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MOLECULAR PROBES IMPROVE THE TAXONOMIC RESOLUTION OF CRYPTOPHYTE ABUNDANCE IN ARCACHON BAY, FRANCE

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RRNA PROBES
CHEMSCAN
CRYPTOPHYTE
TIME SERIES MONITORING

ABSTRACT. – The division Cryptophyta, Class Cryptophyceae, contains ecologically important microalgae that are found in all aquatic habitats. The identification of the Cryptophyta is challenged by a need to examine species in the scanning electron microscope or transmission electron microscope (SEM or TEM) to visualise features needed to identify species. Thus, for routine monitoring programs, this group is not usually identified beyond the level of class and that is done only if the samples are routinely examined by the optic Utermöhl method. Here we present an analysis of field samples taken from Arcachon Bay taken in summer 2006 and analysed with probes made for different clades within the Cryptophyta and counted with a solid phase cytometer, the ChemScan. Our results show that the molecular counts were lower than cell counts made by optic method and this is likely because of cell loss from delay in processing the samples and cell rupture from fixation. Nevertheless, clade 3, composed of the genera *Rhinomonas*, *Rhodomonas*, *Pyrenomonas* and *Storeatula,* were the most numerous in Arcachon Bay in 2006.

INTRODUCTION

Cryptophyceae are important members of the nanoplankton (5-20 μm). There are also picoplanktonic members of the group (Medlin *et al*. 2004). Because they contain phycobilin pigments, they can easily be detected as a group by orange fluorescence coupled with their characteristic shape. However, identification of species requires electron microscopic examination and many species do not preserve well for electron microscopy (EM). In a survey of cryptomonads in the Tyrrhenian Sea, Cerino & Zingone (2006) were able to identify cryptomonads using EM and thus could trace their abundance on a seasonal basis. However, such studies are rare because of the labour intensive EM methods used for species identification. In most time series sites, they are either ignored as a group (Wiltshire & Dürselen 2004) or counted at the class level based on their orange fluorescence. Thus, they are an ideal group for investigation using molecular methods. In the past decade, molecular probe-based methods have proved successful in improving both efficiency and accuracy of the identification of micro-organisms, especially those that are devoid of distinct morphological features or difficult to examine without electron microscopy. Hoef-Emden *et al*. (2002), Hoef-Emden & Melkonian (2003), and Marin *et al*. (1998) have constructed a molecular phylogeny of the group using 18S rDNA markers and have recovered 6 distinct molecular clades, which correspond more or less to families in the Cryptophyceae, although it is clear that a taxonomic revision is necessary (Clay *et al*. 1999, Fig. 1). Metfies & Medlin (2007) demonstrated the effectiveness of molecular probes to identify these clades within the Cryptophyceae. A first approach using hierarchical probes developed at the clade level, which most likely corresponds to the taxonomic level of family or order in the group was successful with one probe for each clade except that one probe (probe 4-6) recognised two of the molecular clades in the phylogenetic tree. These workers presented specificity tests for the probes and an initial application of the probes to field material. In this paper, we apply the probes to field material collected from Arcachon Bay using a Fluorescence in-situ hybridisation with tyramide signal amplification (FISH-TSA) probe detection method with cells being counted by the automated cytometer, the ChemScan, and compared these molecular-based counts to counts made by traditional light microscopy.

MATERIAL AND METHODS

Collecting site: In Arcachon Bay there are two sites that are part of their regular phytoplankton monitoring for toxic algae by the French monitoring network REPHY, IFREMER, LER-Arcachon (Fig. 2). One site, termed Buoy 7, is located just at the mouth of the Bay and is influenced by oceanic waters and the other site, termed Teychan, is located directly in front of the town of Arcachon inside the bay and is influenced by continental nearshore waters that enter the bay. In this study we used samples taken by IFREMER from Teychan from March to August 2006 with the exception of those from June, which were no longer available.

