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Mini Review

Molecular Techniques for Identification and Characterization of Marine Biodiversity

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Submitted: 31 August 2016

Accepted: 21 September 2016

Published: 23 September 2016

Keywords

- Marine biodiversity
- qPCR
- Toxic Algae

Abstract

Molecular methods to detect organisms in the natural environment can be divided into whole cell and cell free formats. Whole cell methods are generally limited by the number of fluorochromes that can be detected, whereas cell free formats offer more possibilities for multiple species detection and multiple methods of detection. This mini review addresses the major tools applied in environmental studies using cell free methods. The methods reviewed include microarrays, biosensors, quantitative real-time PCR (qPCR), and next generation sequencing (NGS).

ABBREVIATIONS

SHA: Sandwich Hybridization Assay; qPCR: Quantitative Polymerase Chain Reaction; NGS: Next Generation Sequencing

INTRODUCTION

Molecular methods are more commonly being used to detect all kinds of organisms in a wide variety of habitats because of the abundance of cryptic species that cannot be differentiated by any other means, the vast amount of microbes that cannot be cultured for any kind of identification, and the extensive training required to distinguish morphologically similar species. Molecular methods are potentially faster and more accurate than traditional light microscopy methods and for prokaryotic organisms are essential because many microbes cannot be cultured. Molecular techniques have been used for identification of phytoplankton in a wide variety of applications [1-6]. The small-subunit (SSU) and large sub-unit (LSU) ribosomal RNA (rRNA) genes have been established as an efficient and effective way to identify organisms and characterize complex microbial samples [7]. Direct cloning and sequencing of the SSU and LSU rRNA genes from natural samples provide a broader view of community structure and composition [8], and have led to the discovery of an enormous amount of hidden biodiversity [9]. Probes from higher taxonomic groups down to the species/strain level can be designed because the rRNA database continues to increase in size and scope [10-13]. Species-specific probes have been applied for the analysis of phytoplankton communities with detection by different methods: 1) whole cell methods in which the cell remains intact and thus also the morphology, or 2) cell free methods in which total nucleic acids are extracted and probes applied directly to the nucleic acid target (SHA, microarrays, biosensors) or used to amplify key barcoding regions (qPCR). In the whole cell methods, the probe is fluorescently labeled, (FISH) and is hybridized to the ribosomes inside the cell for detection by microscopy or cytometry. In the cell free method, probe detection can be made by colorimetry or electrochemistry. A UNESCO manual for quantitative phytoplankton analysis provides detailed step-by-step protocols for nearly all of these methods [14]. In this mini-review, the cell free methods are reviewed and some case studies for toxic marine phytoplankton and freshwater pathogens are included.

Microarrays

When DNA sequences (barcodes) are applied to the surface of a glass slide with special surface properties in an ordered array, this can be termed a microarray and if the barcodes identify species, the microarray is also termed a phylochip. It is based on a minimized form of a dot blot [15-16]. A DNA microarray experiment involves the following steps: microarray production, sample isolation and preparation, hybridization and data analysis. The microarray is produced by immobilizing the probes onto the glass slide. 15 carbon atoms are added to the end of the probe to help raise it above the surface of the slide. Prior to hybridization, the extracted target nucleic acids are fragmented to an appropriate length, then labeled with a fluorescent dye, which can be incorporated directly to the nucleic acid or via indirect labeling of other substances [17-19]. The microarray scanner scans across the printed array to recover fluorescent excitation of each probe to reveal a hybridization pattern [16]. Phylochips have been used to identify phytoplankton, especially

pico-sized cells [4,20], toxic algae [21-33], bacteria [34-39]. Lewis et al. [41] produced a manual that standardized methods for RNA extraction, hybridization, analysis and calibration to convert the fluorescent microarray signal to cell numbers for the monitoring of toxic algae. This is essential for monitoring because nearly all decisions on fisheries closure are based on cell numbers that trigger toxicity testing.

