Marine Microbial Community Fingerprinting: An Analysis between "classical" Microscopy and DGGE

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Abstract:
The world's oceans contain on average 100.000 microbes per milliliter of seawater and despite their size these microbes are the biological motors that drive global and local biogeochemical cycles and processes. Community analysis has always been of importance to the scientific world and helps in understanding the mechanisms and the flow behind diversity. This literature research aims to discuss the differences and commonalities between the microscopy technique and various fingerprinting and identification techniques. The PCR-based method of DGGE is discussed in-depth due to its increasing use in the marine microbial world. Over the past 20 years a lot of research has been done to replace and/or aid the "classical" microscopy techniques due to its limitations especially in marine planktonic communities. Molecular techniques such as DGGE and clone libraries have been adapted in order to easily fingerprint communities through their 16s and 18s ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). Although more and more species are discovered through their respective sequences, it is unclear whether these are different morphotypes of a single species or an actual new species. Because of these uncertainties it is important to realize that morphological information still plays a vital role in community analysis.

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1. Introduction:

Plankton, in particular pico- and nanoplanktons, dominate the photosynthetic biomass in many marine ecosystems. Their range is exceptional as they are found both in oligotrophic and mesotrophic regions and are not even bound to pelagic environments. Pico- and nanoplankton also appear in coastal regions throughout the year where they constitute the 'background' population and can develop episodic phenomena such as spring blooms (*Medlin et al*, 2006). Pico- and nanoplankton are defined by their size. In general all phototrophic and heterotrophic prokaryotes and eukaryotes smaller than 20 µm but larger than 2 µm are considered nanoplankton. All phototrophic and heterotrophic prokaryotes and eukaryotes smaller than 2 µm and larger than 0.2 µm are considered picoplankton (*Medlin et al*, 2006, *Díez et al*, 2001). Plankton numbers are generally high and their concentrations can range between 10^2 and 10^4 cells per ml in the upper photic zone (*Díez et al*, 2001). In general trophic status (*Unrein et al*, 2005) and the hydrographic or a-biotic profile (*Wu et al*, 2009) of a body of water are the most important factors influencing planktonic communities. Diversity and abundance are directly correlated in the sense that in spring diversity is generally highest whereas in the summer abundance increases drastically due to their blooming characteristics but diversity decreases due to competition (*Savin et al*, 2004). These correlations are dependent on the climate of the studied area.