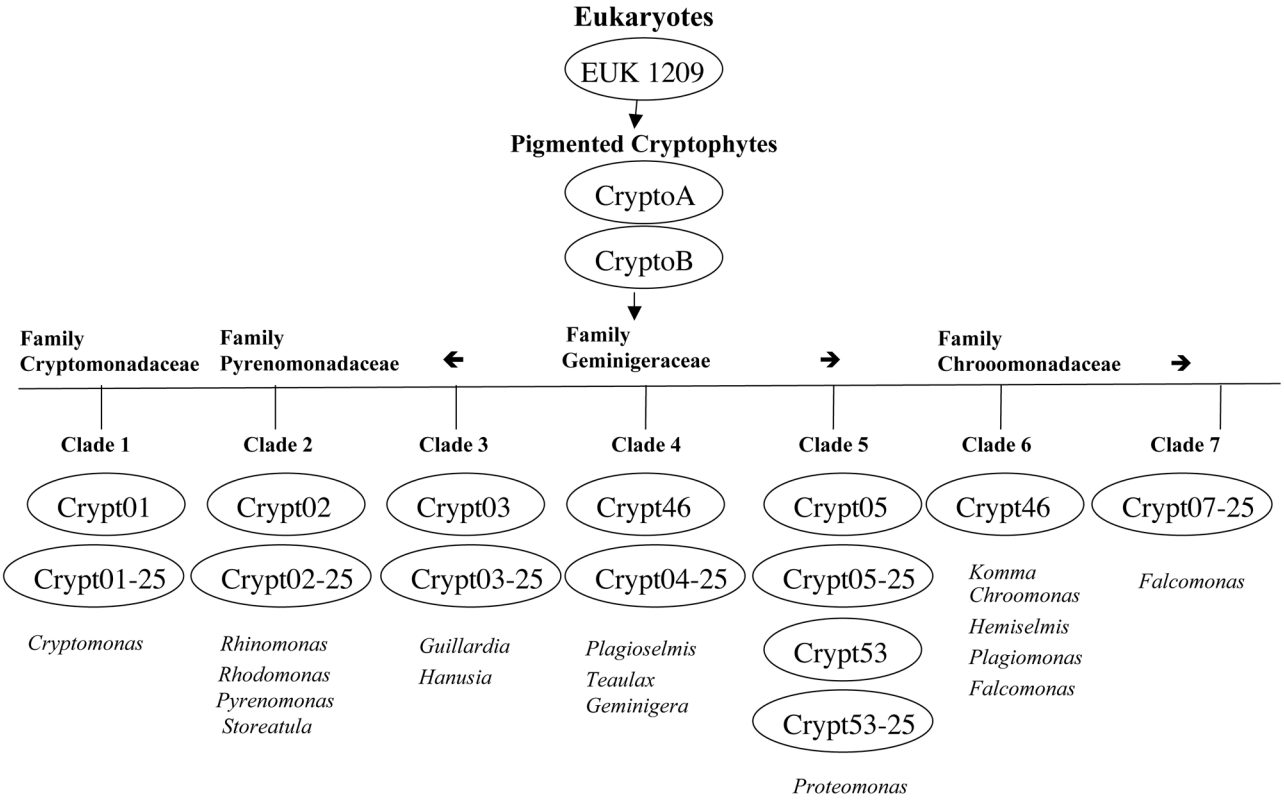


Fig. 1. – Schematic diagram showing the specificity of the probes relative to the phylogenetic tree of the Cryptomonads. Some families are paraphyletic spanning two or more clades.

