

***In situ* hybridisation of phytoplankton using fluorescently-labelled rRNA probes**

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14.1 Introduction

Molecular biological techniques have greatly enhanced our ability to analyse all types of organisms, including the microalgae. This represents a major step forward in marine oceanography, as many microalgae are small in size, lack distinct morphological markers, and are unculturable, which makes it difficult to estimate their biodiversity. The lack of knowledge of their breeding systems also makes genetic or demographic studies difficult, and in addition, long term seasonal studies in aquatic environments are problematic for logistic reasons. This has hindered our understanding of microalgal diversity and their population structure. Despite this, physiological/biochemical measurements have been used to infer the existence of significant genetic diversity within and between microalgal populations (1-3). With these data researchers have speculated on hidden biodiversity and temporal and spatial structuring of genetic diversity or gene flow. Now molecular techniques can present a quantitative framework through which the diversity, structure and evolution of microalgal populations can be analysed, predictive models of the dynamics of aquatic ecosystems formulated, and the idea of functional groups in the plankton proven.

Nevertheless, molecular analysis of microalgal population structure is behind other groups and has been usually inferred from physiological data determined from relatively few clones. This unfortunately is a very naive approach because nearly every physiological measurement has shown that no single clone of any microalgal species can be considered truly representative of that species (4).

The interaction of a species with environmental parameters is influenced by the genetic diversity at the population level of a species. Spatial and temporal partitioning of genetic diversity will occur as these interactions structure the ecosystem. Such

structuring has seldom been measured in the microalgal community and studies of genetic diversity are virtually non-existent in pelagic ecosystems. All evidence of geographically isolated populations would be erased if we continue to assume that microalgae with high dispersal capacities are genetically homogeneous over their entire range. Support for this assumption has come mainly from phenotypic comparisons based initially on net phytoplankton biogeographic studies and later on isozyme studies. It is clear that the same morphotype/species may be endemic or cosmopolitan (5) but it is more likely that cosmopolitan species will exhibit regional differentiation when examined with molecular techniques (6).

Karp *et al.* (7) provide an excellent introduction into the variety of molecular techniques available for use in studying biodiversity at all taxonomic levels, whereas other useful reviews deal with the biodiversity in the marine environment (8) and in the marine phytoplankton (9). We present below a brief review of the progress in analyzing microalgal populations beginning first at the population level and building to higher taxonomic levels with the use of rRNA probes. A detailed protocol of Fluorescent *In Situ* Hybridisation (FISH) methods applicable for most algal cell types so far tested is presented at the end of the chapter.

14.2 DNA fingerprinting

Our current limited knowledge of microalgal genetic diversity is a direct consequence of the difficulties of finding polymorphic markers for ecological genetic studies. Isozymes, the molecular markers used in early studies, evolve so slowly that closely related populations appear identical. The early viewpoints suggesting the absence of genetic diversity in microalgae have undoubtedly been propagated from these studies. The use of high resolution DNA fingerprinting techniques *sensu lato* circumvents these problems and has thus opened areas previously considered unreachable for the microalgae.

DNA fingerprinting is a generic term for different molecular techniques, which have in common that they produce multi-locus banding patterns and can be used to analyse populations down to individuals. The most commonly used techniques are RAPDs (Random Amplified Polymorphic DNAs), AFLPs (Amplified Fragment Length Polymorphisms), VNTRs (Variable Number of Tandem Repeats; minisatellites) and STRs (Simple Tandem Repeats; microsatellites) (7). While these methods are used for population studies in many higher eukaryotic organisms, their employment for

analyzing microalgae is still limited thus far. The few examples of studies of biodiversity and population genetics in phytoplankton include those of the prymnesiophyte *Emiliana huxleyi* (10) and the dinoflagellate *Symbiodinium* (11), both analysed by RAPDs, as well as the dinoflagellate species *Alexandrium tamarense* (12), which was investigated using AFLPs. The latter example showed that these types of fingerprints can provide ironically too much variation in the case of population studies because of the high variability at each locus and the large number of loci. Banding patterns can quickly become so complex that they cannot be analyzed in terms of allele frequencies (the data of population genetic measures). Obviously, more effort is necessary to establish these techniques and more knowledge about the genetic structure of most phytoplankton species must be gained, before DNA fingerprinting methods can be routinely used to investigate phytoplankton biodiversity and population structures. Thus far, DNA fingerprinting with VNTRs hasn't been used in microalgal analysis to our knowledge, but single locus microsatellite markers were applied in analyzing the diatom *Ditylum brightwellii* (13) and *E. huxleyi* (14).

