridization buffer [5× SET, 0.1 % (v/v) Nonidet-P40, no formamide added] at room temperature, and membranes were either used immediately for hybridization or stored at –20°C wrapped in foil.

Hybridization was carried out for 2 h at 50°C in a hybridization oven (Appligene, Germany) in humid chambers, with filters covered with hybridization buffer. The final probe concentration was 5 ng μL^{-1} in hybridization buffer, and the applied probes were labelled with fluorescein-5-isothiocyanate (FITC; Thermo Electron, Germany). After hybridization, the filters were washed in prewarmed 1× SET washing buffer at 50°C for 10 min, air-dried and either directly mounted onto a clean slide using a Citifluor/4′,6′-diamidino-2-phenylindoline (DAPI) mixture [0.5 mL of sterile deionized water, 1 mL of Citifluor (Citifluor, Cambridge) and 1.5 μL of DAPI solution (1 μg μL^{-1}, Invitrogen, Germany, in sterile deionized water)] or stained with DAPI for 10 min at room temperature, washed with sterile deionized water for 1 min, incubated in 80% ethanol for 30 s and air-dried before mounting onto slides with Citifluor. Coverslips were sealed with nail varnish, and slides were stored at –20°C or directly examined microscopically.

TSA-FISH in suspension for automated detection with SPC

To determine the filters best suited for FISH in combination with SPC, we compared black polycarbonate filter membranes (Chemfilter CBO4, Chemunex) with 0.2 μm pore size and 25 mm diameter with white polycarbonate filter membranes (Millipore) with 0.4 μm pore size and 25 mm diameter. The black filters proved unable to withstand the saline ethanol treatment during cell fixation because they were bleached. To be able to use both ethanol fixation and the black filter membranes, the fixation and FISH were carried out in suspension and the labelled cells filtered onto the respective membrane after fixation. For fixation, 10–20 mL of culture was added to 30 mL of saline ethanol (Miller and Scholin, 2000) and incubated for at least 1 h at room temperature or overnight at 4°C. Cells were concentrated by centrifugation at 3000–3500 × *g* at room temperature, using a swing out rotor without brake. The fixative was decanted, the pellet resuspended in 1.5 mL 1× SET and transferred to a 2-mL cup for hybridization in suspension.

The hybridization protocol used for suspended cells was modified after Scholin *et al.* (Scholin *et al.*, 1996) and Biegala *et al.* (Biegala *et al.*, 2003) and a tyramide signal amplification (TSA) was performed according to Schönhuber *et al.* (Schönhuber *et al.*, 1997, 1999) with slight modifications. All centrifugation steps were carried out in a microcentrifuge (Biofuge, Heraeus, Germany) at room temperature with 3000–3500 × *g* for 3–5 min. Cells were washed twice in 1× SET and resuspended in 150 μL of hybridization buffer, containing horseradish peroxidase (HRP)-labelled probe (Thermo Electron, Germany), final probe concentration in hybridization buffer: 5 ng μL^{-1} and higher formamide concentrations than used in the normal FISH protocol (Table II) because of the lowered hybridization temperature. For parallel hybridization with different probes, the

Table II: Specific dot-blot and fluorescence in situ hybridization (FISH) conditions and list of target and reference organisms used for validation of probe PRYM694

Probe name	Specific conditions	Target strains	Non-target strains
PRYM694 in dot blots	53°C, 1× SSC[a]	*P. parvum f. parvum* K-0081	*P. nemamethecum, P. calathiferum, P. annuliferum, P. mediterraneum, Chrysochromulina throndsenii, C. kappa*
		P. parvum f. *patelliferum* K-0082	*Chrysochromulina campanulifera, C. leadbeateri, C. scutellum, C. brevifilum, C. polylepis, C. acantha, C. ericina, C. herdlensis, C. minor, C. cymbium, C. ephippium, Platychrysis pigra, Isochrysis galbana, Imantonia rotunda, Pleurochrysis carterae, P. elongata, Emiliania huxleyi, Coccolithus pelagicus*
		P. parvum f. *patelliferum* K-0252	*P. nemamethecum*
		P. parvum f. *patelliferum* K-0374	*E. huxleyi*
		P. parvum f. *patelliferum* RLpat93	*C. ericina*
		P. parvum f. *patelliferum* RS2pat94	*Isochrysis galbana*
		P. parvum f. *parvum* RL10parv93	
PRYM694 in FISH	Standard-FISH: 50°C, 10% FA	*P. parvum* f. *parvum* K-0081	*Imantonia rotunda*
		P. parvum f. *patelliferum* K-0082	*P. carterae*
	TSA-FISH: 37°C, 20%FA	*P. parvum* f. *patelliferum* K-0252	*Ostreococcus tauri*
		P. parvum f. *patelliferum* K-0374	
		P. parvum f. *parvum* RL10parv93	