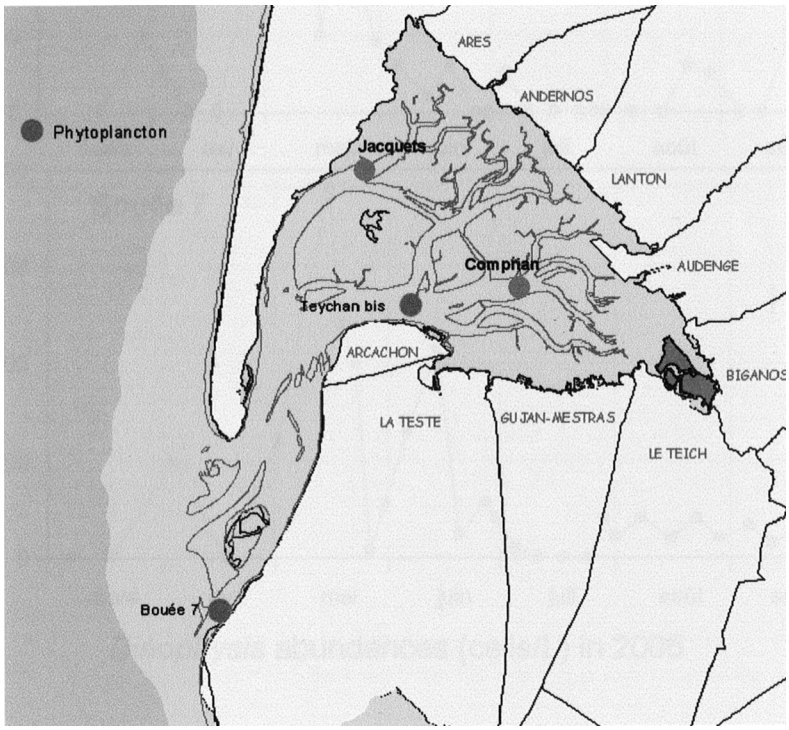


Fig. 2. – Map of Arcachon Bay showing the two sampling sites that are part of the IFREMER monitoring scheme, courtesy of IFREMER.

Traditional Counts by Light Microscopy: A one liter sample was collected at regular intervals at Teychan and fixed with Lugol's fixative. Ten mLs was allowed to settle overnight and used for counting by the optic Utermöhl method (Utermöhl 1958) across one diameter of the settling chamber at 40X. Counts on each sample can be either a partial flora where toxic, harmful or doubtful species and phytoplanktonic blooms observed in concentration higher than 10 000 cells/L were identified and enumerated or a total flora, where everything in the chamber is counted and identified. In the total flora counts, cryptomonads were counted as a class. Total flora counts are irregular because the main goal is to monitor for selected toxic species. Water sampling, identification and counting of phytoplankton were realized by Nadine Masson-Neaud and Myriam Rumèbe from the Laboratoire Environnement Ressources d'Arcachon and were provided by IFREMER. 50 mLs of the original sample were provided for molecular analysis.

Probe Hybridisation: Samples were probed with the clade and class level probes as shown in Table I. 10 mLs of the fixed Lugol's fixed sample were filtered on a 0.2 mm white polycarbonate filter and fixed overnight at 4°C with 2 mls of 2 % paraformaldehyde to allow permeabilisation of the cells (Töbe *et*

Table I. – Probe sequences used in this study.

Probe	Clade according to Marin *et al*. 1998	Clade according to Hoef-Emden *et al*. 2002	Oligonucleotide sequence [5'-3']
CryptoB	n/a	n/a	ACGGCCCCAACTGTCCCT
Crypt01-3	I	CRYP	TCATTACCCCAGTCCCAT
Crypt02 Crypt02-25	II	RHO	GTCCCACTACCCTACAGT CATTACCCCAGTCCCATAACCAACG
Crypt03	III	PROT	TTCCCGCGCACCACGGTT
Crypt4-6	VI	CHRO	CAAGGTCGGCTTTGAATC
Crypt5-3	V	PROT	GTCCCAACGCCCCTCAGT

al. 2001). The filters were rinsed with deionised water and then fixed with saline ethanol (25 mL 100 % ethanol, 2 mL deionized water, 3 mL 25 x SET [3.75 M NaCl, 25 mM EDTA, 0.5 M Tris/HCl, pH7.8] for 1 hour (Töbe *et al*. 2009) to remove the chlorophylls. Prior to TSA-FISH, endogenous peroxidase activity was reduced by a hydrogen peroxide treatment with 100 μL 3 % (v/v) H_2O_2 per filter for 15 min at room temperature. 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 (5 x SET, 0.1 % (v/v) Nonidet-P40, 20 % formamide). The final probe concentration was 5 ng μL^{-1} in the hybridization buffer. Two probes were added to each hybridisation reaction. A clade level probe carried a horseradish peroxidase (HRP) label for the TSA enhancement (Töbe *et al*. 2006) whereas a class level probe CryptoB, carried a Cy5 label (Thermo Electron, Germany). One filter was used for each clade level probe/class level probe combination. Thus, each sample was hybridised with 5 different clade/class probe combinations.

