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Molecular Techniques to Estimate Biodiversity with Case Studies from the Marine Phytoplankton

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1. Introduction

Approximately less than 10% of the known biodiversity in the marine protistan community is known, but among the pico-fraction even less is known with new groups being discovered regularly (Kim et al. 2011). This feature of hidden biodiversity was first recognized in the bacterial community but this phenomenon is now being extended into the eukaryotic fraction. Many cosmopolitan species, which we think we can easily recognize, are now being shown to be species complexes with little or no morphological markers to separate them. Spatial and temporal variation in their abundance and distribution in these complexes are also unknown. With new molecular and analytical techniques, our knowledge of marine species level biodiversity begins to unfold to understand how marine biodiversity supports ecosystem structure, dynamics and resilience. With these techniques, we can augment our understanding of biodiversity and ecosystem dynamics in all areas of the planktonic community, not just the photosynthetic ones. We will review selected molecular techniques and provide case studies to illustrate the use of these techniques.

For the past 30 years scientists have recognised that understanding and preserving biodiversity is one of the most important global challenges facing the world today. There is a science plan for Europe to address the problems associated with a potential loss of biodiversity in the marine environment, which was formulated in 1999 by the Association of Marine Science Institutes.

Biodiversity is strongly affected by the rapid and accelerating changes in the global climate, which largely stem from human activity. There is now common agreement that the world must generate plans to conserve and protect biodiversity to prevent rampant savaging for natural resources. How biodiversity is perceived and maintained affects ecosystem functioning and how the goods and services that ecosystems provide to humans can be used. Recognizing biodiversity at all levels is essential to preserving it. Terrestrial and marine ecosystems are inherently different and the management of their biodiversity requires very different approaches. Often terrestrial ecosystem generalizations concerning biodiversity patterns on both global and regional scales, the processes determining these

patterns (Gosling 1994), and the resulting biodiversity loss are extrapolated to marine ecosystems. However, these extrapolations are generally incorrect because the marine environment experiences many more disturbances than their terrestrial counterparts and their dispersal patterns are not the same (Killian & Gaines, 2003). Medlin and Kooistra (2010) summarized the following fundamental differences between marine and terrestrial biodiversity. The physical environment in the oceans is three dimensional, whereas on land it is essentially two-dimensional. The vast majority of the biomass of marine primary producers is composed of minute and usually mobile micro-organisms, with representatives from most of the eukaryotic crown lineages (sessile macroalgae are only minor players), whereas on land, the bulk of the primary production is carried out by macroscopic and sessile green plants. Climax communities never develop in the ocean as they were once believed to have developed on land. In the ocean, primary production is consumed daily, but on land, most primary production enters the detrital cycle each autumn. Higher-level carnivores often play key roles in structuring marine biodiversity and when exploited heavily, as in over-fishing, there are severe downward-cascading effects on biodiversity and on ecosystem functions. Marine systems are more open than terrestrial and dispersal of species occurs over much larger ranges than on land (Killian & Gaines, 2003). Life originated in the sea and thus has a much longer evolutionary history in the sea than on land (Ormond et al., 1998). There are 14 indigenous marine animal phyla, whereas only one phylum is unique to land, making diversity at higher taxonomic levels higher in the sea. Four new algal phyla have been described in the last twenty years (Moestrup, 1991, Andersen et al., 1993, Guillou et al., 1999, Kawachi et al., 2002). Three new pico-sized classes await formal descriptions (Tomas et al., unpublished, Not et al., 2007, Kim et al., 2011). The sum total of genetic resources in the sea is therefore inferred to be much more diverse in the sea than on land (Grassle et al., 1991). Also on average, genetic diversity within a species (i.e. below the species level) is higher in marine than in terrestrial species. Thus, because of these fundamental differences, our understanding of marine biodiversity lags far behind that of terrestrial biodiversity. There is not enough scientific information to design management and conservation plans for the sustainable use of coastal resources.

Biodiversity can be described in three hierarchical levels: genetic, species, ecosystems. Each has its own spatial scale from single samples to regional and global populations, and temporal scales changing from short time intervals (days to weeks) to long (years to decades). On land, the full range of these scales can be sampled, but not in the ocean. In the ocean the planktonic population that is sampled at any one point in time will not be same population at that location the next day. Each scale can be affected by loss but loss at any of these scales is rarely calculated and the knock-on effect of any loss at one scale to another scale is unknown. Marine biodiversity is more widely commercialized than that on land because of the many species used as food stocks, whereas fewer species are used as food stock in terrestrial ecosystems. Exploitation of marine biodiversity is not well regulated and harvesting and fishing technology is so advanced that many marine species are now driven to extinction or near extinction.

Global biodiversity projects must first characterize the existing biodiversity as fully as possible (from genetic to ecosystem level) in selected key (flagstone) habitats across broad geographical ranges. However, this is a monumental task to compile comprehensive inventories even at a few sites. The Census of Marine Life (*Http://www.coml.org/*) is a global network of researchers from over 70 countries that tries to answer the questions "What lived

in the oceans?" "What lives in the oceans?" and "What will live in the oceans?" Molecular methods have proven to an indispensable tool to answer these questions.

The world's oceans cover 70 percent of the Earth's surface, and their dominant populations, both numerically and biomass-wise, belong to microscopic protists and prokaryotes. The marine phytoplankton are major components of both these groups and are assumed to be high dispersal taxa with large population sizes. Small photosynthetic organisms are responsible for the bulk of primary production in oceanic and neritic waters. These organisms play pivotal roles in many biogeochemical processes that regulate our global climate. Net samples and bulk process measurements, such as chlorophyll *a* and ^{14}C biomass estimates have historically provided most of our knowledge about marine phytoplankton. However, whole water samplers and new analytical methods, e.g., flow cytometry, epifluorescence microscopy and HPLC (high pressure liquid chromatography) have found previously unrecognised groups (such as *Prochlorococcus*), size classes (the picoplankton < 3 μm) and hidden biodiversity (new algal classes, e.g., Bolidophyceae, Pelagophyceae, picobiliphytes). Although the global importance of picoplankton was unknown 30 years ago, they can contribute up to 90% primary production in oligotropic oceanic waters (Waterbury et al., 1979, 1986, Chisholm et al., 1988).