[a] For a specific signal, the washing of the membranes after the hybridization was done at hybridization temperature only, no room temperature step.

suspension was divided into triplicates. Hybridization was carried out in a thermoshaker at 37°C for 2 h and stopped by adding 100 μL of 1× SET prewarmed to 37°C and then centrifuged. The pellet was resuspended in 175 μL of 1× SET and washed for 5–10 min at 37°C with agitation, centrifuged and was additionally washed with 100 μL of TNT-buffer [0.1 M Tris–HCL, (pH 7.5), 0.15 M NaCl, 0.05% Tween-20] for 10 min at room temperature. For the TSA reaction, TNT-buffer was discarded by centrifugation, and cells were resuspended in 30 μL of a 1:50 mixture of fluorescein tyramide (TSA-direct Kit, Perkin Elmer, USA) and amplification diluent [2× amplification diluent (TSA-direct Kit, Perkin Elmer, USA)], diluted with 40% dextran sulphate (Sigma-Aldrich, Germany, w/v, in sterile deionized water) to reduce unspecific staining during signal amplification, incubated for 30 min at room temperature in the dark and afterwards centrifuged. The pellet was resuspended in 1 mL of TNT-buffer and washed twice at 55°C in prewarmed TNT-buffer in the dark for 5 and 15 min with agitation to remove unbound residual fluorescein tyramide. TNT-buffer was removed by centrifugation, and for counterstaining, cells were resuspended in 20 μL of Citifluor and 5 μL of DAPI solution (2 μg mL^{-1} in sterile deionized water) and filled to ~300 μL of 1× phosphate-buffered saline (PBS) (depending on the cell concentration) prior to filtration onto membranes for detection with the ChemScan. The cells can be kept at 4°C for several months without a loss of signal or analysed directly with the ChemScan.

TSA-FISH on membrane filters for automated detection with SPC

Filtration and fixation onto polycarbonate membrane filters (Millipore) 0.4 μm pore size, 25 mm diameter was conducted as described above. Prior to TSA-FISH endogenous peroxidase activity was reduced by a hydrogen peroxide treatment with 100 μL of 3% (v/v) H_2O_2 per filter for 15 min at room temperature according to Pougnard *et al.* (Pougnard *et al.*, 2002). Membrane filters were washed with sterile deionized water to remove the H_2O_2, incubated in 96% ethanol and air-dried. Hybridization was carried out at 37°C with the HRP-labelled probe (final probe concentration in hybridization buffer: 5 ng μL^{-1} with the adequate formamide concentration, for probe PRYM694 see Table II). After the last washing step of FISH, filters were rinsed in sterile deionized water and equilibrated for 10 min in TNT-buffer. One hundred microlitres of a 1:50 mixture of fluorescein tyramide and amplification diluent (set up as

described above) were applied per filter and incubated for 30 min at room temperature in the dark. Filters were rinsed in prewarmed TNT-buffer for 5 and 15 min at 55°C with agitation, rinsed with sterile deionized water, air-dried and counterstained with DAPI as described above.

Preparation of environmental samples

Samples of a *P. parvum* bloom from Lake Colorado (TX, USA) were kindly provided by Carmelo Tomas (University of North Carolina, USA) and Joan Glass (Texas Parks and Wildlife Department, USA). Samples were fixed in 4% formaldehyde on site and stored at 4°C. Prior to FISH, samples were fixed with saline ethanol as described above to ascertain a better probe penetration and to reduce autofluorescence. After TSA-FISH in suspension, 100 μL to 1 mL of sample was filtered onto white polycarbonate filters (0.4 μm pore size, 25 mm diameter, Millipore).

Preparation of spiked samples

A workshop designed to calibrate molecular techniques against traditional light microscopic counting methods was held in Kristineberg Marine Research Station, Sweden from 22 to 26 August 2005. Eight different molecular methods were compared against nine traditional counting methods (Godhe *et al.*, in press). Laboratory cultures of the target species *A. fundyense* were counted with a Sedgewick rafter cell chamber and then inoculated to seawater samples with and without an environmental background matrix in four single-blind tests. In the first experiment, *A. fundyense* was added in a dilution series to the samples to determine the limits of detection and dynamic range of each different method. In the second experiment, the ability to discriminate the closely related species *A. fundyense* and *A. ostenfeldii*, which co-occur in many locations, was tested. In the third and fourth experiments, the accuracy of each counting method was examined when the target organism was in the presence of different amounts of other phytoplankton and detritus (i.e. matrix effects). In the third experiment, the target inoculum was held constant in increasing concentrations of the environmental matrix, whereas in the fourth experiment, the amount of the environmental matrix was held constant and the cell inoculum was increased. We used the probe ATNA02 (5′-AAC ACT CCC ACC AAG CAA-3′; John *et al.*, 2005) recognizing the North American *A. tamarense* species complex to detect the *A. fundyense* cells with 40% formamide in the hybridization buffer as described above.