The probes were processed for hybridization to their target molecule using a TSA-FITC labelled enhancement as required for examination with the solid phase cytometer, the ChemScan (Töbe *et al*. 2006). Hybridization was stopped by adding 100 μL 1 x SET prewarmed to 50°C to each filter. Filters were rinsed in sterile deionized water and equilibrated for 10 minutes in 100 μL of TNT-Buffer (0.1 M Tris-HCL, [pH 7.5], 0.15 M NaCl, 0.05 % Tween 20) at room temperature. One hundred μL of a 1:50 mixture 1:50 mixture of fluorescein tyramide (TSA-direct Kit, NEN Life Science Product Inc., Boston, USA) and amplification diluent (2 x amplification diluent [TSA-direct Kit, NEN Life Science Product Inc., Boston, USA], diluted with 40 % dextran sulphate [w/v, in sterile deionized water]) to reduce unspecific staining during signal amplification was added to the filter, incubated for 30 mn at room temperature in the dark. The filters were washed twice at 55°C in pre-warmed TNT–buffer in the dark for 5 and 15 mn with agitation to remove unbound residual fluorescein tyramide. Cells were counterstained by adding 20 μL Citifluor and 5 μL DAPI solution (2 μg mL^{-1} in sterile deionized water) and air-dried. Each filter was then scanned with the ChemScan. A set of discrimination criteria were applied to the scanned filter that allowed the differentiation between autofluorescent particles, unlabelled cells and labelled target cells (Roubin *et al*. 2002). This discrimination was based on optical characteristics, such as particle size and signal shape (Bauer *et al*. 1996) and was carried out automatically by the ChemScan software. Positive signals were analyzed with MatLab software (Matworks, Natick, Mass.), which compared Gausssian curves and removed 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. Each signal on the scan map was microscopically validated by epifluorescence microscopy after the transfer of the filter to the motorized stage of the microscope, which was connected to the Chem*Scan* (Roubin *et al*. 2002, Pougnard *et al*. 2002). A positive count was recorded if there was both a TSA-enhanced FITC label and a Cy5 label on the cell (Fig. 3).

RESULTS

Manual Counts

The manual counts for the Cryptophyceae are presented in Fig. 4. Basically, the cells are present in Arcachon Bay year round, with variable abundances throughout the summer, with some dates with particularly high numbers. Counts are made using the optic Utermöhl method based on the shape of the cells across one diameter of the counting chamber (10 mL) and extrapolated to total cells per litre. No attempt was made to identify the cells below the level of Class Cryptophyceae by IFREMER. The highest numbers in April coincide with the *Dinophysis* bloom in April (IFREMER-LER/ARCACHON 2006).

Fluorescent Counts

The counts made from the IFREMER samples using the Chemscan are also presented in Fig. 4. Although not all of the samples from 2006 were available for processing for the probe/ChemScan analysis, samples were counted from April to August 2006, with samples missing from June 2006. Cryptophytes were present in all the samples and the counts ranged from 1000 to 310,000 cells per litre. Not all clades were present in every sample (Fig. 4). In general, it can be said that the crypto-