Because of these recent discoveries about phytoplankton biodiversity, we must ask the questions: Do we know all of the groups in the phytoplankton? Do we know how they are related to one another? Do we know their spatial and temporal changes in their abundances? Do we know the extent of their genetic diversity? The answer to these questions is an unequivocal NO.

In picoeukaryotes, where there are far too few morphological markers explored upon which to determine species identification, α-level taxonomy is lacking. A new group of picoplankton was only discovered this year (Kim et al., 2011). In addition, we know the population structure of the phytoplankton in only a few isolated cases and many of these belonging to the toxic dinoflagellate genera. It is likely to be very different from that on land because marine planktonic organisms live in an ever-changing three-dimensional environment. Many taxa may have little genetic structure over very large geographic areas. However, where population structure has been studied in the marine phytoplankton, global populations have appeared fragmented with some adjacent areas with limited gene flow between them (see review in Medlin et al., 2000). Admittedly, most of these studies have not sampled the phytoplankton species over their entire range, but if their population are fragmented on a local scale, then by inference, they are fragmented on a global scale. Further, recent evidence suggests that speciation and dispersal mechanisms in marine planktonic organisms may be very different from those on land (Killian & Gaines, 2003).

The advent of molecular biological techniques has greatly enhanced our ability to analyse all populations (Parker et al. 1998), not just the marine phytoplankton. The small size and paucity of morphological markers of many phytoplankton species, the inability to bring many into culture, and the difficulty of obtaining samples for long term seasonal studies in open ocean environments has hampered our knowledge of phytoplankton diversity and population structure. The idea of a single globally distributed species or of temporal stasis is no longer valid. Temporal genetic change may often be greater than spatial change or change between species (Brand, 1982, 1989, Gallagher, 1980, Hedgecock, 1994) and may very well apply to bloom populations. Because the rate of genetic change can and does occur on

ecological time scales (Palumbi, 1992), this suggests that mechanisms are in place to determine how local adaptations and speciation can occur in apparently homogeneous populations (Gosling 1991). Now molecular techniques can present a quantitative framework through which the diversity, structure and evolution of marine phytoplankton populations can be analyzed, predictive models of the dynamics of ocean ecosystems formulated, and the idea of functional groups in the plankton proven.

2. Determining biodiversity in environmental samples by sequence analysis

The most exact method to assess biodiversity down to the species level in environmental samples is by sequencing clones from such samples. The SSU rRNA gene is often the gene of choice for cloning and is the gene most commonly used as a phylogenetic yardstick. This is best achieved by isolating total DNA from the sample followed by full-length SSU gene amplification using PCR and universal primers, then cloning and sequencing. The method allows the exhaustive description of biodiversity in a sample down to the species level. Also the resulting sequence information may serve as a basis for developing specific oligonucleotide probes necessary for subsequent methods like FISH. It should be noted though that even universal PCR primers might only amplify a subset of all organisms and therefore bias the result. It has been shown that different groups of organisms were detected when different primers have been used and if possible the analysis of an environmental sample should always include the use of different primers to get a more complete picture of its diversity.

2.1 Clone libraries

The first assessments of ecosystem biodiversity were made using clone libraries from DNA and in every case far more diversity was revealed than expected (see review in Bull 2004). However, these early clone libraries were limited by sequencing capacity and most statistical analysis revealed that coverage of the diversity of the clones had not reached a plateau. This problem has more or less been eliminated with new age sequencing. Also clone libraries made from RNA and not DNA are not identical (Lami et al., 2009).

2.2 454 sequencing and the rare biosphere

The culture independent 454 pyrosequencing is rapidly gaining favor for environmental analysis because it allows a rapid attainment of around 400 bp in a 10-hour run from an exhaustive search of a library. This exhaustive search has revealed many sequences (operational taxonomic units, OTUs) that are represented by only a single clone in the library. With traditional methods of making and sequencing clone libraries, these single sequences would not have been recovered to a large extent. This plethora of single occurring OTUs has been termed the “rare biosphere” [Sogin et al., 2006] and much effort is now being concentrated to recover this aspect of many communities with 454 sequencing or pyrosequencing as it is often referred to. The reason for this rare biosphere is unknown but it is clear that the same species are not repeated in different geographic areas (Brazelton et al. 2010). Also this technique has enabled more genes to be explored and community analysis is now moving into the age of metagenomic and metatranscriptomic analysis (Cuvelier et al., 2010). However, until the length of the sequence read is increased, full phylogenetic assignment is not attainable.

2.3 Barcoding

The barcode is defined as a short gene sequence from a standardized region of the genome (the "barcode") that can characterize, and distinguish species, and to assign unidentified individuals to species. Basically, this method is not different from the sequencing methods mentioned before but what is new here is the scale at which international consortia and scientists try to analyze biodiversity in a standardized way. The Consortium for the Barcode of Life (*Http://barcoding.si.edu/index_detail.htm)*, for example, has started initiatives to develop DNA barcodes for all fish and bird species on Earth, and many other groups of organisms are targeted the same way. The primary opposition to barcoding is that it could lead to the elimination of taxonomy but this is not justified.