SPC

A ChemScan RDI (Chemunex) was used for SPC. An overlapping scan of the whole filter membrane surface was carried out with a 40-mW water-cooled argon laser at a wavelength of 488 nm and therefore suited to detect probes or tyramides, which are labelled with FITC. Signals were detected by three photomultipliers (Bauer *et al.*, 1996), with wavelength windows set for green (500–530 nm), green-yellow (540–570 nm) and yellow-amber (570–585 nm) regions of the light spectrum. A set of discrimination criteria allowed the differentiation between autofluorescent particles, unlabelled cells and labelled target cells (Roubin *et al.*, 2002). This discrimination was based on optical characteristics like particle size and signal shape (Bauer *et al.*, 1996) and was carried out automatically by the computer software. Positive signals were analysed with MatLab software (Matworks, Natick, MA, USA), which compares Gaussian curves and removes non-Gaussian signals, often generated by autofluorescent non-target microalgae or autofluorescent particles (Pougnard *et al.*, 2002).

The software allowed a comparison of the scan results before and after the application of the automated discrimination step on a representation of the filter on the computer screen, termed a scan map. The signals on the scan map were microscopically validated by epifluorescence microscopy after the transfer of the filter to the motorized stage of the microscope, which was connected to the ChemScan (Pougnard *et al.*, 2002; Roubin *et al.*, 2002). Cursor highlighting of a signal on the scan map on the computer screen then moves the motorized stage to the corresponding position on the filter for validation of each event (Roubin *et al.*, 2002). The function of the ChemScan system was controlled with 100 μL of Standard C3 solution (FITC-labelled latex beads, diameter 2–3 μm, Chemunex) filtered onto black polycarbonate filters (Chemunex). As a support for the filters, a black support pad was mounted with 100 μL of ChemSol B16 (both Chemunex) on the membrane filter holder. After FISH, membrane filters were analysed directly or stored at –20°C until analysis. Immediately after the scan, signals were validated using an epifluorescence microscope (Nikon, Eclipse E 800) equipped with filter blocks for FITC (Nikon Filter Block B-2A), DAPI (Nikon Filter Block UV-2A) and a motorized stage (Prior Scientific, UK). Images were captured with a digital camera (CCD-1300CB,

Vosskühler, Germany) and analysed with the Nikon software Lucia G.

RESULTS

Probe design and validation

For specific detection of *P. parvum,* the variability of the 18S rRNA gene was insufficient. Repeated probe design with the 18S rDNA dataset and dot-blot tests of the resulting oligonucleotides revealed that it was not possible to distinguish *P. parvum* from its next relative, the non-toxic *P. nemamethecum* using the 18S rRNA gene. Therefore, the more variable D1/D2 region of the 28S rDNA gene was sequenced from different *P. parvum* f. *parvum* and *P. parvum* f. *patelliferum* strains and their taxonomic relatives. Probes were designed from this ribosomal region. From the resulting oligonucleotide list of 51 probes, probe PRYM694 with the sequence 5′-CAG CCG ACG CCG AGC GCG-3′ was chosen for further evaluation.

The specificity of this probe was tested by dot-blot and whole-cell hybridization assays, choosing closest taxonomic relatives to the target group and organisms with the lowest number of mismatches to the probe sequence as non-target references. Conditions were assumed to be specific if only the target cells had a positive signal and if the signal intensity of the target cells was decreased by using higher stringency conditions (Fig. 1). Probe PRYM694 was specific to *P. parvum* in dot-blot and whole-cell hybridization at the conditions listed in Table II.