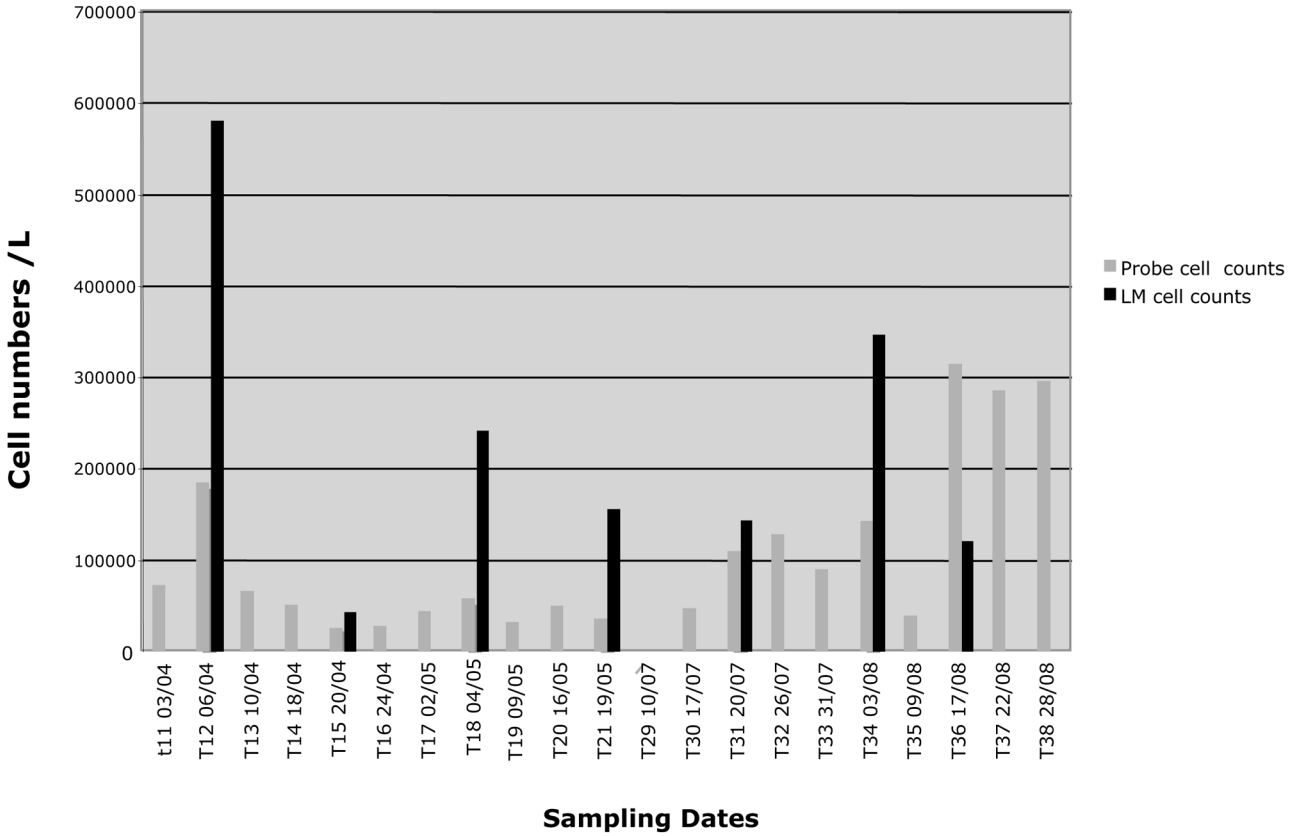


Fig. 3. – Comparison of the manual and molecular counts from site Teychan from Arcachon Bay.