For barcoding to work, the "barcoding community" must agree on the gene fragment to use so that barcodes from different species are comparable. The mitochondrial COI gene (cytochrome oxidase I) is most often used for DNA Barcoding, especially in animals, but it cannot be used in many groups of phytoplankton because of the non-specificity of primers. Therefore other gene fragments, i.e., RUBISCO and ITS have been explored (Evans et al., 2007). It is likely that DNA Barcodes will be developed using many genes. DNA Barcoding will be a powerful taxonomic tool to analyze marine biodiversity. The high-throughput sequencing approach and the comparability of data will address many questions regarding cryptic and invasive species, and to identify quickly microbial diversity in any sample. Again, the main limiting factor is that barcodes of all possible organisms in the biosphere must be determined first. Is the barcode of a single individual representative of the species because different individuals in a population, let alone individuals in different geographic populations could possess slightly different barcode sequences? So again, we need to know the extent of intraspecific variation, and this variation should remain far less than differences among species. Yet, if all these problems can be solved at least in part, barcoding provides a very powerful tool of obtaining semi-quantitative data on the species composition of e.g., large numbers of environmental samples in a rapid and cost-effective way.

3. Fingerprinting methods as applied to environmental samples

Often it is not possible or necessary to get a full assessment of biodiversity but instead it may be enough to identify temporal changes or spatial differences among samples. In this case, DNA fingerprinting methods can be used. These are several PCR based methods of determining population structure. All these methods exploit differences in the length and base composition of specific gene segments which result in different banding patterns after electrophoresis – the "fingerprint" of the sample. Many of the methods work with any sequence that can form a secondary structure. Fragments of identical size but different base composition can then be separated in either denaturing or non-denaturing polyacrylamide gels, depending on the method.

DNA polymorphisms between individuals can, e.g., be found by Restriction Fragment Length Polymorphism (RFLP), a technique in which DNA is digested by restriction enzymes and then the presence or absence of restriction sites in different individuals is compared as well as insertions or deletions in their genome between these restriction sites. A slightly different RFLP method consists of the PCR amplification of a specific gene, e.g., the SSU rDNA, followed by restriction digestion with enzyme and gel electrophoresis. Because it uses only a limited number of fragments this method avoids the need of blotting and

probing for visualisation and is much faster and easier than the "classical" RFLP. On the other hand, the limited number of possible bands leads also to a very small number of possible polymorphisms and one needs luck to find a usable marker. Nevertheless, there are examples where this kind of RFLP marker has been used with success, e.g., for discriminating species and strains of the toxic dinoflagellate genus *Alexandrium* (Scholin et al., 1994a).

Two well-established methods for assessing diversity in environmental samples are Temperature Gradient Gel Electrophoresis (TGGE) and Denaturing Gradient Gel Electrophoresis (DGGE) (Muyzer et al., 1993). These methods allow the qualitative and semi-quantitative determination of biodiversity in environmental samples through amplification of a short segment of DNA that is electrophoresed either through a temperature or a density gradient gel. With either system bands of different base composition do not migrate to the same location, thus generating the sample fingerprint. Bands can be cut out of the gel and sequenced.

Single-stranded-conformation polymorphism (SSCP) uses the fact that single stranded DNA fragments fold into secondary structures depending on their base composition. Small fragments of ca. 300 bp are most useful. This method does not require gradient gels or temperature gradient electrophoresis and can be used in normal sequencers with fragment length analysis programs. Also the fact that one of the two strands is degraded reduces the variability obtained from communities because it avoids heteroduplex formation, a problem known in community analyses based on DGGE.

The first widely used PCR marker technique was Random Amplified Polymorphic DNA (RAPD) or Arbitrary Primed PCR (AP-PCR) with the former being the most commonly used name for this kind of method (Hadrys & Balick 1992, Lynch & Milligan 1994). These methods use a single short random primer in a PCR reaction, most often a decamer, to amplify the DNA, which produces a fingerprint of multiple bands and polymorphisms between individual samples are derived from single nucleotide changes that prevent or allow primer binding and therefore lead to different banding patterns between individuals. This method became quite popular because it could be carried out in a short time without previous knowledge of the organism under investigation. Nevertheless, RAPDs have been shown to have some drawbacks: The use of short primers gives not only the possibility of random binding in all kind of genomes and therefore makes this method working at all, but it also makes it unreliable, too, and susceptible even to small changes in the PCR conditions. Unfortunately, RAPD markers are hard to reproduce even within the same laboratory. Also, RAPDs are normally dominant markers by which they give less information than other, mostly co-dominant markers. RAPDs should only be used when time and resources are limited and no previous information about the species under investigation are known, otherwise other markers should be targeted. Populations of the dinoflagellate *Gyrodinium catenatum* among Australian and global populations displayed spatial and temporal differences using RAPD fingerprinting data (Boalch et al., 1999). Despite this, it was not possible to define the route of introduction into Australian waters, although the introduction is judged to be quite recent based on fossil sediment records. RAPDS were used to assess populations of *Emiliania huxleyi* (Barker et al., 1995) and was one of the first studies to show that blooms were not clonal and high diversity could be shown in relatively small bodies of water, viz., mesocosms.

Recently AFLP (Amplified Fragment Length Polymorphism) has become a popular marker technique for studying biodiversity in the marine environment. It combines the advantages