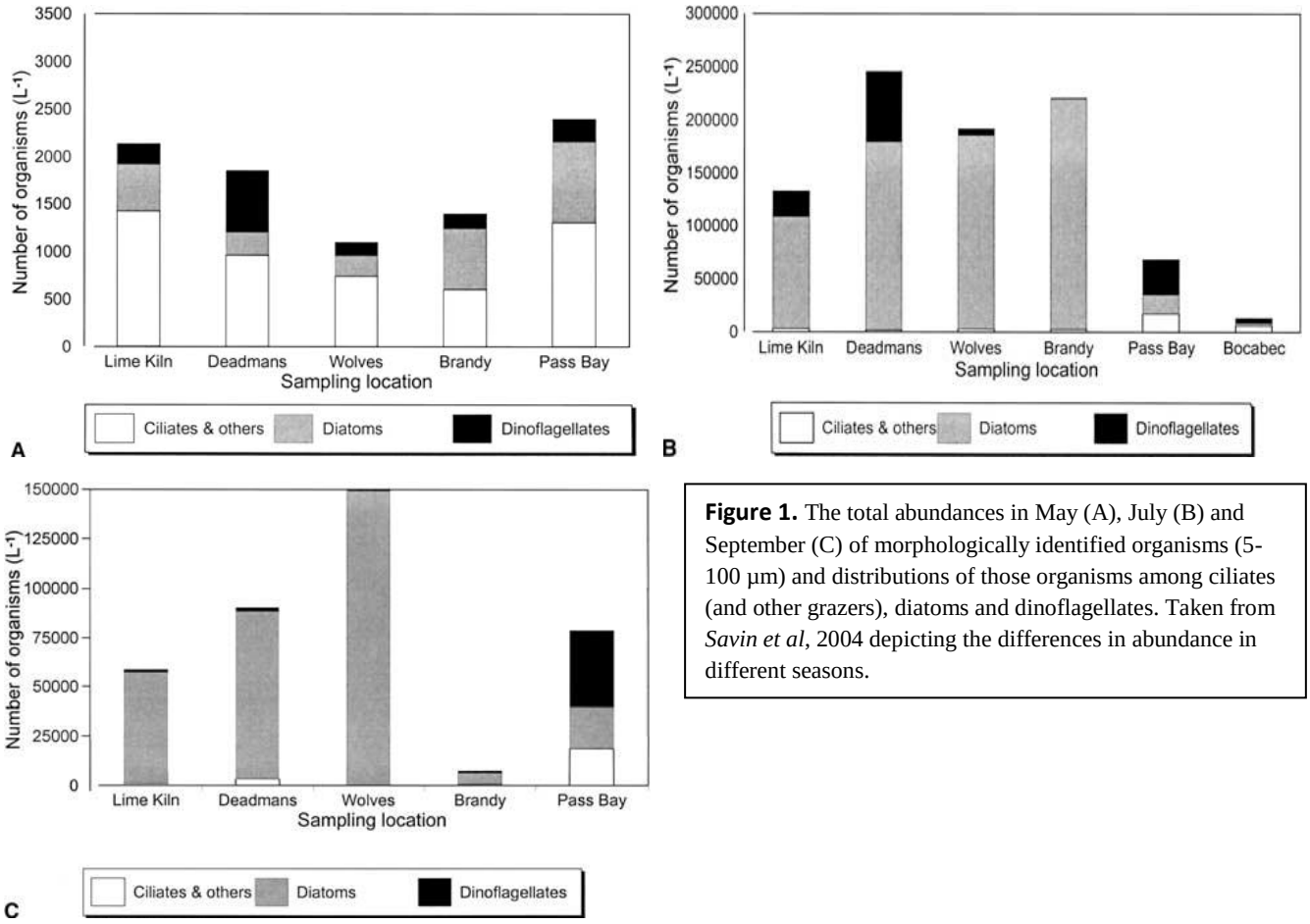

Figure 1. The total abundances in May (A), July (B) and September (C) of morphologically identified organisms (5-100 µm) and distributions of those organisms among ciliates (and other grazers), diatoms and dinoflagellates. Taken from *Savin et al*, 2004 depicting the differences in abundance in different seasons.

Due to the size and lack of distinctive taxonomic differences of pico- and nanoplankton, direct identification by microscopy is often problematic. However both prokaryotic and eukaryotic plankton identification can take advantage of several culture independent techniques (*Díez et al*, 2001) such as denaturing gel gradient electrophoresis (DGGE) (*Roelfsema et al*, 2005)(*Yan et al*, 2007)(*Díez et al*, 2001)(*Savin et al*, 2004)(*Wu et al*, 2009)(*Unrein et al*, 2005)(*Tian et*

al, 2009)(*Piquet et al*, 2009), single-strand conformation polymorphism (SSCP)(*Medlin et al*, 2006), terminal restriction fragment length polymorphism analysis (t-RFLP) (*Schütte et al*, 2008), high performance liquid chromatography (HPLC) (*Kazakevich et al*, 2007, *Savin et al*, 2004) and fluorescent *in situ* hybridization (FISH) (*Daims et al*, 2007). These techniques could confirm the results of the morphological analysis. These techniques are used for the screening of a community and further analysis of the results through DNA microarrays (*Medlin et al*, 2006) or clone libraries is needed in order to fully identify an organism to the species or genus level (*Díez et al*, 2001). Methods like DGGE are currently still under heavy development in order to, in the end, solely use these techniques to define single species, because in its current state further analysis is needed in the form of clone libraries and or sequencing.

With other microorganisms this is also done through cultivation and although that has led to the discovery of previously unidentified organisms, only a small percentage of the planktonic diversity can currently be cultured (*Savin et al*, 2004).

A paper by *Pedrós-Alió* (2006) discusses the magnitude of the range of microbial species on earth. He quotes ecologist Robert May: "we have a catalog of all the celestial bodies our instruments can detect in the universe, but we ignore how many living beings share the Earth with us" (ISME meeting, Barcelona, 1992) stating this is particularly true for life in the oceans. The truth to the statement that very little is known about the diversity in the oceans is confirmed by the fact that some articles discussed in this literature research (*Díez et al*, 2001)(*Savin et al*, 2004)(*Wu et al*, 2009)(*Medlin et al*, 2006), that used a molecular technique, have reason to believe they have discovered a novel organism/species.

Because of all these novel organisms and species being discovered science gains a growing understanding of the molecular and genetic features of these organisms. However without morphological identification a plethora of questions arise when comparing sequences to the GENBANK (http://www.ncbi.nlm.nih.gov/Genbank/). In order to get a clear picture of current fingerprinting techniques and their applications it is vital to look at all aspects of this field of research and to answer several important questions. These questions are:

- Which techniques can be applied in order to screen the planktonic communities in marine environments?
- Why are certain techniques favorable to specific situations?
- Is the classical approach redundant when compared to the fingerprinting techniques or is it still a viable method?

This literature research aims to compare and assess the classical way of community analysis (microscopy) with a "new age" molecular approach, in this case DGGE. This technique is currently favored by the scientific community to perform marine microbial community fingerprinting.