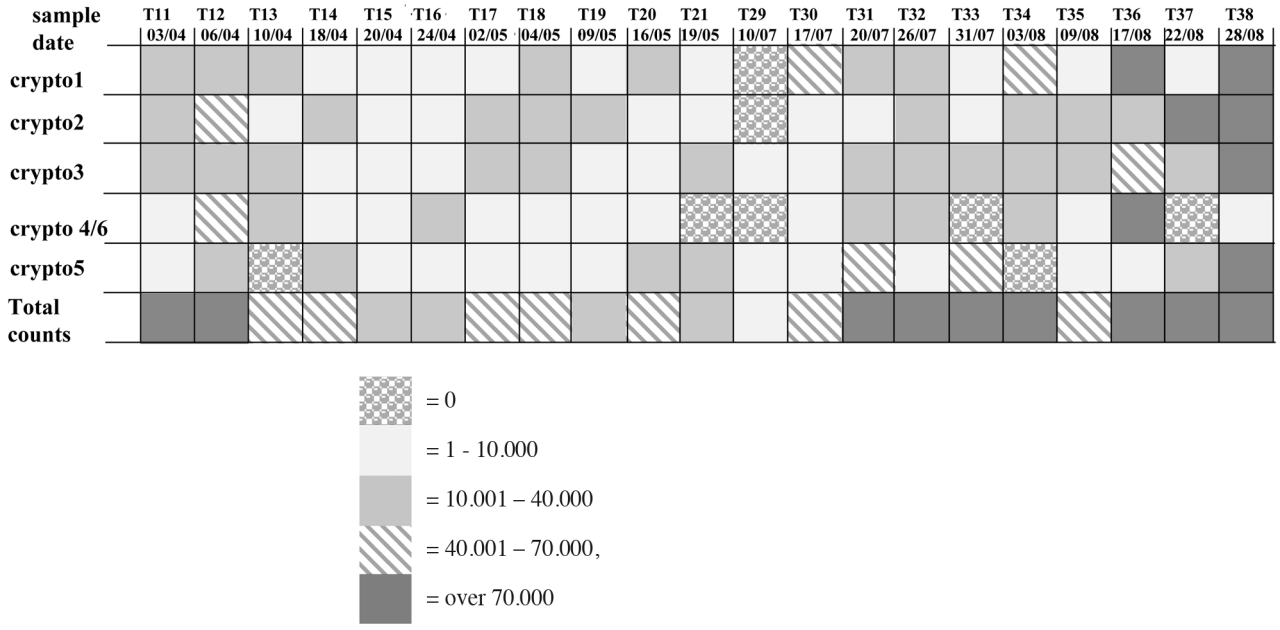
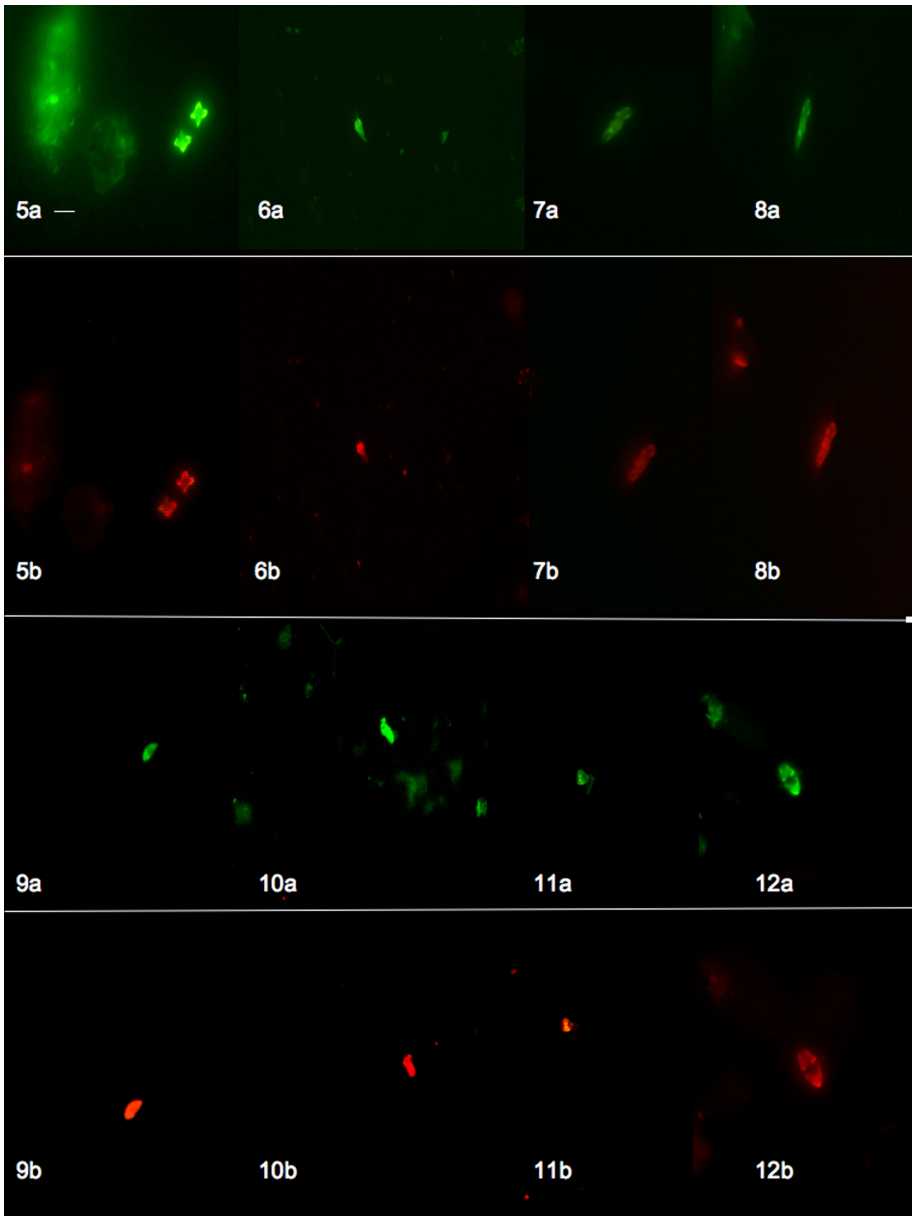