14.3 Sequence data

Sequence data for both coding and non-coding regions of the genome can be used to reconstruct the evolutionary history of organisms and to examine relationships at all taxonomic levels. The ribosomal RNA genes are usually used for phylogenetic analyses, although many genes are potentially available. The rRNA genes have special attributes that make them ideally suited as molecular markers (15). They are of a relatively large size, contain both variable and highly conserved regions to address both close and distant evolutionary relationships, respectively, and are of an universally conserved function with no evidence to suggest that they are laterally transferred (15).

Non-coding regions separating genes are termed spacer regions. In some operons, such as in the ribosomal operon, they function in the final processing of the mature rRNA molecular but in most other genes their function is not well understood. They can evolve at a faster rate because they are not subjected to the same evolutionary constraints as coding regions. To resolve closely related species or population level genetic structure, these faster-evolving non coding regions are best

used but even they can be conserved at the genus level or higher in some algae (reviewed in 6).

Analysis can be performed on the sequences obtained from mixed natural samples/communities. Whole DNA extraction of a water sample, followed by cloning of PCR-amplified rDNA sequences and screening of random clones from this library can provide insights into the genetic diversity, which are unobtainable using more traditional means of community analyses. This is especially true for unculturable groups. In every such study novel groups have been found such that the biodiversity of the picoeukaryotic fraction in oceanic samples is considerably underestimated as has found to be the case for the prokaryotic fraction.

Biases in the phylogenetic results obtained will vary with choice of gene used, the geological age of the taxa investigated, the rate of evolution in the gene of choice, the number of nucleotides used in the analysis, the number of outgroups, the evolutionary model and the taxa selected for analyses. The Model test program (http://inbio.byu.edu/Faculty/kac/crandall_lab/Computer.html) tests 56 different types of models of evolution to determine the model that best fits the data so that this model and all of its parameters can be imported into PAUP for analysis. But nevertheless phylogenies have been generated for most microalgal groups and they are under frequent refinement. In the last 10 years, three new microalgal classes have been either defined or recognised from sequence analysis (16-18).

14.4 Oligonucleotide probes for the detection of phytoplankton

The fast and secure identification of phytoplankton, especially of toxic species, is important from an ecological and economical point of view, but the aforementioned problems for most pico- and nanoplankton species makes this difficult. Identification usually necessitates other time and cost intensive techniques, such as electron microscopy, pigment analysis with high-performance liquid chromatography (HPLC) or sequencing of conserved genes before a definitive identification can be made of particularly difficult taxa. Phytoplankton species identification by whole-cell hybridisation with specific fluorochrome-labelled probes followed by fluorescence microscopy or flow cytometry offers a faster alternative for species identification. Based upon conserved and variable regions of the RNA of the ribosomal small and large subunit (SSU, LSU rRNA), signature sequences of varying specificity can be found, which have been used to develop probes for the identification of phytoplankton

at various taxonomic levels from classes down to species or strains. The vast amount of rapidly accumulating sequence data for all kinds of organisms makes it possible to develop these probes for a broad spectrum of taxa. Although these techniques have been largely used for Bacteria (i.e., 19, 20) (see also Chapter 8) there is already a growing number of probes for eukaryotic pico- and nanophytoplankton. To date, they have been developed for classes including Chlorophyceae (21, 22), Prymnesiophyceae (22, 23), Pelagophyceae (22), Dinophyceae (24) and Bolidophyceae (25), taxonomic clades like those for toxic and non-toxic *Chrysochromulina/Prymnesium* species (26) and for ecologically- and economically-important species like *Chrysochromulina polylepis* (26), *Alexandrium tamarense* (24), *A. ostenfeldii* (24), *Phaeocystis globosa* (23), *Emiliana huxleyi* (27) and various *Pseudonitzschia* species (28). As mentioned before, even more probes are available for numerous prokaryotic groups and strains, including many marine and limnic bacteria. An overview of the analysis of these groups by rRNA probes is given by Amann et al. (29). Successful application of molecular probes to field samples have already demonstrated their use in characterization of phytoplankton abundance, e.g., for *Pseudo-nitzschia* species in coastal waters from Louisiana, USA (30), Bolidophyceae in the Mediterranean Sea and the Pacific Ocean (24) or groups of Prymnesiophytes also in the Pacific Ocean (28).