of RAPDs and RFLPs into a powerful tool (Vos et al., 1995). First, genomic DNA is digested with two different restriction enzymes, a rare and a frequent cutter. Then matching adapters are ligated to the digested fragments. Afterwards, a PCR is performed with primers homologous to the adapters plus up to four additional random bases at its 3' end. By using these selective bases, only a subset of digested DNA fragments is amplified, giving distinct bands instead of a smear and making it possible to analyse the bands on a polyacrylamide gel. The major advantage of this technique is the large number of bands it produces, giving a very good chance of finding a large number of polymorphic bands among them. The polymorphisms detected by this method come from the same sources as in RFLPs, insertions, deletions and point mutations leading to the presence or absence of restriction sites, but compared to RFLPs, AFLPs are normally scored only as dominant markers, even when some researchers give possible methods for using them co-dominantly. The use of longer PCR primers that anneal to the adapters and a few bases of the genomic DNA make the whole reaction much more reliable than RAPDs, because higher annealing temperatures can be used. The greatest advantage of the RAPD technology on the other hand remains, because no previous sequence information of the species under investigation is needed and PCR reactions are fast to be carried out. Nevertheless, AFLPs are technically demanding, sensitive to the purity and quantity of DNA to be digested, need some experience to be performed and data analysis of the hundreds of amplified bands should be done by computer analysis. Since 1995 when AFLPs were first introduced, there has been an increasing number of publications using this technique, but most of them deal with population studies or the development of genetic linkage maps for higher plants. Among algae, the multicellular red alga *Chondrus crispus* was the first organism to be analyzed by AFLPs, and more seaweeds have been investigated since then (e.g., *Caulerpa*, *Chara* and *Porphyra* species), but the method has since then also been used for phytoplankton, e.g. the marine dinoflagellate *Alexandrium tamarense*, the diatom *Asterionella formosa* and the chlorophyte *Chlorella vulgaris*, both freshwater algae. AFLP banding patterns in isolates of the dinoflagellate *Alexandrium tamarense* from the Orkney Islands were correlated with toxin patterns as determined by HPLC analysis (John et al., 2004), but a later study in the same area with more isolates and depending on the spatial scale investigated, AFLP patterns did not correlate with allelopathic capabilities (Alpermann 2009). A preliminary study of *Phaeocystis antarctica* indicated that the gyres around the Antarctic were not isolated from one another and it was likely that the ACC provided the vehicle for dispersal around the continent (Gaebler et al., 2007).

4. Analysis of population structure using molecular markers

The first molecular markers to be used in all fields were isozymes. These are proteins that show only small differences in their size or iso-electric point and therefore can be separated by starch gel electrophoresis but are still able to catalyse the same biochemical reaction. Their advantages of quick and easy isolation and detection made them the markers of choice for many investigations. But the requirement that isozymes must still be functional in the biochemical pathways strongly limits the number of possible mutations and therefore the number of alleles and the heterozygosity of this marker type. Another disadvantage of this kind of marker is also that protein content of cells and following the detectability of isozymes is strongly influenced by the environment and as a consequence, marker types were developed that directly used environment-independent DNA.

The goal of most early molecular studies concerning microalgae using isozyme analysis was to resolve species-level issues among species with conflicting or little morphological resolution rather than to study genetic structure within bloom populations. The recognition of cryptic species or the recognition of previously discounted morphological markers that can be used for separation of a species complex was the most common results of early isozyme studies. For example, different in isozyme banding patterns in neretic, shelf and oceanic populations of *Thalassiosira pseudonana* prompted Murphy & Guillard (1976) and Brand et al. (1981) initially to suggest that this species was composed of clinal populations but later detailed morphological investigations separated each ecological population into a different species (Hasle, 1978, 1983) for *Thalassiosira guillardii, oceanica* and *pseudonana*). There are many examples among the dinoflagellates where significant insights into species complexes have been made with isozymes that show in some areas populations are unique and in others they are not (*Alexandrium tamarense/fundyense/catenella*, Cembella & Taylor, 1986, Cembella et al., 1989; Hayhome et al., 1989; *Gambierdiscus toxicus* Chinain et al., 1997 and *Peridinium volzii*, Hayhome et al., 1987). In most of these cases, the isozyme conclusions were supported by further studies with sequence analyses. *Alexandrium* species have been studied in more detail using sequence analysis of rapidly evolving genomic regions, such as the ITS and the D1/D2 region of the LSU rRNA gene. Using these regions, isolates of the *Alexandrium tamarense/fundyense/catenella* species complex were shown to be related by geographic origin rather than by morphological affinities (Scholin et al., 1994b), which was originally indicated by the isozyme analysis. The world-wide biogeographic dispersal of ancestral population from the Pacific into the Atlantic has been hypothesized from these data. Furthermore, *Alexandrium* isolates will interbreed more successfully if they have similar isozyme patterns from two different locations than will isolates from the same locations but with different isozyme patterns (Sako et al., 1990). We now suspect that in these areas where isolates do not interbreed, they likely originate from different geographic clades that are overlapping in their distribution. For example, on the east coast of the UK down to about the Firth of Forth along the North Sea coast of Scotland, the non-toxic Western European clade of *Alexandrium tamarense* will overlap with the toxic North American clade. In contrast, other dinoflagellates, such as isolates of *Gambierdiscus toxicus* from similar geographical regions were not shown to be closely related, which suggested a multiclonal origin (Chinain et al., 1997). Populations of the green freshwater alga, *Gonium pectorale*, also appear from several locations to be multiclonal (Sako et al., 1991).

Microsatellites (MS) or simple sequence repeats (SSR), are the most powerful molecular markers available (Burke et al., 1991; Wright et al., 1994). In the beginning, MS were mainly from the field of fisheries sciences with most if not all economically important fish and shellfish species covered, but by now microsatellite markers and their use are available for macroalgae (e.g., *Gracilaria gracilis*, *Laminaria digitata*) and microalgae (e.g., *Chlamydomonas reinhardtii*, *Emiliania huxleyi*, *Ditylum brightwellii*, various *Pseudo-nitzschia* and *Alexandrium* species). It is from the microalgal studies that we find the strongest evidence for fragmentation of oceanic populations.

Microsatellites are short sequences of one to six nucleotides, e.g., $(CT)_n$ or $(CAG)_n$, that are repeated five to dozens and sometimes hundreds of times and are found in great abundance dispersed all over the genomes of all organisms investigated so far. This abundance together with the large number of alleles, resulting from high mutation rates because of their special, regular structure, makes them highly useful molecular markers at the population level. Microsatellite polymorphisms can be revealed where other marker types have failed and

therefore they are especially useful for species that otherwise lack a high degree of polymorphism, such as inbreeding species like important crops as soybean, or clonal species like planktonic algae that do not have a regular sexual cycle. Comparisons of different marker types have shown that microsatellites have the highest degree of polymorphism of all commonly used marker types. Both genetic diversity and gene flow can be calculated from this marker. Microsatellite markers usually fail to resolve any genetic structure only when the populations are very recently diverged and in this case AFLPs will usually provide better resolution (Alpermann 2009).