2. The goal of the fingerprinting approaches:

Taxonomic identification and analysis has historically been a difficult task to master. Even the seasoned taxonomist is prone to err due to the lack of distinguishing features, especially in many not yet described, yet abundant micro-organisms (*Yan et al*, 2007). This "classical" approach is highly valuable in order to examine single species because it aims to describe a certain species individually, providing ways to distinguish it from other species in its family or genus.

As mentioned previously there is no real estimate of the amount of species that exist on this planet today. *Pedrós-Alió* (2006) compared the total amount species on earth (biodiversity) to the amount of stars in the universe; we are unable to make an estimation of the numbers and therefore we should not. Biodiversity is defined as the total genetic information on Earth or any part of it. Diversity would then be the components that are abundant and active at one particular time and place (*Pedrós-Alió*, 2006). By these definitions studies aiming to describe biodiversity would have no real conclusion or result until all genetic information on this planet has been identified and processed. Although these statements are based on all the genetic information on this planet it is most likely equally true for the marine microbial systems (*Pedrós-Alió*, 2006).

Diversity however, is something that can actually be investigated at this time and that is what the fingerprinting techniques are aiming at. When it comes to investigating diversity or biodiversity the routine microscopy approach is no longer favored due to the fact that classical microscopy is a very time consuming method and when one aims to describe and compare the diversity between numerous samples classical optical microscopy is just not very effective. Molecular approaches on the other hand also aim to discover the diversity in a given sample and claim to be faster. This is not always true since DGGE and other molecular approaches alike rely on additional methods like clone libraries to be actually definitive in their conclusion. However it is true that they are less labor intensive allowing for more time efficiency.

There are a lot of marine microbial studies that benefit from molecular techniques. These researches for instance investigate the spatial and/or temporal shifts in diversity (*Piquet et al,* 2009). It has been used to compare the diversity between the spring, summer and fall months to see if there is any substantial difference in species composition and abundance (*Piquet et al,* 2009). Paired with the a-biotic profiles of the locations it is possible to gain a deeper understanding of the driving forces behind changes in the system throughout the year (*Piquet et al,* 2009). Not only temporal changes but also different locations can be analyzed and easily compared through the use of these molecular techniques. The effects of glacial melting on arctic marine microbes (*Piquet et al,* 2009) could also be identified through use of these techniques. In order to compare the effects on species diversity, in relation to differences in stratification in glacier systems, fingerprinting techniques could provide a clear overview.

Unfortunately a way to quantify organisms efficiently has yet to be discovered. The method that gives the best estimate is to count the organisms by hand using a microscope. However given the lack of distinctive features of some species, these counts are only accurate for a limited number of species (the ones that are distinguishable). Several suggestions have been made to apply polymerase chain reaction (PCR) and DGGE or T-RFLP as methods to quantify organisms. However, there is one major flaw in this method. The number of gene copies is not convertible to cell numbers because the target gene copy number per genome is unknown and the number of genome copies per cell can vary in different growth phases (*Daims et al*, 2007)(*Yu et al,* 2009).

3. The different techniques

Prokaryotic and eukaryotic plankton can be identified effectively by utilizing several different techniques. Some of these techniques are favored because of their ease of use and others for their accuracy. In order to explain the specific choice of comparing and discussing PCR-DGGE with classical optical microscopy, a clear picture of DGGE and the other molecular techniques like SSCP and t-RFLP needs to be created and discussed. Keep in mind that none of the non-microscopy techniques discussed in this literature study give a decisive answer as to what organisms are present within the samples that are studied. Only techniques such as DNA microarrays (*Medlin et al,* 2006), clone libraries, culture dependant studies and/or genomic sequencing will be exact enough to identify an organism to their species level. These techniques will also be touched on briefly although they are not the focus of this literature research.

When screening a planktonic community it is essential to realize that not every method will yield the same results. For instance DGGE will give a measure of diversity but only for the specific primer set used. Specific primer sets influence the size and the location of the DNA/RNA fragment that is investigated and could therefore yield a different banding pattern between primer sets. But, if the goal of the research is to obtain the (taxonomic) class specific abundances, HPLC is the way to go even though it lacks in accuracy when compared to the other methods.

Before the majority of molecular techniques can be executed the DNA/RNA fragments that are going to be analyzed have to be amplified because usually a given sample has too little genetic material to be investigated properly. Polymerase chain reaction (PCR) is a method designed to amplify DNA/RNA. The full DNA/RNA is extracted from the sample and then merged with a primer that cuts the DNA/RNA into the pieces that are to be investigated. These sequences are put into a PCR together with primers that regulate transcription of the particular sequence (for both the 3' to 5' and 5'to 3' direction). During a number of cycles the strings of DNA/RNA are denaturated (pulled apart, usually at high temperature (50-55 ºC)) and then annealed by the sequence specific primer. Then transcription occurs using nucleotides present in the PCR mixture and the sequence is rebuilt. This rebuilding is done in order to increase the number of copies. The principle is that when starting with 1 double-stranded copy it will denaturize into 2 single-strand copies. These 2 will each be transcribed into 2 new double-stranded pieces of DNA/RNA. Those 2 will become 4 pieces of double-stranded DNA/RNA and so on (*Vosberg,* 1989).