Experimental considerations

The broad diversity we face in the phytoplankton makes it difficult to develop an *in situ* protocol capable of analysing all kinds of algal cells. For example, different types of cell walls and membranes may require different conditions for probe penetration. Also, cell autofluorescence, especially from chlorophyll, can become a problem when it is very strong and therefore masks the probe signal. Taking these problems into account, we adapted an existing protocol (31, 32) for *in situ* hybridisation with specific, fluorescent-labelled probes to suit a broader range of phytoplankton species.

For the fixation and hybridisation of phytoplankton using fluorescent-labelled probes, various protocols are in use (i.e., 22, 31), that are mainly derived from those developed for bacteria and often use paraformaldehyde (PFA) as the fixative (20). Comparing different protocols we found that one published by Scholin *et al.* (31, 32) gave the best results with most species tested. This method eliminates paraformaldehyde and replaces it with a saline ethanol fixative. Nevertheless, this

method was originally developed for probes for two genera only and including at least two mismatches between target and non-target sequence. We found the conditions in our own experiments not to be stringent enough for a broader range of species and probes. Thus we modified the hybridisation conditions to increase stringency by addition of formamide to the hybridisation buffer and reducing the salt concentration in the last washing step. Formamide concentrations must be established empirically for every probe and normally range between 0 and 50%.

During the testing of different fixation/hybridisation protocols it became clear that one of the most important components was the type of detergent used. Sodium dodecylsulfate (SDS), for example, which is often used in hybridisation buffers (i.e., 22) destroys the more fragile cells like unarmoured dinoflagellates, whereas IGEPAL-CA630 (or the chemically identical NONIDET-P40) maintains cell stability whilst enabling efficient probe penetration into the cell. The use of the latter detergent in the hybridisation buffer, enables the investigation of some of the most delicate dinoflagellates, such as *Gymnodinium mikimotoi* (Fig. 1 a & b).

In addition to the fixation, the saline ethanol in the Scholin protocol (31, 32) extracts the chlorophyll from the cells and bleaches them, thus enabling good visualisation of probe signals. This is an advantage for *in situ* hybridisation experiments with phytoplankton in which autofluorescence can be problematic. Nevertheless, sometimes the autofluorescence of some species is so strong and persistent that a sole ethanol treatment, even for an prolonged time, is not sufficient for probe detection using fluorescence microscopy (Fig. 1 c). In these cases, an additional treatment with 50% dimethylformamide (DMF) can help, because it bleaches the chlorophyll from the cells far better than ethanol alone (Fig. 1 d). As DMF is toxic, this procedure should be added to the protocol only if autofluorescence of cells is expected to be a problem.

Using this modified protocol it is possible to analyse a very broad range of laboratory cultures and field samples with *in situ* hybridisation using probes ranging from single to multiple base mismatches. This provides researchers with a powerful tool for investigating the occurrence and biodiversity of phytoplankton.

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Protocols

MATERIALS

- Reagents:** 25 x SET buffer: 3.75 M NaCl, 25 mM EDTA, 0.5 M Tris/HCl (pH 7.8), filter-sterilize through 0.2 µm pore-size filters and store at room temperature
- Saline EtOH fixative: 100% ethanol: distilled water: 25 x SET (25:2:3) (v/v), prepare fresh for every experiment
- Hybridization buffer: 5 x SET, 0.1% (v/v) IGEPAL-CA630 (or Nonidet-P40), X% (v/v) Formamide, filter-sterilize through 0.2 µm pore-size filters, add 30 µg ml⁻¹ Poly A and store at room temperature, the percentage of formamide (X) in the buffer depends on the probe requirements

50% Dimethylformamide (DMF): not mandatory; dilute DMF with de-ionized water, use glassware only as concentrated DMF can dissolve plastic

Citifluor/DAPI-Mix: Citifluor (Citifluor Ltd, Cambridge, UK): distilled water (2:1) (v/v) containing DAPI at a final concentration of $1 \mu\text{g ml}^{-1}$