All MS studies have shown high genetic diversity in planktonic populations (see review in Medlin et al. 2000). Rynearson and Armbrust (2000, 2004, 2005, 2006) studied the diatom *Ditylum brightwellii* in the Puget Sound estuary. Four genetically distinct and highly diverse populations were identified that differed in the timing and localisation within the estuary over the course of seven years. Distinct physiological characteristics were associated with each genetically distinct population. Genetically distinct populations in the upper basin of the estuary were never found in the lower basin of the estuary despite a constant flushing rate from the upper basin to the lower basin. In a study of more localised area, the flagellate *Heterosigma akashiwo* around Japan was composed of distinct populations with little evidence for gene flow between them even though tidal currents would permit natural dispersal of the cells from one area to another (Nagai et al., 2007). The global cosmopolitan coccolithophore, *Emiliania huxleyi* is highly diverse (Iglesias-Rodriguez et al., 2006) with disjunct global populations and little gene flow between populations in North Atlantic and Norwegian fjords. The Norwegian fjords were resampled 10 years apart with a shift in the genetic structure and only one genotype being shared by the population sampled in 1990 and in 2000. An estimate of the number of unique genotypes of *E. huxleyi* on a global basis was 9.4×10^{20}, a number scarcely believable when most oceanographers think that blooms are clonal and modellers only use one strain of a species in their models for climate change. More recently, the level genetic polymorphism of one phytoplanktonic eukaryote, *Ostreococcus tauri* has been estimated in the Gulf of Lion by using a population genomic approach to target neutral evolving genomic regions (Piganeau et al., 2010) that showed no spatial structure of these species in the Gulf of Lion and provided evidence for recombination in the ancestry of 17 isolates. With the development of Next Generation Sequencing, genetic diversity of whole communities will be available from metagenomic data. The planktonic cosmopolitan diatom *Pseudo-nitzschia multiseries* also contains genetically distinct and highly diverse and distinct gene pools between North American and European populations (Evans et al., 2004), whereas a morphologically similar cosmopolitan species, *Pseudo-nitzschia pungens*, is also highly diverse but with little population structure in local areas (Evans et al., 2005) but globally with distinct populations corresponding to major oceanic water masses (Casteleyn et al., 2010). However, all global isolates can interbreed (Chepurnov et al., 2005) and thus this species is the only example of a planktonic protist so far tested with a global gene pool with distinct population structure. In the toxic dinoflagellate, *Alexandrium tamarense*, microsatellites revealed four populations in the study area around the Orkney Islands, which were assumed to be temporal populations that had resulted form the inoculation of different year classes from the cyst beds in the current year's bloom (Alpermann 2009). All clones were phenotypically distinct. In the freshwater diatom, *Sellaphora capitata*, MS revealed that only a small number of alleles from water bodies in Scotland, England, Belgium and Australia could be found in all isolates (Evans et al., 2009), indicating a limited dispersal between populations, although all isolates could still

interbreed. In the antarctic haptophyte *Phaeocytis antarctica*, each gyre in around the Antarctic had a unique genonotype and the Antarctic Circumpolar Current disperses genotypes from one gyre to another (Gaebler-Schwarz 2009). It is obvious that not only lakes but also oceans have genetically distinct populations with varying amounts of gene flow between them, some separated temporally and others spatially (Medlin 2007).

5. Molecular probes for identification and characterization of marine phytoplankton

Quite often morphological features as seen by light microscopy are not sufficient to distinguish clearly between species or groups of phytoplankton or marine bacteria. Therefore, more expensive methods, such as electron-microscopy or analysis of specific chemical components by HPLC, are needed to identify with certainly any species, but these are laborious, time-consuming and expensive. An alternative approach is to use specific molecular probes. Probes are short oligonucleotides of normally 16-24 bp length that are hundred percent homologous only to a complementary sequence in a gene of the species of interest and differ by at least one position to all other organisms. In hybridisation experiments, these probes can therefore be used to identify species of interest by binding to the target's sequence and later detection by a probe-attached label, e.g., Digoxigenin (DIG) or a fluorochrome like Fluorescein (FITC, Fig. 1). The application range of these probes extends from answering ecological questions, such as species composition and its change through space and time to the development of an early warning system for harmful algal blooms using probes for toxic species.

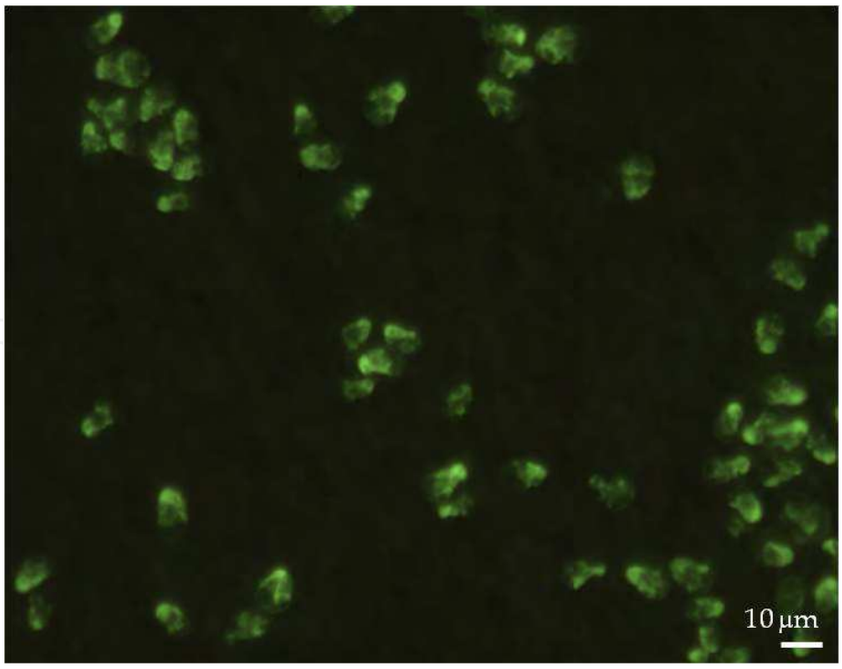

Fig. 1. FISH of the pear shaped toxigenic dinoflagellate *Azadinium spinosum*, x40.