This literature research will discuss 5 "modern" techniques used to screen species composition and diversity in aquatic samples. The techniques discussed here are: DGGE, SSCP, t-RFLP, HPLC and FISH**.**

3.1 Denaturing gel gradient electrophoresis (DGGE): DGGE is a PCR based method that was designed to detect point mutations in the human genome. DGGE was designed to analyze various genes involved in genetic diseases (e.g. colorectal cancer, muscular dystrophy). DGGE is also able to detect deletions and insertions in DNA sequences. This allows it to be very precise and to be utilized in the highly conserved regions of both 18s and 16s DNA. The process of DGGE detects the difference in denaturing temperature in the PCR product. The PCR products migrate through the porous gel at a given speed determined by the size and sequence. If denaturation occurs at a "low" temperature the PCR products does not migrate far through the gel (*Roelfsema et al*, 2005). In order to stop the double-stranded DNA from fully separating a GC-clamp is attached to the PCR primer. This will attach a string of 40-60 nucleotides composed of only guanine and cytosine with a very high denaturing gradient. This will create a Y-shaped conformation that will stop migration through the gel.

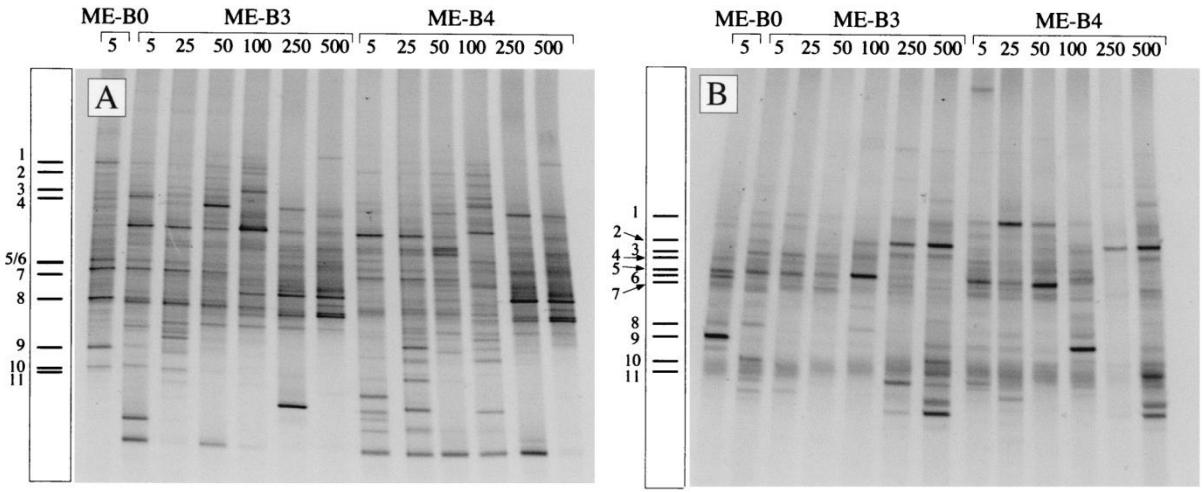

Figure 2. An example of a DGGE profile from *Díez et al* (2001) displaying the banded pattern created by the samples. The vertical lanes depict single samples and each band depicts a single species. The fingerprints were obtained with primer set A (A) and primer set B (B).

3.2 Single-strand conformation polymorphism (SSCP): SSCP was initially developed for the analysis of bacterial communities but through the adaptation of the PCR product through the use of different primer sets even eukaryotic DNA (18s) could be processed. SSCP is a PCR-based technique in which a fraction of the 16s/18s is amplified and a single strand product is created by the digestion of the opposite strand. The single strands are separated in an acrylamide gel. This is because the single-strand DNA is undergoing conformational changes and/or folding and thereby differentiating its flow through the gel. Since all the species in the sample have different nucleotide sequences between the primer regions the strands all fold differently and thereby produce a unique band in the gel. Just like with the DGGE method the primer sets 'cut' at a region where the DNA sequence is most preserved. In contrast to DGGE the separation takes place in non-denaturing gels, which are easier to handle (*Medlin et al*, 2006). It is assumed that each band represents a single species and bands that occur on the same visual height are considered to be the same species. This has been tested and confirmed by excising the bands and sequencing the fragments (*Medlin et al*, 2006). The graphical output of the SSCP analysis is similar to the DGGE output depicted in figure 2.