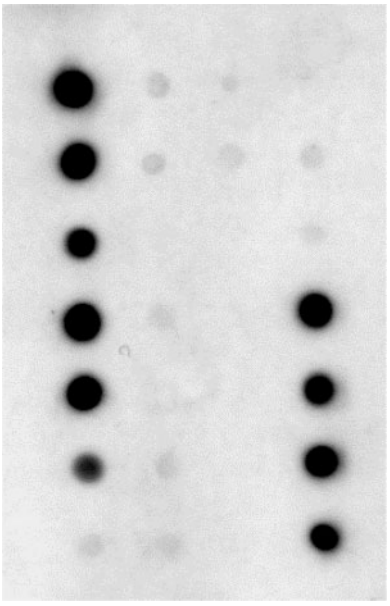

Fig. 1. Specificity tests of the probe PRYM694 recognizing *P. parvum* in dot blot hybridization with PCR amplified 28S rDNA of different target and non-target microalgae. A1: *Prymnesium parvum* f. *parvum* RL10parv93 A2: *P. parvum* f. *parvum* K-0081, A3: *P. parvum* f. *patelliferum* K-0082, A4: *P. parvum* f. *patelliferum* K-0252, A5: *P. parvum* f. *patelliferum* RLpat93, A6: *P. parvum* f. *patelliferum* RS2pat94, A7: *P. nemamethecum*, B1: *Prymnesium* sp. HAP52bis (Algo), B2: *P. faveolatum,* B3: *P. calathiferum*, B4: *P. zebrinium*, B5: *P. annuliferum*, B6: *Prymnesium sp.* HAP_Pt (Algo), B7: *Chrysochromulina camella,* C1: *C. ephippium,* C2: *C. polylepis,* C3: *C. kappa,* C4: *C. ericina,* C5: *C. herdlensis,* C6: *C. minor,* C7: *Chrysotila lamellosa* HAP 17 (Algo), D1: *Pleurochrysis carterae,* D2: *P. elongata,* D3: *Platychrysis pigra,* D4: *P. parvum* f. *parvum* K-0081, D5: *P. parvum* f. *patelliferum* K-0082, D6: *P. parvum* f. *patelliferum* K-0374, D7: *P. parvum* f. *parvum* RL10parv93.

Adaptation of FISH procedure for detection with the solid-phase cytometer

The manufacturer of the ChemScan machine recommends the use of a black membrane filter. Our initial results showed that our standard saline ethanol fixation directly on the black polycarbonate filters caused sufficient bleaching of the black filter colour to increase background fluorescence. We then compared the use of black polycarbonate (0.2 μm pore size, 25 mm diameter, Chemunex) and white polycarbonate membrane filters (0.2 μm pore size, 25 mm diameter, Millipore) for the detection with the ChemScan cytometer, and conducted fixation with saline ethanol in suspension rather than directly on the filters. After the fixation step, cells were washed in PBS and hybridized in suspension, and then the cells were filtered onto the black or the white membrane filters. This allowed the use of saline ethanol as a fixative without damaging the black filters especially adapted to fit into the filter holder. However, it turned out that white filters were superior to the black Chemunex filters in our experiments, because FISH signals were visibly stronger against the white membranes with less background when viewed under epifluorescence microscopy. Additionally, when working in suspension, cells were lost in centrifugation steps and more debris, for example from broken cells accumulated in these samples, increasing the number of fluorescing particles in the sample. This often led to an abort of the scan by the computer because of too many fluorescent events on the filter. Using the white filters, we could collect the cells directly onto the filter, fix them and then hybridize with the probes. This combination afforded us the best signal and the minimal cell loss. In addition to the tests for the best-suited fixation method and filter membrane for the ChemScan, it had to be verified if the signal intensity of labelled cells was sufficient to be detected by the ChemScan and to determine the peak intensities of the fluorescence signals. We found that the fluorescence signals of standard FITC-labelled cells were generally too weak to adjust the discrimination settings to

distinguish positively labelled cells from fluorescing particles and autofluorescent cells (data not shown). However, cells labelled with HRP probes by FISH and amplified using TSA were easily detected with a ChemScan solid-phase cytometer.

The computer program supplied with the ChemScan calculates the signal ratios of the positive signals detected at the three wavelength intervals. Thereby, the oligonucleotide probe-labelled cells can automatically be distinguished from particles or autofluorescent cells, if the program parameters are set correctly. The negative controls (no probe, no probe together with the fluorescein-labelled tyramide and hybridization with a probe that does not react with the target species) always gave low mean peak intensity (PI, green fluorescence intensity) values of <400. Another discrimination parameter is based on the ratio of the total fluorescent light detected in the orange/red channel (secondary channel, S, signals derived from autofluorescent particles), divided by the total fluorescent light (green fluorescence generated by the bound probe in the primary channel, P) detected in the green channel. This S/P-value was above 1 with negative controls, and the expected red/green value for positive labelled cells is in the order of 0.7.