Harmful algal blooms (HABs) are becoming more frequent as climate changes. Many tropical species are moving northward and toxic algal blooms are being reported in areas where none were reported in previous decades. Monitoring programs/ tools that detect the presence of toxic algae before they bloom are of paramount importance to protect aquatic ecosystems, aquaculture, human health and local economies. The toxic algal microarray (MIDTAL) detects 32 toxic algal species and was field-tested for 2 years in 5 EU countries that regularly monitor for toxic algae showing good correlations with standard cell counting methods (Figure 1).

Monitoring drinking water quality is also an important public health issue. The EU Project µAQUA made novel tools for the early and sensitive detection of water-borne pathogens (bacteria, cyanobacteria and protozoans) and toxins. Pathogenic organisms occurring in lakes and rivers used as drinking water reservoirs represent a serious health-hazard. River water is usually contaminated with bacteria (*E. coli, C. perfringens, etc.*), viruses (adenoviruses) and pathogenic protozoa (*G. duodenalis, C. parvum, etc.*). Cyanobacteria are more of a problem in lakes where they can bloom. The microarray for freshwater pathogens (mAQUA) was developed to evaluate impacts on water quality and was field tested in 7 countries in two EU projects (mAQUA and MicroCokit). In the latter project, the entire length of the Tiber River, Italy was sampled over a two year period from pristine, agricultural, industrial and anthropogenic impacted sites (Figure 2). The microarray successfully detected target pathogens in the Tiber River. The four sampling sites appeared to be unique, with T2, the agriculturally polluted site and T4, the site receiving anthropogenic impact, likely responding to nutrient loading, which increased the presence of the pathogens at those sites. Throughout the Tiber, bacterial load was high with some evidence of seasonality: higher signals in the autumn than in the spring, when more rain and runoff occurs. Total community (both eukaryotes and prokaryotes) hybridizing to the microarray were more abundant by 3-4 times in October 2015 than any other time, and this was reflected in the total RNA extracted. In the Oct 2015, T4 had the highest bacterial and eukaryote signal. Marcheggiani et al., [42] also sampling the Tiber River with bacterial plate count confirmation, also found higher signals in the autumn, after the dry summer season.

Within the last two years, the microarray method has received an ISO number (ISO 16578:2013(en)) and thus is now a fully accredited method for determining the concentration of DNA in any environmental sample. For the microarray we have used hollow fiber ultra filtration to concentrate fifty litres to a concentrated one litre, which has proven to be a rapid method with minimal cell loss to provide a concentrate for downstream analysis. The 70 Da cut-off of the filter ensures many organics, such as toxins, can be concentrated for downstream analysis [43].

Biosensers

Biosensers are attractive candidates to overcome traditional detection and quantification limitations because they are simple, fast and have allowed the manufacture of compact and inexpensive devices [44-45]. Biosensors can be adapted to an electrochemical or chemiluminescent detection and those developed for toxic algae involve a sandwich hybridization assay (SHA). In SHA, one probe, the capture probe, is immobilized onto any surface (membrane, microtiter plate or an electrode)