FLUORESCCEIN-labelled oligonucleotide probes, stored at -20°C in the dark

Nail varnish

Equipment:

Filtration unit with vacuum pump

Polycarbonate Filters ($0.2 \mu\text{m}$ or $3.0 \mu\text{m}$ pore size)

Moisture chamber (i.e., plastic box with its inside covered by 3MM Whatman paper that is soaked with hybridization buffer)

Hybridization oven

Microscopic slides and cover slips

Fluorescence microscope equipped with UV lamp and appropriate filter sets for probe label and DAPI

METHODS

Fixation of samples

1. Set up filtration unit. Use polycarbonate filters with pore sizes matching the expected cell sizes ($0.2 \mu\text{m}$ pore size for

picoplankton and bacteria, 3 μm pore size for larger phytoplankton). The cells should be filtered onto the shiny side of the filter.

2. Pour sample into filtration unit, use enough liquid to evenly cover the whole filter.
3. Filter sample through by using the lowest amount of vacuum possible in which a constant flow still occurs to prevent breakage of delicate cells
4. Close connecting piece to pump with parafilm so that no liquid is dripping through the filter during incubation and the filter does not run dry.
5. Add 5-15 ml of freshly prepared saline EtOH fixative so that the whole filter is covered and incubate at room temperature for 1 h. The solution will normally show strong precipitation, but this does not influence the fixation. For samples that contain species with strong autofluorescence incubate for 2 h. Afterwards remove the solution by vacuum filtration.
6. Add 5-15 ml of hybridization buffer (without formamide), incubate at room temperature for 5 min and filter buffer through.
7. If strong autofluorescence is expected, add 50% DMF to cover the filter and incubate at room temperature for one hour. Repeat step 6 afterwards.
8. Briefly air dry the filter on Whatman paper. Wrapped filters can be stored at 4°C for at least 3 weeks, or processed immediately.

Hybridization

1. Cut filter into pieces. A filter of 25 mm diameter can easily be cut into six pieces if necessary, one of 47 mm diameter into at least 12 pieces.
2. Put a filter piece onto a microscope slide, apply 54 μl hybridization buffer with 6 μl probe (50 $\mu\text{g l}^{-1}$ stock)

directly onto the filter piece. Ensure that the whole filter piece is covered with liquid and if necessary use more buffer-probe mixture. The fluorescent labelled probe is light sensitive, so keep the filters in the dark for the rest of the procedure, e.g., cover them during incubation times, and expose them only shortly to light when handling them.

3. Hybridize slides at hybridization temperature (normally 50°C in a moisture chamber in the dark for 1 h).
Formamide (FA) concentration for new probes need to be determined empirically. Hybridize a filter piece each with 0, 10, 20, 30, 40 and 50% FA in the buffer. Choose the highest FA concentration for which target cells from defined lab cultures give clear positive signals while non-target species show no signal. A moisture chamber can be made using hybridization buffer without formamide.
4. Wash filter briefly in a small tray in 1 x SET buffer pre-warmed to hybridization temperature and the dry the filter on Whatman paper. Put the filter back onto a clean slide.
5. Apply 100 µl of pre-warmed 1X SET buffer onto the filter and incubate again in the moisture chamber at the hybridization temperature for 5 min.
6. Briefly dry the filter on Whatman paper, then put it back onto a clean slide.
7. Apply 20-30 µl of Citifluor/DAPI mixture directly onto the filter, mount cover slip carefully so that the liquid covers the filter piece without any air bubbles and seal the sides of the cover slip with nail varnish.
8. View slides by fluorescence microscopy. Look at the specimens using the filter for the DAPI stain and then switch to the one for the fluorochrome, only target cells should give positive, emerald green signals whereas non-target cells show a brown-yellowish colour.