Using the cell size and general shape of the hybridized microalgae *P. parvum* (8–11 μm long and 4–6 μm wide, cell shape from elongate, pyriform to almost spherical, Green *et al.*, 1982; Moestrup and Thomsen, 2003) and *A. fundyense,* (normally between 27 and 37 μm long, width varies with the length; Balech, 1995), we judged whether the positive signals recognized by the ChemScan were accurate. Because of the high PI of TSA-FISH labelled cells, the ranges for the PI and S/P discriminants were set at 1000–20 000 and 0.2–0.9, respectively, to distinguish labelled from non-labelled cells or autofluorescent particles. In addition to these two parameters, the number of lines that the laser passed through a cell was also used as discriminants (of size) to eliminate autofluorescent particles, which often was much smaller in size than positive algal cells. If there are too many positive signals, the memory capacity of the computer presently supplied with the ChemScan is exceeded and the scanning of the filter is aborted. However, it is possible to extrapolate to total cell counts from a partial, aborted scan.

Detection limits achieved with the ChemScan

Using the staining procedure and the configuration given above, we achieved detection limits of 1 cell per filter when scanning diluted pure cultures. In mixtures of different proportions of target and non-target cells, both total and partial cell numbers were redetected with high reproducibility. By defining the parameters for automated analysis according to the cell morphology of the target organism, the time needed for microscopic verification of the automated counting could be reduced remarkably. With these settings, particles of the wrong size and/or exhibiting autofluorescence were excluded from the automated counts.

Automated detection of target cells in environmental samples with the ChemScan

Target cells were detected in an environmental sample of a *Prymnesium* bloom from Lake Colorado, using our three hierarchical probes: (i) the class-level probe, Prym03, recognizing all Prymnesiophytes (G. Eller *et al.*, unpublished data) (ii) the genus-level probe PrymGl01A for *Prymnesium* (G. Eller *et al.*, unpublished data) and (iii) the species-specific probe PRYM694.

Repeated cell counts with the ChemScan gave reliable results, which were validated microscopically. Hybridization with HRP-labelled probes resulted in a bright signal, easily detectable with the ChemScan (Fig. 2A-H). Negative controls show no green fluorescence signal and were not counted by the ChemScan (Fig. 2I-J). Cell counts of *P. parvum* were 2–8 $\times$ 10^3 cells mL^{-1} with the ChemScan machine and were somewhat lower than the light microscopical cell counts of 6–9 $\times$ 10^3 cells mL^{-1} of the same samples (Carmelo Tomas, personal communication). Nevertheless, the microscopic validation showed that the ChemScan counted only the brightly labelled *P. parvum* cells. This made validation very easy, because after testing different positive signals on different areas on the filter and an evaluation if the positive signals are indeed the target cells, it was not necessary to recheck all positive signals. However, this is only the case, when bright and clear FISH signals are present.

Automated detection of target cells in spiked samples with the ChemScan

Intercalibration workshop

The complete cell counts are summarized in Table III. The cell counts of experiment 1 were close to the inoculated cells, except for the first sample. In this case, the SPC counted more positive cells than inoculated cells were given to the sample. On the first day, we did not have the possibility to validate the positively counted cells because there was no connected motorized microscope present; thus, it is logical that without validation of the results, it is likely that the SPC overestimated cell numbers. The cell counts of experiment 2 consistently underestimated the inoculated cells but showed an increasing trend with the inoculated cells. The cell counts of