The use of rDNA sequences has also other advantages for probe design. First, this molecule has regions with different degrees of conservation, which makes it possible to develop

probes for higher taxonomic groups (class level probes, e.g., for prymnesiophytes Simon et al., 2000, probes for groups of related species ("clades") [clades of toxic or non-toxic *Chrysochromulina/Prymnesium* species (Simon et al., 2000), genus-specific probes for *Phaeocystis* species (Lange et al., 1996) down to species- or even strain-level probes for *Chrysochromulina polylepis (*Simon et al., 2000) and the toxic North American clade of *Alexandrium tamarense* (John et al., 2003). This hierarchical approach makes it easier to analyze field samples because higher level probes can be applied to the samples and then, depending on these results, only probes of a corresponding lower level need be used, therefore, reducing the number of necessary experiments. Because of the limited number of fluorochormes, usually two different, e.g., FITC and Cy5, are all that can be used in a single experiment especially if the taxa under investigation are photosynthethic. Second, the use of probes for rDNA allows them also to bind to the rRNA of ribosomes *in situ*, making it possible to use fluorochrome-labelled probes in whole-cell hybridisation experiments (FISH). The thousands of ribosomes provide enough targets for probe binding and therefore, strong enough signals to be detected. If this is not the case, i.e., in picoplankton and also in bacterial cells, which often show weaker signals because of their small size and therefore lower ribosome content, techniques like catalyzed reporter deposition–fluorescence *in situ* hybridization (CARD-FISH, Fig. 2) can be used to boost the signal strength up to a detectable level (Schönhuber et al., 1997; 1999). This method combined with FISH increases the intensity of fluorescence and thus raises the detection limit and the signal/noise ratio, which is critical for small cells and results in a strong signal enhancement of the hybridized cells up to 20 times compared to probes with a single fluorochrome (Fig. 2). CARD-FISH has been shown to be very useful in the detection of cyanobacteria (Schönhuber et al., 1997; 1999; West et al., 2001), picoplankton cells (Biegala et al., 2003, Not et al., 2004; 2002) and bacteria associated with micro algae (Biegala et al., 2002, Alverca et al., 2002).

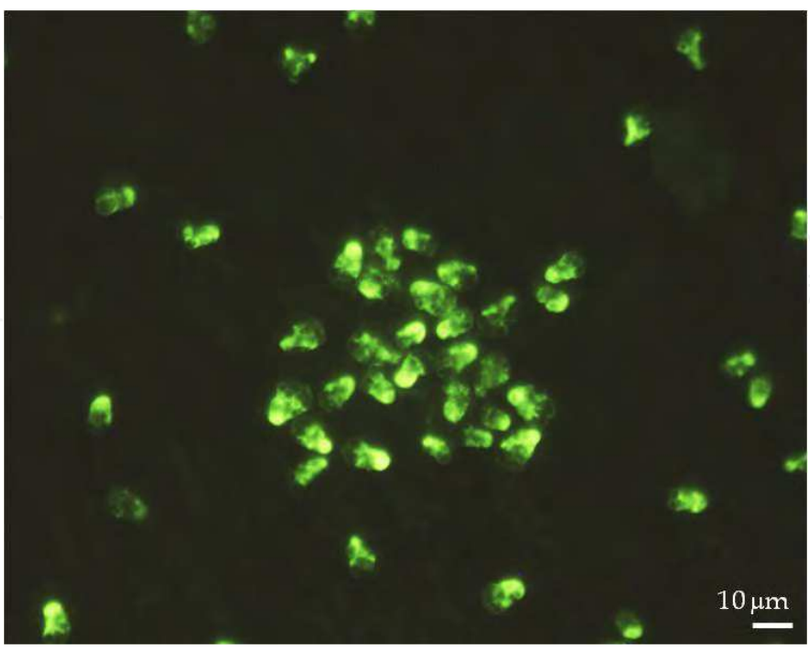

Fig. 2. CARD-FISH of the pear shaped toxigenic dinoflagellate *Azadinium spinosum*, x40.

Fluorescence *in situ* hybridisation targeting ribosomal RNA molecules has been often used for identification of harmful species in field samples. This molecular biological detecting tool is often deployed together with other molecular biological techniques, like quantitative PCR, to visualize the morphology of harmful algal species and for morphological comparisons using traditional methods, such as Utermöhl counts. Another important benefit of (CARD-) FISH is the potential detection and morphological visualization of unculturable harmful microalgae, such as the diarrhoeic shellfish poising causing dinoflagellate *Dinophysis*.

Higher group level probes, species, or even strain specific oligonucleotide probes for FISH are available for many taxa and enable the differentiation of morphological similar co-occurring species, especially for harmful algal bloom (HAB) species. Probes are available for detecting toxic species of the marine pennate diatom genus *Pseudo-nitzschia* that are associated with domoic acid in natural samples (e.g., Scholin et al., 1996 & 1997) as well as for toxic species in the important bloom forming microalgae, *Chrysochromulina* (Simon et al., 2000) and *Prymnesium* (Simon et al., 2000; Töbe et al., 2006) from marine and brackish environments. Probes targeting the different species of the toxigenic paralytic shellfish toxin (PSP) producing dinoflagellate genus *Alexandrium* include those detecting the North American *Alexandrium tamarense* ribotype (Miller and Scholin 1998; John et al., 2003), the toxic Temperate Asian ribotype (Hosoi-Tanabe and Sako 2005), the non-toxic Western European ribotype (John et al., 2005, Touzet et al. 2008), the non-toxic Mediterranean species complex (John et al., 2005), *A. minutum*, the non-toxic co-occurring morphologically similar species *A. andersoni* in Irish coastal waters (Touzet and Raine 2007; Touzet et al. 2008) and *A. peruvianum* (Touzet et al., 2011) and the spirolide producing taxa *A. ostenfeldii* (John et al., 2003, Touzet et al., 2011) All of these probes have been applied in field studies (John et al., 2005; Anderson et al., 2005). A set of FISH probes are also developed (John et al., in prep.) for the newly described genus *Azadinium*, (Tillmann et al., 2009) which comprises three different species, the toxigenic species *A. spinosum* and the non-toxic representatives. *A. obesum* & the very recently described species *Azadinium poporum* (Tillmann et al. 2011). *Azadinium spinosum* has been shown as the culprit for Azaspiracids, the most recently discovered group of lipophilic marine biotoxins of microalgal origin associated with human incidents of shellfish poisoning (Tillman et al., 2009 & 2011).