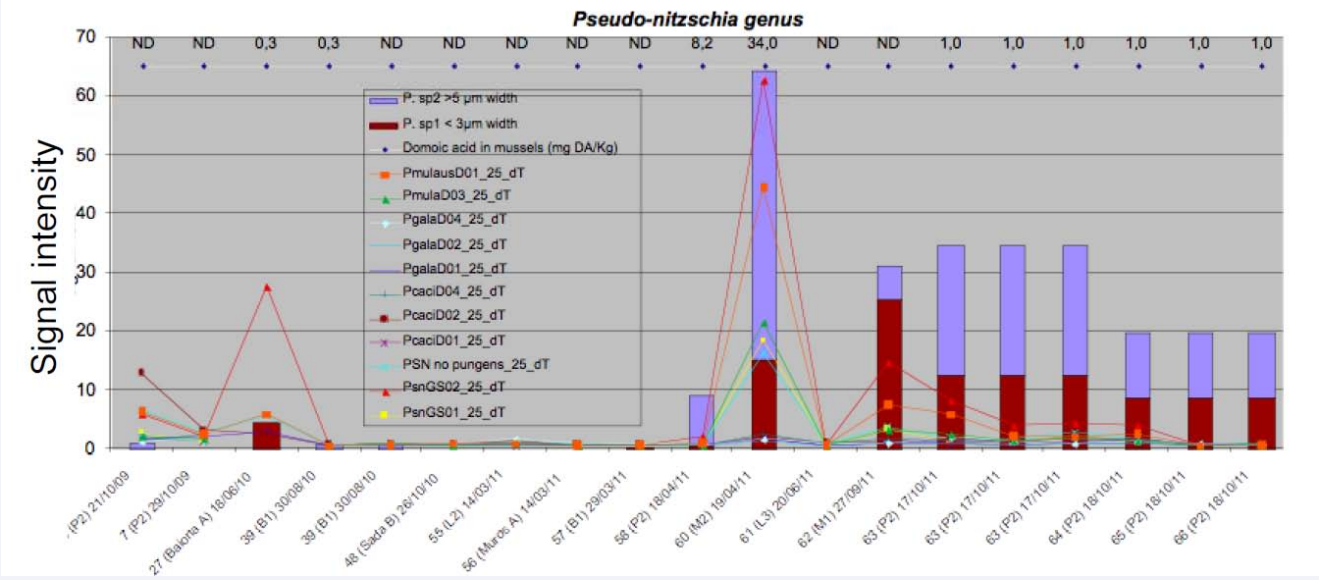

Figure 1 Comparison of microarray signals for Pseudo-nitzschia spp. with cell counts for two size categories and mussel toxicity from the Galician Rias in Vigo, Spain. Bars = cell counts. Lines over the bars are probe signal intensities. Mussel toxicity is expressed across the top of the graphs. Data courtesy of Y. Pazos.

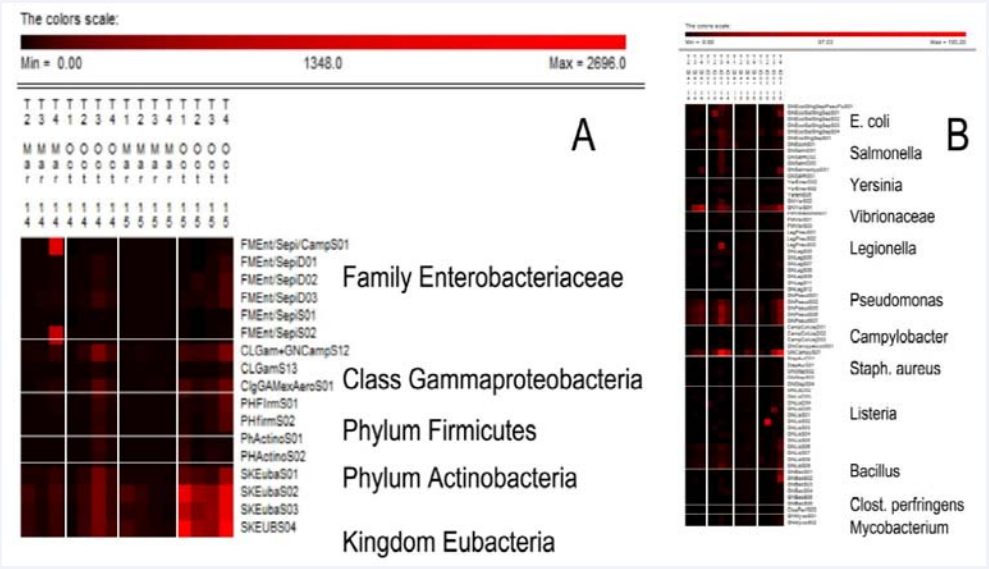

Figure 2 Heatmap of the relative abundance of A) the bacterial hierarchical probes from family to Kingdom and B) from genus to species. Vertical lines separate sampling sites and dates. Samples taken along the length of the Tiber River, Italy in the MicroCokit pr.