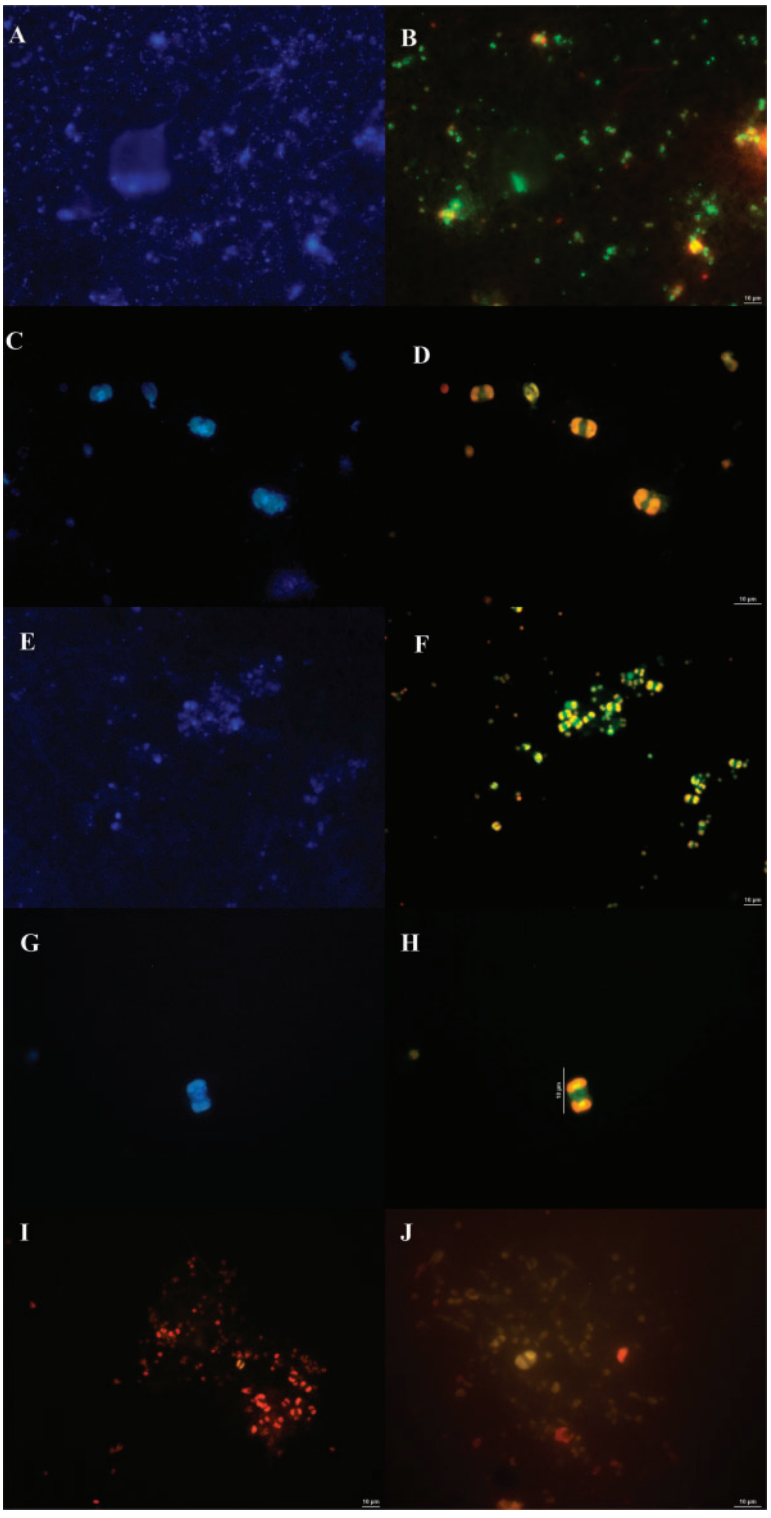

Fig. 2. Whole-cell hybridization of environmental samples from Lake Colorado, TX, USA, hybridized with different hierarchical probes. (**A**) 4′,6′-Diamidino-2-phenylindoline (DAPI) counterstain. (**B**) Environmental sample hybridized with Prym03 (×40). (**C**) DAPI counterstain. (**D**) Environmental sample hybridized with PrymGl10a, horseradish peroxidase (HRP)-labelled and tyramide signal amplification (TSA) (×60). (**E**) DAPI counterstain. (**F**) Environmental sample hybridized with PRYM694, HRP-labelled and TSA (×40). (**G**) DAPI counterstain. (**H**) Environmental sample hybridized with PRYM694, HRP-labelled and TSA (×100). Negative controls: (**I**) Environmental sample hybridized without a probe and addition of the fluorescein tyramide (×40). (**J**) Environmental sample hybridized without a probe (×100).

Table III: Alexandrium fundyense cell numbers detected in spiked samples with solid-phase cytometry (SPC) in combination with tyramide signal amplification-fluorescence in situ hybridization (TSA-FISH)

	Day 1		Day 2		Day 3		Day 4	
	Mean and SD of three SPC counts	Inoculum for the single-blind test	Mean and SD of three SPC counts	Inoculum for the single-blind test	Mean and SD of three SPC counts	Inoculum for the single-blind test	Mean and SD of three SPC counts	Inoculum for the single-blind test
Sample 1	1100 ± 624	100	164 ± 67	1000	300 ± 200	700	1555 ± 1667	500
Sample 2	633 ± 252	500	267 ± 177	3000	800 ± 361	700	4222[b] ± 6332	5000
Sample 3	1100[a]	1000	547 ± 284	7000	733 ± 603	700	15 022[b] ± 4991	25 000
Sample 4	11 933[b] ± 8860	10 000	690 ± 213	9000	667 ± 416	700	11 422[b] ± 7018	100 000

[a] Only one sample was counted because of filter problems

[b] Samples for which the final cell concentration had to be extrapolated because of an aborted scan.

experiment 3 more or less showed a consistency over the four inocula and were sometimes very close to the inoculated cell concentrations (samples 2, 3 and 4). The cell counts of experiment 4 again underestimated the inoculated cells, except in sample 1, which overestimated cell numbers.