3.3 Terminal restriction fragment length polymorphism analysis (t-RFLP): t-RFLP is just like SSCP and DGGE applied to screen/fingerprint a community of prokaryotic or eukaryotic species. This technique amplifies a small subunit of either 16s or 18s ribosomal RNA genes from the sample DNA using PCR where one or both of the primers are labeled with a fluorescent dye. The resulting mixture of rRNA gene amplicons is then digested with one or more restriction enzymes that have four base-pair recognition sites. These fluorescent labeled T-RFs are then determined by size and relative abundance and then sequenced by an automated DNA sequencer. The fingerprint is based on the differences in size between the sequences and the differences in the sequence itself (*Schütte et al*, 2008).

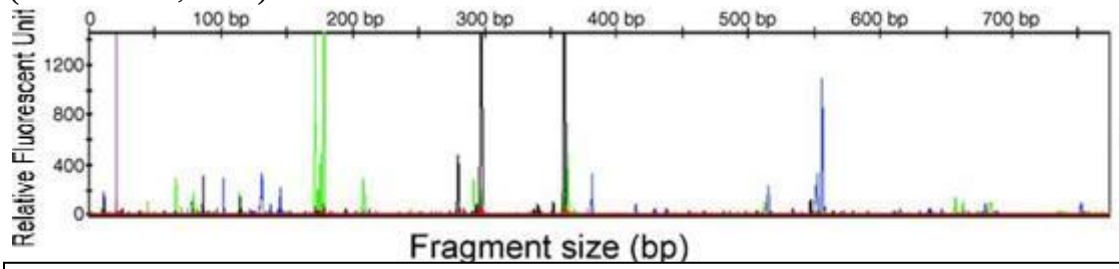

Figure 3. An example of a M-TRFLP profile from *Schütte et al* (2001) displaying profiles obtained for archaeal, bacterial and fungal communities for a single sample. Bacterial (*green*), fungal (*blue*) and archaeal (*yellow*) obtained from PCR products.

3.4 High performance liquid chromatography (HPLC): HPLC is the method of pigment separation in a column. A liquidized sample is introduced to a high pressure compartment in the machine. The sample is then being forced through the column (a tube filled with porous material also known as the stationary phase) that will separate the pigments via another liquid (mobile phase). The different pigments react different to the phases (physically and chemically) and get separated. The separated pigments move through the stationary phase at different speeds (according to their affinity for the different phases) passing through the detector. The detector measures a chemical or physical property of each now relatively pure compound and creates a proportional electronic signal. By calibrating with a standard mixture with known compounds the nature of the sample can be identified (*Kazakevich et al,* 2007). HPLC can group plankton by pigment characteristics but placement is at a family level and is not species or even genus specific (*Savin et al,* 2004). The algorithms proposed to transfer pigment counts to biomass are powerful and satisfactory approximations when the ecological setting and biogeographic area have been defined and investigated previously, but are not reliably enough to be extrapolated to other ecosystems (*Lemaire et al,* 2002).

3.5 Fluorescence *in situ* hybridization (FISH): FISH is one of the most widely used fluorescence method used today (*Daims et al,* 2007). The goal of this method is to attach a fluorescent probe to 16s or 23s rRNA with rRNA-targeting oligonucleotide probes. Depending on the base sequence conservation, FISH can detect a single species or a larger phylogenetic target group and it is not PCR dependent. Although not really a method for fingerprinting it can be used to identify several species at once and it does give an easy way of counting the organisms. FISH allows for the simultaneous detection of up to 7 different populations in a single experiment. However it is not the most accurate technique due to the great amount of preservation present in the sequences of the targeted small and large subunit rRNA. This technique is mostly used to efficiently count cells under the microscope (*Daims et al,* 2007). By targeting a specific organism and attaching a fluorescent probe to its surface it is easy to distinguish it from the other organisms in the sample, simplifying the cell counting.

These techniques all provide a relatively basic to very exact fingerprint of a sample. However, they are far from absolute and further investigation needs to be done in order to identify the exact species composition of a given sample. Banding techniques like SSCP and DGGE all require the excision of a band which is then re-amplified using the PCR and then purified before it is sequenced but it is not always possible to excise every band needed resulting in more bias. This sequence will then give the definitive result when compared to a Genbank-like database. Not only excision of bands and then sequencing is an option if further information is needed, the construction of clone libraries will also yield detailed information about the species. The construction of clone libraries on its own is also a way to check for community diversity but it is not nearly as easy to compare as the fingerprinting methods described previously. The clone libraries are created from the DNA/RNA information in the samples. Where DGGE, t-RFLP and SSCP only look at a part of a particular gene, clone libraries aim to investigate (in the micro-eukaryotic case) the full 18s DNA/RNA (*Medina-Pons et al,* 2009). This is done to acquire a higher accuracy than the other molecular techniques. These pieces of DNA/RNA are amplified by use of the PCR method and then ligated into competent bacterial cells (e.g. pMD19-T *E. coli* DH% or JM 109 (Promega) (*Medina-Pons et al,* 2009). These bacterial cells with the, in this case, 18s inserted DNA/RNA vector are then selected for ampicillin resistance after being plated on a medium. If the ligation was a success the ampicillin resistance will be activated and several colonies will be