Fig. 4. – Relative abundances of cells giving both a positive clade signal and class probe signal for the cryptomonads and counted in the Chemscan

monads had two blooms: a smaller one in spring and another larger one in late summer, although no samples after August were available for manual or probe counting. The class achieved their highest numbers in August among the samples counted where all clades except clade 46 reached their highest abundances. The highest counts were recorded for clade 3 over the entire sampling season, followed by clade 2 throughout the samples counted with



Figs. 5-12. – Cells hybridised with the probes for each clade (A = FITC labelled probe) and for the entire group (B = Cy5 labelled probe). 5a, 5b, 6a, 6b = crypto1; 7a, 7b, 8a, 8b = crypto2; 9a, 9b= crypto3; 10a, 10b, 11a, 11b = crypto4/6; 12a, 12b = crypto5.

the CHEMSCAN (Fig. 4). Taxonomically, these clades contain the genera *Rhinomonas*, *Rhodomonas*, *Pyrenomonas* and *Storeatula* and *Guiardia* and *Hanusia*, respectively (Fig. 1). However, if you view micrographs of the cells detected by these probes, you cannot see any obvious difference in the cells detected by any of the clade level probes (Figs. 5-12). However some cells belonging to Clade 2 were particularly large (Figs. 7, 8).

DISCUSSION

In nearly every case, the molecular counts were lower than the traditional counts where the two counting dates coincided (Fig. 4). The two counts followed the same trend, even though the changes in magnitude were not the same. Both counts showed an early spring bloom followed by a lower population in mid summer and a second peak in August. Exceptions to this are those dates in late August where there were no traditional counts but the samples were available for the molecular counts and once where the probe count exceeded the manual count. During August, the cryptomonad populations maintain a high population. Perhaps at this time, the pico-fraction of the cryptomonads dominated and were detected by the more sensitive probe counting method used here. These pico-sized flagellates could easily be overlooked or disregarded as non-specific particles in a manual count. This may account for the one date in August where the probe counts exceeded the manual counts.

At the Helgoland time series station, total flagellates are counted, among which would be the cryptomonads

(Wiltshire & Dürselen 2004). Here, flagellates also show a bloom in early spring and another smaller one in August (see Medlin *et al*. 2004). The pico-sized cryptomonads were present in the clone libraries in 2000 from the Helgoland time series site in the spring and in August but had disappeared by October (Medlin *et al*. 2004). It is likely that the pico-sized cryptomonads contributed to the difference between probe and cell counts in August.