DISCUSSION

FISH and SPC

Larsen and Medlin (Larsen and Medlin, 1997) showed with two molecular markers that *P. parvum* and *P. patelliferum* were genetically identical, and it was concluded that the two taxa were the same species. Further investigations into ploidy states of the two species were performed using flow cytometry, and *P. patelliferum* was found to be haploid and *P. parvum* to be diploid or haploid. Therefore, it was assumed that these two taxa belong to the same haplo-diploid life cycle (Larsen and Edvardsen, 1998) and because *P. parvum* is the oldest species name for this species, *Prymnesium patelliferum* was relegated to a form of *P. parvum*, *P. parvum* forma *patelliferum* (Larsen, 1999). Therefore, a single probe was designed to target a single species *P. parvum* with its two varieties, both of which are toxic.

Probe RYM694 was shown to be specific to this species in dot-blot hybridization and FISH and was successfully used for the automated counting by SPC. The beneficial use of fluorescence labelling (FISH) of marine microalgae for a rapid detection has been reported several times (Scholin *et al.*, 1994b, 1996; Knauber *et al.*, 1996; Simon *et al.*, 1997; Tyrrell *et al.*, 2001; John *et al.*, 2003b, 2005; Groben *et al.*, 2004; Groben and Medlin, 2005). However, the combination of FISH with epifluorescence microscopy limits the number of samples to be analysed and the accuracy of counting at low cell densities. For a high sample throughput, FISH has been combined with flow cytometry (Simon *et al.*, 1995; Rice *et al.*, 1997; Marie *et al.*, 2000, 2005; Biegala *et al.*, 2003), which allows the analysis of different cell parameters. Nevertheless, a combination of flow cytometry and light microscopy of the single particles detected is difficult, because particles have to be sorted and examined afterwards microscopically. The solid-phase cytometer has the advantage of a direct combination of automated counting and epifluorescence microscopy, allowing the microscopic verification of each single cell detected. A comparison of the detection of bacteria by microscopy, flow cytometry, and SPC showed that the SPC is merely useful at low cell densities (Lemarchand *et al.*, 2001). West *et al.* (West *et al.*, 2006) successfully used this device for the detection of *P. parvum* labelled with fluorescent monoclonal antibodies. However, to our knowledge, the ChemScan has not been used in combination with FISH for the detection and enumeration of microalgae previously.

The effectiveness of our TSA-FISH technique in combination with SPC was investigated in the intercalibration workshop in Sweden in 2005, where molecular and traditional methods for concentrating, preserving and counting HAB species were compared (Godhe *et al.*, in press). The target species, the toxic *A. fundyense* (strain CA28), is widespread in North America and in the Orkney Islands (UK) and is responsible for seasonal HABs in these regions. There are few morphological differences between *A. fundyense* and other *Alexandrium* species (Balech, 1995), and therefore, this species was a good choice for this comparative workshop. In general, our results underestimated cell numbers, but in each experiment our counts, albeit lower, followed the same trend as the inoculum. We could have encountered cell loss because we washed our filters and performed the TSA steps with the filters removed from the filter manifold and placed into a small washing container. Up to 30% cell loss has been noted where an analysis of the washing solution has been undertaken (Cembella, personal communication). This potential source for cell loss can be avoided in future by performing all of the hybridization steps including the washes on the filter manifold. A more detailed comparison of washing techniques on and off the filter manifold should be undertaken to assess accurately this potential cell loss. Overestimation on the first day can be explained because we were unable to verify our positive signals. An overestimation can happen if false-positive particles are counted by the SPC, especially when a microscopic validation is not performed and/or the filter material is not suited for FISH and SPC. In addition, it must be accepted that there will be a natural variability in the inoculums in the single-blind test and that the true inocula is likely to be an average of all methods. The underestimation of some of the highest counts could be the result of the abortion of the scan by the computer and having to extrapolate to total numbers from partial counts. Field samples also contained a high amount of fluorescing non-positive particles, which overloaded the computer memory. It must be remembered that the ChemScan was initially developed for counting rare events in microbiological applications and was not designed for counting large numbers of positive signals. If cells are not evenly distributed over the filter, our extrapolation will also be in error. However, this problem can easily be solved with a more powerful computer system to record all of the signals. Because of these computer problems, we sometimes filtered only a small amount of the given samples, which could also lead to a higher or

lower cell distribution on the filters, even if the samples were well mixed before filtration.

Examination of formaldehyde fixed environmental samples of a *Prymnesium* bloom showed differences in cell counts between traditional methods and quantification with the ChemScan method. A reason for the difference in cell numbers could be the sample fixation method, which may have influenced cell numbers. Lower cell numbers for *P. parvum* were also found in the formaldehyde-fixed samples with antibody labelling followed by ChemScan detection in samples of these *Prymnesium* bloom, whereas glutaraldehyde-fixed samples of this bloom and antibody labelling followed by ChemScan detection were closer to the cell counts by traditional methods (West *et al.*, 2006). However, glutaraldehyde fixation in combination with FISH detection is not possible because of the high autofluorescence of glutaraldehyde. Fixation with Lugols iodine is also possible for later FISH probing (Töbe *et al.*, 2001), but this method was not used here.