formed on the plates. These are then extracted and sequenced to gain information about the full 18s gene (*Medina-Pons et al,* 2009) (*Tian et al,* 2009).
Other techniques like HPLC are not always able to identify an organism to the species level and are mostly used in combination with the other techniques.
The reason why only DGGE and classical optical microscopy are the 2 techniques that are being compared to each other is actually quite simple. Where SSCP and t-RFLP look at a bigger part of the 16s and 18s DNA/RNA gene when compared to DGGE and could therefore be described as more accurate these techniques are also being utilized less frequent in the world of marine microbial fingerprinting.

4. Microscopy and DGGE: a comparison

4.1 Microscopy

The biggest issue is that only species that have been previously described through cultivation based methods can be identified by use of microscopy. Further confirmation is done by light, phase contrast, or scanning electron microscopy (*Savin et al*, 2004). However, many organisms cannot be cultivated yet and lack distinct morphological features and therefore there is high potential for encountering undescribed species in a given sample. As an example there are the unarmored dinoflagellates that are poorly described by morphological methods. They lack well-defined thecal plates and are hard to identify because of that. Therefore their taxonomy has changed only a little since the early 1900's (*Savin et al*, 2004). Because of the difficulty of morphological identification it has happened that even though that particular organism is the most abundant in a given sample, in the end of the research it remains a mystery as to what species that particular organism is. The biggest problem of identification by microscope is that only real experienced taxonomists are able to analyze samples properly. There are over a thousand species (*Savin et al,* 2004) of eukaryotic plankton currently described by their 18s rDNA sequence and even more in the prokaryotic plankton. This is not even close to the amount that is morphologically described and a lot of sequences are missing from the database. For the morphological identification it takes a lot of time and practice to be able to know all these species, or to be able to quickly look them up in a guide is a huge investment.
Microscopy is not only used for identification it is also used for cell counts and abundance checks. However because of the size of these organisms it is hard to identify them and count them at the same time while sustaining some sort of efficiency. Microscopic counts are usually performed at a magnification of 400 times. However as an example in the research of *Unrein et al* (2005), *Chrysophyceae* unid 2 and *Ochromonas*-like species are very similar in size. Their main morphological difference is the presence of 1 or 2 flagella. Observation under a 1000 times magnification allows for perfect identification. Unfortunately this is not an advisable magnification to perform cell counts under (*Unrein et al*, 2005).

4.2 DGGE

DGGE is one of the oldest molecular techniques and utilized in many different ways. As previously mentioned it is used to check for point mutations in genetic diseases and it is also used to fingerprint microbial (and micro-eukaryotic) communities. DGGE is dependant mainly on 2 factors; the PCR and the primers that are used. The PCR is the process that is of most influence. The biggest problem with PCR is that it does not amplify all sequences at the same rate, there is something known as a PCR bias. This phenomenon has been described in for instance *Savin et al* (2004) where the microscopy approach found an abundance of diatoms in the given sample but very few were registered on the DGGE. They believed this was the cause of preferential PCR amplifications. Preferential PCR amplifications is basically

just what it sounds like, the primers used in the PCR are more likely to attach themselves to perfect match sequences in the DNA or RNA in the sample than to sequences that are very similar to the primer sequence but not a perfect match. This has nothing to do with any error since it has been proven that the primers actually do attach themselves to the imperfect sequences of the diatoms as long as there are very little perfect matches in a sample. This problem however can be solved by utilizing different primer sets when it is deemed necessary. The banding pattern on the DGGE seems to be very consistent. When performing a DGGE and comparing the banding patterns *Unrein et al* (2005) found that 2 or more bands on the same height gave virtually the same sequence after they had been excised. This proves that DGGE is an accurate method to screen a population, but only when taking into account all the different primer sets.
Currently (March 2010) there are only a couple of primer sets "standard" for eukaryotic investigations using DGGE: Euk1A (*Díez et al,* 2001), Euk516r (*Díez et al,* 2001), Euk1209f (*Díez et al,* 2001), Uni1392r (*Díez et al,* 2001), 1427GC (*Savin et al,* 2004) and R1616 (*Savin et al,* 2004). For prokaryotic and bacterial RNA there are several other primer sets like: F357GC R518 (*Yan et al,* 2007), F1427GC R1616 (*Yan et al,* 2007). Primers can be designed specifically for groups of organisms in order to avoid PCR bias by using computer programs like Winmelt from Bio-Rad Laboratories and Meltingeny from Ingeny International (*Roelfsema et al,* 2005).
DGGE is capable of differentiating sequences with a 99.6% similarity (*Unrein et al,* 2005) and usually only sequences of between 300 and 500 base pairs are considered (due to the decrease in accuracy with less base pairs) (*Unrein et al,* 2005). Because of the relatively small amount of base pairs that are considered in DGGE it has some clear limitations. The biggest problem is that there might not be a difference in band height when the sequences are too much alike causing 2 bands to overlap. There also seems to be a limit on the amount of bands a gel can produce with great accuracy. This could lead to only the showing of the most abundant species and even though novel species are found almost every time it could be that extremely rare species are not detected (*Unrein et al,* 2005).