Fixation is a critical point in FISH applications and a suitable fixation method for species that are difficult to fix should be developed prior to any FISH application The harmful radiophyte *Heterosigma akashiwo*, which causes mass mortalities of cultured fish, alters its morphology dramatically after fixation with various commonly used fixatives. Chen et al. (2008) found that the gentle, but effective saline ethanol fixation method described by Miller and Scholin (1996 & 2000) is suitable for the fragile cells of *H. akashiwo* and does not cause clumping or breaking of the cells as does formalin or glutaraldehyde fixation. However, even this gentle fixation method can slightly change the cell's morphology, but this distorted morphology does not have a negative effect on the FISH signals of the newly developed species specific probes for *H. akashiwo* (Chen et al., 2008).

CARD-FISH has been deployed to a lesser extent in HAB studies, most probably because of the higher cost of the labelled probe, the chemicals used for signal enhancement and the additional needed time for the signal enhancement. Rehnstam-Holm et al. (2002) developed a genus specific probe for *Dinophysis* and applied it successfully in CARD-FISH experiments with field samples containing different *Dinophysis* species. This FISH technique has considerably alleviated a persistent autofluorescence of species, such as that exhibited by

Prymnesium parvum cells (Töbe et al., 2006) or to detect ingested cells in the guts of other species.

Despite the numerous advantages of FISH applications in HAB studies, the use of other techniques, such as high sample throughput techniques to analyse bulk environmental samples in a shorter time have not been widely applied to field samples. One such method of detection is by flow cytometry, which is not *per se* a molecular biological method, but can be used in combination with molecular probes to great advantage to analyze large numbers of cells (Wallner et al., 1993). CARD-FISH has been successfully applied for the identification and enumeration of phytoplankton cells by flow cytometry (Biegela et al., 2003). Probes have been used with the solid phase cytometer to scan and enumerate all cells on a filter (*Prymnesium parvum* in Töbe et al., 2006, cryptomonads in Medlin & Schmidt 2010). In Medlin & Schmidt (2010), a hierarchical probe approach was used to study the cryptomonads in Arcachon Bay, France and they were able to show that genera belonging to Clade 3 were dominant in this bay system.

When extracted DNA is available, another method for the use of oligonucleotide probes is as PCR primers. A specific oligonucleotide in combination with a matching primer from a highly conserved region of the same gene should only amplify a product if the DNA comes from the species for which the oligonucleotide probe was designed. Nevertheless, when a probe can be used this way, this method is much faster than a dot blot hybridisation in detecting the presence of a certain type of organism, which can even be quantified through the use of quantitative real-time PCR (qPCR).

qPCR enables a high sample throughput and several species can be detected at a time even when only small sample volumes are available. Data are collected over the entire PCR cycle by using fluorescent markers that are incorporated into the PCR amplicon during amplification and directly in the exponential phase where PCR is precise, thus avoiding the problem of the amplification plateau of qualitative PCR experiments. The increasing fluorescence is measured and the change in fluorescence is directly proportional to the amount of starting material (Demir et al. 2008). Different qPCR chemistries are available, depending on fluorescent dyes binding to double stranded DNA (dsDNA) or the application of fluorescently labelled species-specific oligonucleotide probes. SYBR Green is the most commonly used methods used in qPCR applications using primers specific for the target DNA. In another more sensitive and specific approach primers together with a specific fluorigenic oligonucleotide probe are used. This probe based qPCR approaches enables the detection of several different original templates in one sample, whereby the number of detectable target genes in one sample is limited by the number of available fluorescence reporter dyes for the separate probes, which can be excited by the qPCR instrument. However, these multiplex qPCR experiments have to be carefully optimized (Kudela et al. 2010). However, results of qPCR experiments could be hampered by external influences, e.g., different DNA extraction yields depending on the extraction method used and the presence of humic substances that could influence or even inhibit the PCR reaction, possibly resulting in discrepancies between traditional cell counts and qPCR determined cell counts. Therefore, the (quantitative) species composition of the investigated habitat could be incorrectly recorded. These problems could be resolved or at least minimized by applying a standardized DNA isolation method generating high quality DNA samples and the use of an internal standard in some of the environmental samples to monitor the amplification efficiency of the qPCR experiment.

There are already examples where the combination of real-time PCR and species specific primers/probes has been successfully applied, e.g., for the detection and enumeration of the

A. catenella/fundyense/tamarense species complex (Dyhrman et al. 2006 & 2010), and with the rapid progress in technology, this will likely be a promising method for routine monitoring of selected species.