and captures the target DNA/RNA from any sample. A second probe, the signal probe, binds to the target, hence the term sandwich hybridization because the target barcode is bound by both the capture and the signal probe. An antibody to the signal probe is coupled to a horseradish peroxidase (HRP) enzyme for signal amplification and forms the final complex. HRP converts inactive substrates to a product that can be detected electrochemically or colorimetrically. The SHA method has been widely used for the detection of toxic algae [45, 46] (Figure 3). An automated Universal Assay Processor (Saigene Biotech, Inc.) has been constructed to provide user with flexibility and control over various assay parameters (e.g., sequence, duration, and temperature of individual steps) [47].

Electrochemical SHA detection has low power requirements, which has made this method sensitive, accurate, and versatile. Moreover, the ability of electrochemical sensors to identify nucleic acids directly in complex samples is a valuable advantage over other approaches, such as PCR, which requires target purification and amplification [48] and sensitive to enzyme inhibitors. Biosensors are powerful tools for species detection. Among them, those based on the direct electrochemical detection of nucleic acid target molecules have successfully applied the SHA method to detect toxic algae [49]. The reactions are rapid, easy to execute and amenable to automation. Quantification of the target species can be performed by using smaller, portable and less expensive instrumentation.

The colorimetric SHA offers the cheapest and fastest way to test the specificity of primer pairs [2] (Figure 4) and has been optimized for lab based and buoy based applications (EU SMS, Villa, unpubl.). Oligonucleotide probe detection assays involving the amplification of hybridization signals through enzyme tracer molecules have the advantage of being potentially ultrasensitive. This assay format maximizes discrimination of the target sequences and purification of target molecules (e.g., RNA) is not

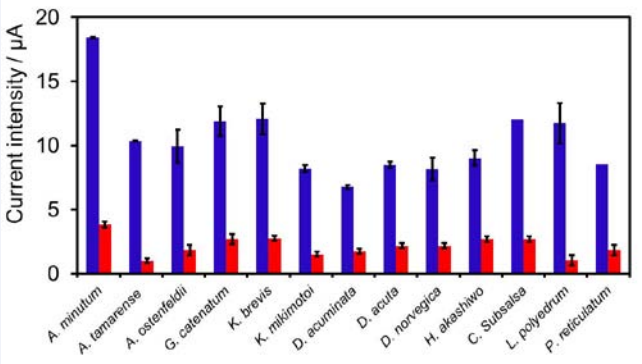

Figure 3 Current intensity of 13 toxic algal species tested under optimal conditions. The current intensities correspond to positive target in blue (synthetic DNA) and negative controls in red (non-target DNA), Reproduced courtesy of Elsevier [54].

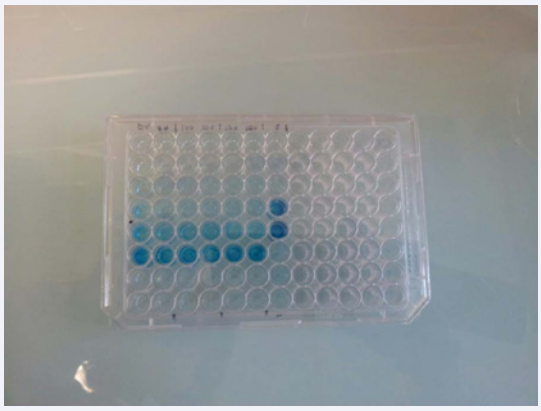

Figure 4 Colorimeter SHA assay. Intense color indicates maximum amount of RNA bound in the SHA. Figure courtesy of Elisa Villa and EU SMS.