At first sight both these techniques seem to have little or nothing to do with each other. However when looking back in time, biologists started out with doing community fingerprinting with a microscope. They would investigate a sample by screening the sample image by image and then both counting and identifying the organisms. Both classical and molecular approaches aim to discover the diversity in a given sample and the molecular approach claims to be the fastest. This is only true when the construction of clone libraries and the sequencing of the pieces of DNA/RNA are not taken into account. Techniques like DGGE are certainly faster when it comes to the point of purely stating diversity. Having 20 bands in one lane and only 12 bands at another lane and none or only a few are at the same height indeed indicates a difference in community structure, but that is simply not enough information. Science aims to be as conclusive as possible and where the identification of the individual species is already incorporated in the microscopy, DGGE and molecular approaches alike rely on additional methods like clone libraries to be conclusive. These methods are, just like microscopy, very time consuming. The problem with microscopy was not only that it took up too much time but that it required expertise in a particular field of morphology. The unavailability of this expertise caused people to go looking for identification methods elsewhere and start looking into techniques that were not designed for the identification of organisms. Of course there was skepticism from the microscopy side but in the end most research agrees that the data and results yielded by both microscopy and DGGE are very much alike (*Yan et al,* 2007). Both techniques yielded species the other did not reveal and both techniques failed to recognize certain species. Most studies also agree on that DGGE

fingerprinting is a valid way of community analysis and that it yields statistical viability as long as all the DGGE gels were done in the exact same way with the same solutions and primers. Inter primer comparison is not possible due to the differences in the nucleotide sequences and the length of the fragments (*Tourlomousis et al,* 2009).

5. Discussion:

Community analysis is a very important subject in the field of marine biology. It gives detailed information about organisms in a system and how certain a-biotic factors like location and seasonality affect these systems. However the big picture is still not clearly visible (*Pedrós-Alió,* 2006). There are a lot of organisms that have not been identified yet, both molecular and morphological, and it is not even sure what magnitude of novel organisms is missing from the databases. This could be hundreds of species, but more likely thousands or even millions (*Pedrós-Alió,* 2006). The modern molecular techniques are trying to replace the somewhat more classical approach and in a way there is nothing wrong with that. Several studies discussed in this thesis give undisputable evidence that molecular techniques are not yet at a stage where microscopy can be neglected. Although it is clear that microscopy still is a very information rich method, the major aspects of it that withholds researchers from applying this method, is that it requires a lot of time, energy and expertise. While DGGE and other molecular techniques itself take relatively little time in comparison, the techniques used to analyze the outcome are not quite as fast. Developing clone libraries and sequencing the strands of DNA and RNA yielded from the excision of bands are also time consuming. Personally I think that the reason most studies neglect classical research is because of the added factor of observer bias. In a perfect environment all samples under the microscope would be examined and analyzed by the same individual, avoiding an observer bias. But when analyzing over twenty samples it is an almost impossible task to complete on your own. Bias does in fact also exist in the PCR based methods but can easily be solved by using specific primer sets derived from programs and databases.
Clone libraries in itself are very valuable and could be used even without a fingerprinting technique like DGGE, but that is also its weakness. Because of the amount of information clone libraries could yield (the full sequence of a gene) it is hard to actually fingerprint a community with only the clone libraries. The research of *Medina-Pons et al* (2009) even states that morphological identification of organism is already obsolete due to the information yielded by clone libraries. In my opinion this is far from the truth. Morphological identification is just too important to neglect, because how much use is it when one realizes he has 49 different species in a sample but when taking a look under the microscope he can identify only 10? Microscopy or at least visual confirmation is in my opinion needed to determine whether a species is novel no matter what its sequence indicates.

What is important to realize here is that for this kind of in-depth research one technique is never enough. Even now if a researcher only utilizes a DGGE fingerprint you still have to create either a clone library or sequence excised bands in order to actually know what organisms are present in a given sample.
At the current level of research and technology I think that researchers are a little blunt in their way of thinking. Given the inaccuracy of both the molecular and the microscopy coupled with the limited knowledge about these kinds of organisms at this moment in time should make it apparent that not one single technique is enough to precisely fingerprint or screen planktonic diversity. *Medlin et al* (2006) claimed to have found several novel sequences that bore a close resemblance to other sequences in the database but without further investigation they seemed to claim they found a new sequence and therefore a new organism. Microscopy

(both light and electron) were deemed too laborious and therefore were not performed. These claims are in my opinion ungrounded until you can actually point at an organism and prove that the plankton you are pointing at has never been morphologically described and that the sequence found in your DGGE, SSCP, t-RFLP or clone library research matches its sequence.
For future research I highly recommend that we put more research into not only molecular techniques but also in the ability to morphologically distinguish species based on visual interpretation. It is this combination that would give power to the results because without visual confirmation I believe one has no ground to state that they have found a novel species.