The previously described techniques are powerful and highly quantitative tools for the identification of microbial organisms. However, they all have the drawback that they are single probe approaches that are limited to the analysis of only one or a few targets at a time. The introduction of the concept of DNA microarrays about ten years ago suggests an option to void the limitations of single probe approaches. DNA microarray-experiments are multiplexed assays that provide the possibility for high throughput analysis of molecular probe based species identification without a cultivation step. This is of special interest for the identification of prokaryotic and eukaryotic cells with very small sizes and few distinct morphological features. This provides an aid to science because taxonomists are not being trained because this field is perceived to be no longer needed with the new age of molecular analyses. Therefore, DNA microarrays might be of special value for phycological studies because they represent a tool that does not require a broad taxonomic knowledge to identify cells. Consequently there are a growing number of publications that report the use of microarrays bearing molecular probes that target the rRNA for the identification of microbial species. They have been used successfully in combination with an amplification of the rRNA-gene for the identification of phytoplankton, bacteria, bacterial fish pathogens, and sulfate reducing prokaryotes (Gescher et al.,, 2008, Manz et al., 1992, Giovanonni et al., 1990, Pace et al., 1986, Rehnstam et al., 1993). DNA-microarrays allow the parallel analysis of almost infinite numbers of probes at a time in just one experiment. The technology is based on a DNA-microchip that contains an ordered array of molecular probes on its surface (Fig. 3).

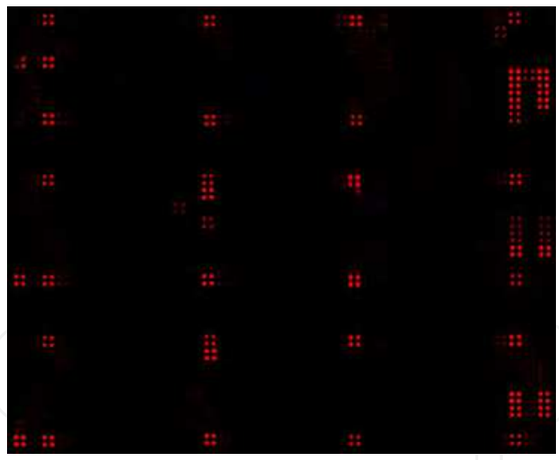

Fig. 3. Image of a scanned DNA-microarray from field sample taken in OsloFjord, Norway. Each cluster of 4 dots represents replicate probes specific for one species spotted onto the glass slides and hybridised to fluorescently labelled RNA from the field sample. The DNA-chip contained probes for various toxic phytoplankton taxa. Photo taken by Dr. S. Dittami for the EU MIDTAL project.

All these methods have, despite their high set up costs for the machines, the disadvantage of requiring quite bulky pieces of equipment, hence, making them difficult to be used in the field and on-board ship. This is of particular interest for the monitoring programs for toxic algae, where samples are taken regularly at places that are not in close proximity of

laboratories that host the previously described equipment. DNA-biosensors could serve the needs of monitoring programs for an easy-to-handle and inexpensive tool. Electrochemical readings of DNA-biosensors are unambiguous and even for a scientific layperson easy to use and interpret. There are a number of examples for the application of DNA-biosensors that have been developed for the identification of organisms, including toxic algae. The application of a DNA-biosensor for the identification of organisms is again based on taxon specific molecular probes, of which a large number already exists for toxic algae.

A DNA-biosensor has been adapted for the electrochemical detection of the toxic dinoflagellate *Alexandrium tamarense, ostenfeldii* and *minutum* (Diercks et al., 2008, Metfies et al., 2005; Diercks et al. & 2011, respectively). The DNA-biosensor detection reaction is a sandwich-hybridisation that takes place on a carbon electrode on a disposable chip. A sandwich-hybridisation is based on a set of two specific molecular probes that bind in close proximity to the target nucleic acid. One probe, termed the capture probe, is immobilised via biotin on the carbon electrode, which is coated with avidin. The second probe, termed the signal probe, mediates the detection reaction, if target DNA is captured by hybridisation to the capture probe on the carbon electrode. The second probe is recognized by an antibody that is coupled to a horseradish-peroxidase that catalyses the red/ox reaction of hydrogen peroxide to water. The electron-transfer during the red/ox-reaction can be measured as a current, which is only possible if the target nucleic acid as a link between the capture and signal probes is present in the system. Experiments with RNA isolated from laboratory strains showed, that the electrochemical signal is proportional to the amount of target RNA applied to the sensor. The device was expanded in the EU ALGADEC project to regional chips for up to 14 harmful algal species at a time. In order to serve the needs of the monitoring programs that aim to count all potentially harmful algae in a geographic area it would be indispensable to adapt the present DNA-biosensor to a bigger range of toxic algae. This device is not yet available to the general public, nevertheless the method has a high potential to become a powerful monitoring tool in the future.

As it can be realised, there are numerous techniques possible for analysing multiple samples with specific probes automatically and more are surely to come. With them the way is open for mass screening of water samples for the detection of interesting marine species like toxic algae, even as there are still some problems to be solved and methods to optimise before they can be routinely used for this kind of purpose.

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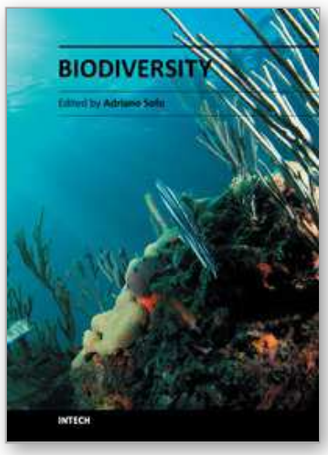

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Biodiversity is strongly affected by the rapid and accelerating changes in the global climate, which largely stem from human activity. Anthropogenic activities are causing highly influential impacts on species persistence. The sustained environmental change wildlife is experiencing may surpass the capacity of developmental, genetic, and demographic mechanisms that populations have developed to deal with these alterations. How biodiversity is perceived and maintained affects ecosystem functioning as well as how the goods and services that ecosystems provide to humans can be used. Recognizing biodiversity is essential to preserve wildlife. Furthermore, the measure, management and protection of ecosystem biodiversity requires different and innovative approaches. For all these reasons, the aim of the present book is to give an up-to-date overview of the studies on biodiversity at all levels, in order to better understand the dynamics and the mechanisms at the basis of the richness of life forms both in terrestrial (including agro-ecosystems) and marine environments.

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