Figure 5 Amplification plot of 18S rDNA from *Alexandrium ostenfeldii* OKNL 11 using the TaqMan approach and probe Aost213. The excited fluorescence is plotted against the cycle number. The delta Rn is the magnitude of the signal generated by the given PCR conditions relative to astandard. Figure courtesy of Dr. Kerstin Toebe.

required.

qPCR: The polymerase chain reaction (PCR) is one of the most powerful technologies in molecular biology. Traditional qualitative "endpoint" PCR cannot generate any information about the quantity of starting material in the sample. In qPCR, data are collected over the entire PCR cycle by using fluorescent markers that are incorporated into each PCR product during amplification. Thus, the quantity of the amplified product is proportional to the fluorescence generated during each cycle. This is monitored with an integrated detection system during the linear exponential phase of the PCR [50]. The change in fluorescence that is measured as the PCR amplicon is accumulated during each cycle is directly proportional to the amount of starting material (Figure 5). Closely related species or populations can be distinguished because qPCR can discriminate base pair differences. External standards for quantifying the amplified DNA come from either a dilution of plasmids or DNA derived from laboratory cultures with a known concentration of the target template. Concentration curves must be constructed for each species and is required for the analysis of environmental samples because of differences in DNA content per cell [51]. The copy number of the rDNA genes may vary among different strains of an organism and species [52] and that must be taken into account when concentration curves are generated. Several approaches for qPCR are available: SYBR Green, TaqMan and digital qPCR. Potential drawbacks and limitations of qPCR could be that different DNA extractions yield different amounts depending on the extraction method used and that the presence of humic substances could inhibit the PCR reaction. These problems can be resolved or minimized by applying a high quality DNA isolation method. qPCR can be easily performed immediately after in-situ sampling onboard ship or on shore, but preserved samples can also be used, although this may also be accompanied by inhibition problems. No preservation, or preservation using ethanol, coupled with freezing are preferred strategies, because it is still possible to detect and quantify target cells after three years from field samples processed in this way [53]. Preservation considerably lowers the sensitivity of qPCR. As always, enzyme-based assays can be inhibited by natural products in the waters.

NGS: Next-generation sequencing technologies have recently inspired almost all life science studies using techniques, such as full genome sequencing (de novo sequencing and resequencing), amplicon sequencing, transcriptome sequencing, and metagenomics. NGS techniques with pyrosequencing generate much higher throughput data, by which millions to billions of sequencing reactions take place at the same time, in small reaction volumes [54]. Ebenezer et al., [54] summarized the NGS technologies available and their major features. In field sample studies, NGS technologies gather DNA data from both environmental DNA and/or PCR products amplified from environmental DNA. NGS does not require cloning of template DNA into bacterial vectors because DNA templates are bound to substrates and amplified by PCR to generate clonal representatives. The number of sequence reads by the NGS

methods are continually increasing hence and with upwards of 500 bp reads, NGS is fast becoming the tool of choice for the identification and detection of microbes from environmental samples [54]. However the long time to process data is still a major concern and makes the use of microarrays more attractive as a means of analyzing large volumes of sequence data. Phylochip®, a universal microarray for all prokaryotic organisms is commercially available and circumvents the long analysis time to perform community analysis for the prokaryotes.

SUMMARY

Beyond the traditional microscopic methods, many molecular techniques have been developed as alternative methods to discriminate all species, especially microbial ones. Whole cell methods retain the cell's morphology but have greater limitations in terms of numbers of species that can be discriminated and how many samples can be handled at any one time than cell free methods, which are more versatile. Each molecular technique has its own particular strengths and limitations in detecting species. However the accuracy of these methods to discriminate closely related or even cryptic species cannot be challenged. The cost of these techniques is being reduced all of the time. This means that the frequency of monitoring can be increased. Thus our spatial and temporal resolution of community changes becomes almost real time.

ACKNOWLEDGEMENTS

This work was supported in part by the following EU projects: MIDTAL, µAQUA, MicroCokit, SMS.

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Cite this article

Medlin LK (2016) Molecular Techniques for Identification and Characterization of Marine Biodiversity. Ann Mar Biol Res 3(2): 1015.