Reasons for the possible differences in the two counts, where the manual counts exceed the probe counts are most likely because of cell loss. A systematic comparison of traditional cell fixation methods for use with FISH hybridisation methods with cryptomonads was performed after this study and it was found that saline ethanol can rupture cells but affected all clades equally (Medlin & Strieben 2010). The molecular counts were done one year after the traditional counts and two additional preservatives (one including saline ethanol) were added to the cells to facilitate probe penetration and loss of chlorophyll that could mask the rRNA signal. Not only time differences between the two analyses but also cell rupture from the preservatives could account for the cell loss and thus the discrepancies in the two counts. Despite the differences in the magnitude of total cells present, the same trend between the two counts can be visualised. John *et al*. (2003) found differences between probe and manual counts for *Alexandrium ostenfeldii* in the Orkney Islands. Manual counts were up to one order of magnitude above probe counts, but the counts followed the same trend. The station with the highest probe counts also had the highest manual counts. They attributed differences in the cell numbers to cells with low ribosome content that could produce such a low positive signal that it wasn't recognised by the observer as positive signal. We likely avoided this problem because we used two probes and counted only those cells with a positive signal with both probes and more importantly all of our clade probes had a signal amplification and were found by the Chemscan, which is far more sensitive than the human eye is to weak fluorescent signals. It is much more likely that our differences are caused by the fixation that likely ruptured the cells and the delay in counting the cells with the FISH-TSA method.

Nevertheless, the added advantage of the molecular counts is that we can now determine which genera were present in the molecular counts; however, we do not know if there was a selective loss in the genera as well as in the cell numbers following the treatment with the preservatives. Our study to determine the best preservative for use with FISH for the cryptophytes did not detect any difference in the degree of cell rupture among the clades when fixed with saline ethanol (Medlin & Strieben, 2010). In a similar study of the Helgoland time series site, the same probes were applied to the total phytoplankton community but in a microarray cell free format (Gescher *et al*. 2009, Metfies *et al*. 2011). In that study, clades 4 and 6 were the dominant clades with major abundances being recorded in spring. These workers attributed zooplankton grazing to the decrease in cryptophyte abundance during the summer. In Arcachon Bay, the highest numbers of cryptophytes occurred in April, which coincided with the *Dinophysis* bloom in April. After this, both *Dinophysis* and cryptomonad numbers rapidly declined. *Dinophysis* is known to feed on cryptomonads and the presence of certain cryptomonads have been used to signal the onset of a toxic event for *Dinophysis (*Adolf *et al*. 2008). Cerino & Zingone (2004) also found two periods of peak abundance in April and in August in the Gulf of Naples, A spring maxima has also been reported in Oslo fjord (Throndsen 1969) and in the Kiel Bight (Jochem 1990) using serial dilution to estimate cell numbers.

Cerino & Zingone (2004) have reviewed the studies of the abundance of cryptophytes where attempts have been made to identify the group below the class level in a seasonal study. They concluded that as a group they were dominant in spring-summer and sometimes in autumn. They found that many of their isolates were new to science emphasising how poorly known the cryptophyte flora is. They found that *Hemiselmis* and *Plagioselmis* belonging to clade 6 and clade 4, respectively (probe clade46) were dominant in the spring, whereas *Hemiselmis* also produced a peak in summer. There are several differences between the Gulf of Naples, Arcachon Bay and the Lower German Bight, which include salinity, clarity of the water and nutrient load, any of which could result in the preferential growth of one clade over another. It is interesting that this group of phytoplankton, which is seldom investigated, appears to have distinct seasonal patterns and clades that seem to prefer distinct ecological niches. The important feature of this work is that we have demonstrated the usefulness of molecular-based probe methods to enhance the taxonomic resolution of routine time series data for phytoplankton identification without having to resort to electron microscopic studies.

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