Besides the differences in the final cell numbers, the fixation with formaldehyde could cause problems for the detection by FISH together with SPC, because our FISH protocol works best with initial saline ethanol fixation. Additionally, cell loss during washing stages is also likely as mentioned. Nevertheless, FISH signals were clearly visible even in the environmental samples fixed with formaldehyde and counted by the ChemScan. Consequently, this result proved that the counting of microalgae in environmental samples is possible with this solid-phase cytometer. Our preliminary results suggest that SPC has the potential to become a rapid semi-automated detection and enumeration method of spherical (e.g. *Alexandrium*) and pyriform (e.g. *Prymnesium*) microalgal cells.

Additionally, it is necessary to modify and optimize the available software of the SPC for microalgal applications. It will be better to have more possibilities to change the discrimination patterns and to include more information such as cell size and morphological features such as cell shape. So far, we were able only to use the existing parameters provided by the program, which uses the fluorescing intensity of the cells as a discrimination parameter, but not the cell size or shape. In collaboration with the company Chemunex, we will work on developing new discriminating parameters for counting microalgae. After resolving these problems, this new detecting system should be a powerful tool for counting microalgae and discriminating closely related morphological identical microalgae in an easy-and-fast way without the need of a highly trained person. Specific oligonucleotide probes are now available for nearly every HAB species, and it is only matter of time until these probes can be incorporated into a fully automated monitoring scheme. The protocols that we have presented are only one possibility for routine monitoring. The ChemScan will always be most useful for the detection of rare events and, in the case of HABs, the detection of a potential bloom.

Benefits and limitations of the ChemScan detection method

The SPC in combination with TSA-FISH enables the efficient detection of a single cell in a filtered volume in <5 h, whereas the time involved to count an environmental sample will vary with the diversity of the sample and the skill of the operator. All traditional counting methods using the Ütermohl method involve an overnight sedimentation, thus making total time for traditional methods over 24 h.

The detection limit of positively labelled target cells is one cell per filter. However, FISH in suspension is not suitable for quantitative analyses with the ChemScan system because of the high cell loss and the resulting above-average fluorescing particles. It is preferable to use white membrane filters instead of the specially designed black filters for the ChemScan to allow fixation and probe application directly on the filter. Cell concentration in a microcentricon tube could be a useful alternative, but this has not been tested.

For a reliable automated detection of target cells with the ChemScan, a signal amplification was necessary. This was achieved by using the Tyramide Signal Amplification system, TSA, which enhances the fluorescent signal of hybridized cells up to 20 times (Schönhuber *et al.*, 1997) and was proven to be very useful in the detection of cyanobacteria (Schönhuber *et al.*, 1999; West *et al.*, 2001), picoplankton cells (Not *et al.*, 2002, 2004; Biegala *et al.*, 2003) and bacteria associated with microalgae (Alverca *et al.*, 2002; Biegala *et al.*, 2002). With TSA-FISH, discrimination settings of the ChemScan system could be set for a successful differentiation of target and non-target cells and autofluorescent particles. The threshold for the fluorescence intensity (PI) could be increased, because the TSA-FISH-labelled cells had very high PI. This was mostly not possible with standard FISH- and FITC-labelled cells because of their low PI.

The detection and enumeration with this new detection method of TSA-FISH-labelled microalgae has a great potential for their application in analysing field material, where fast and reliable detection and enumeration of toxic species is of great importance as in toxic algal monitoring programs. However, the shape of the

algae could cause problems, for example the ChemScan cannot count long filamentous chain-forming cells like *Pseudo-nitzschia* cells (data not shown) at present. The adaptation of improved software could help overcome this problem. Additionally, the actual high price of this machine and the additional costs of an epifluorescence microscope and other required equipment is a limiting factor. An additional time limiting factor is the actual need for manual validation of the ChemScan results to determine whether the system counted only positive target cells. However, in most cases, it is sufficient to recheck only a representative part of the filter to determine the quality of the FISH-labelled positively counted cells. Thus, the combination of specific cell labelling by TSA-FISH, automated detection by the ChemScan and verification of the results by epifluorescence microscopy allows to achieve the high sample throughput and low detection limit needed for monitoring harmful marine microalgae in aquatic ecosystems. In combination with probes developed for other taxonomic groups of microalgae, even studies of population dynamics and ecological significance are possible.

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