6. References:

Daims H., Wagner M. (2007) Quantification of uncultured microorganisms by fluorescence microscopy and digital image analysis. Appl Microbiol Biotechnol Vol. 75: 237-248

Díez B., Pedrós-Alió C., Marsh T.L., Massana R. (2001) Application of Denaturing Gradient Gel Electrophoresis (DGGE) To Study the Diversity of Marine Picoeukaryotic Assemblages and Comparison of DGGE with Other Molecular Techniques. Applied and Environmental Microbiology Vol.67: No. 7

Kazakevich Y.V., LoBrutto R. (2007) HPLC System. HPLC for Pharmaceutical Scientists Chapter 1.5.2

Lemaire E., Abril G., De Wit R., Etcheber H. (2002) Distribution of phytoplankton pigments in nine European estuaries and implications for an estuarine typology. Biogeochemistry Vol. 59: 5-23

Medina-Pons F.J., Terrados J., López-López A., Yarza P., Rosselló-Móra R. (2009) Evaluation of the 18S rRNA clone library approach to study the diversity of the macroeukaryotic leaf-epiphytic community of the seagrass *Posidonia oceanica* (L.) Delile. Marine Biology Vol.156: 1963-1976

Medlin L.K., Metfies K., Mehl H., Wiltshire K., Valentine K. (2006) Picoeukaryotic Plankton diversity at the Helgoland Time Series Site as Assessed by Three Molecular Methods. Microbial Ecology Volume 52: 53-71

Pedrós-Alió C. (2006) Marine microbial diversity: can it be determined? TRENDS in Microbiology Vol.14 No. 6

Piquet A.M.-T., Bolhuis H., Buma A.G.J. (2009) Shifts in Antarctic Marine Microbial Communities due to Breakdown of Surface Stratification. Mild Climate, Harsch Times For Polar Marine Microbes. Chapter 4

Piquet A.M.-T., Scheepens J.F., Bolhuis H., Wiencke C., Buma A.G.J. (2009) Variability of Prostistan and bacterial Communities in two Arctic fjords (Spitsbergen). Mild Climate, Harsch Times For Polar Marine Microbes. Chapter 5

Roelfsema J.H., Peters D.J.M. (2005) Denaturing Gradient Gel Electrophoresis (DGGE). Medical Biomethods Handbook Chapter 8

Savin M.C., Martin J.L., LeGresley M., Giewat M., Rooney-Varga J. (2004) Plankton Diversity in the Bay of Fundy as Measured by Morphological and Molecular Methods. Micobial Ecology Vol. 48: 51-65

Schütte U.M.E., Abdo Z., Bent S.J., Shyu C., Williams C.J., Pierson J.D., Forney L.J. (2008) Advances in the use of terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA genes to characterize microbial communities. Appl Microbiol Biotechnol Vol. 80: 365-380

Tian F., Yu Y., Chen B., Li H., Yao Y., Guo X. (2009) Bacterial, archaeal and eukaryotic diversity in Arctic sediment as revealed by 16S rRNA and 18S rRNA gene clone libraries analysis. Polar Biol. Vol. 32: 93-103

Tourlomousis P., Kemsley E.K., Ridgway K.P., Toscano M.J., Humphrey T.J., Narbad A. (2009) PCR-Denaturing Gradient Gel Electrophoresis of Complex Microbial Communities: A Two-Step Approach to Address the Effect of Gel-to-Gel Variation and Allow Valid Comparisons Across a Large Dataset. Microbial Ecology Published Online

Unrein F., Izaguirre I., Massana R., Balagué V., Gasol J.M. (2005) Nanoplankton assemblages in maritime Antarctic lakes: characterisation and molecular fingerprinting comparison. Aquatic Microbial Ecology Vol.40: 269-282

Vosberg H. (1989) The polymerase chain reaction: an improved method. Human Genetics Vol. 83: 1-15

Wu Q. L., Chatzinotas A., Wang J., Boenigk J. (2009) Genetic Diversity of Eukaryotic Plankton Assemblages in Eastern Tibetan Lakes Differing by their Salinity and Altitude. Microbial Ecology 58: 569-581

Yan Q.Y., Yu Y.H., Feng W.S., Deng W.N., Song X.H. (2007) Genetic Diversity of Plankton Community as Depicted by PCR-DGGE Fingerprinting and its Relation to Morphological Composition and Environmental Factors in Lake Donghu. Microbial Ecology Vol.54: 290-297