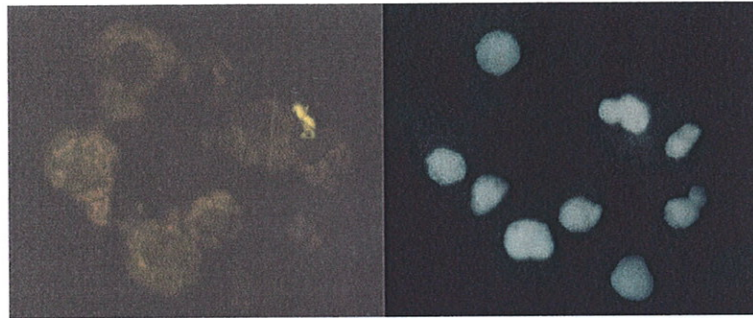
Figure legend

Figure 1a: Cells of the dinoflagellate *Gymnodinium mikimotoi* hybridized with a dinoflagellate-specific FLUORESCHEIN-labelled probe (left) and same field seen with DAPI staining (right). Cells were preserved with PFA and SDS detergent was used in the hybridization buffer. The cells were ruptured after the hybridization and no signals could be detected.

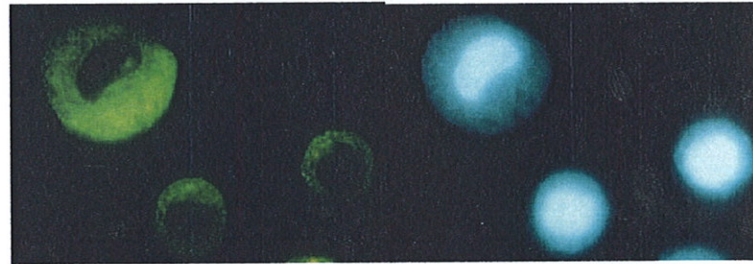
Figure 1b: Cells of the dinoflagellate *Gymnodinium mikimotoi* hybridized with a dinoflagellate-specific FLUORESCHEIN-labelled probe (left) and same field seen with DAPI staining (right). Cells were preserved with saline-ethanol fixative and IGEPAL-CA630 detergent was used in the hybridization buffer. The cells were intact and gave clear hybridization signals.

Figure 1c: Cells of the dinoflagellate *Alexandrium tamarense* hybridized with a dinoflagellate-specific FLUORESCHEIN-labelled probe (left) and same field seen with DAPI staining (right). Without DMF treatment, cells show strong autofluorescence that masks the probe signal and make them impossible to analyse.

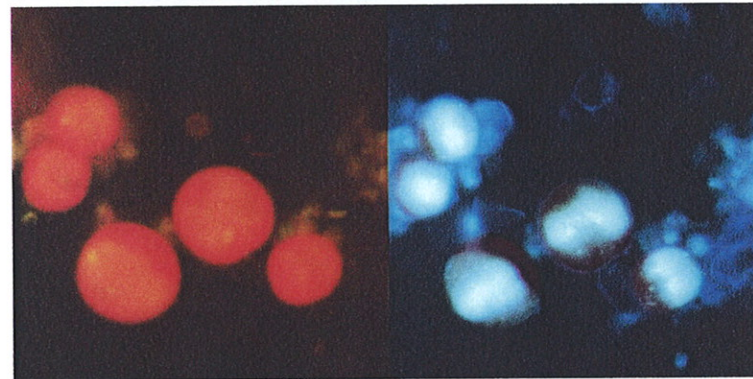
Figure 1d: Cells of the dinoflagellate *Alexandrium tamarense* hybridized with a dinoflagellate-specific FLUORESCHEIN-labelled probe (left) and same field seen with DAPI staining (right). After DMF treatment, autofluorescence is mainly reduced in nearly all cells and probe signals are clearly visible.



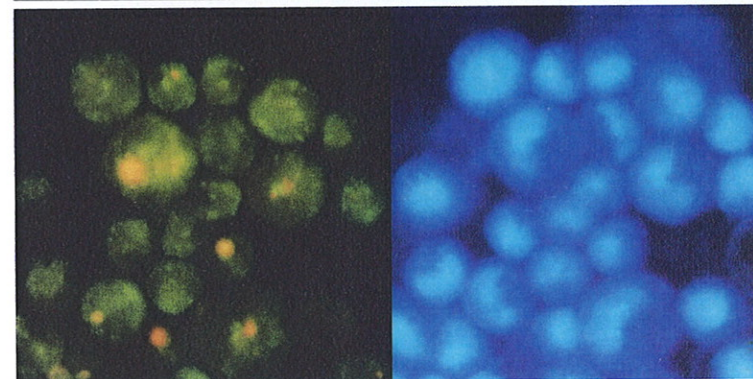
A



B



C



D