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REVIEW ARTICLE

# Molecular Detection, Quantification, and Diversity Evaluation of Microalgae

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**Abstract** This study reviews the available molecular methods and new high-throughput technologies for their practical use in the molecular detection, quantification, and diversity assessment of microalgae. Molecular methods applied to other groups of organisms can be adopted for microalgal studies because they generally detect universal biomolecules, such as nucleic acids or proteins. These methods are primarily related to species detection and discrimination among various microalgae. Among current molecular methods, some molecular tools are highly valuable for small-scale detection [e.g., single-cell polymerase chain reaction (PCR), quantitative real-time PCR (qPCR), and biosensors], whereas others are more useful for large-scale, high-throughput detection [e.g., terminal restriction length polymorphism, isothermal nucleic acid sequence-based amplification, loop-mediated isothermal amplification, microarray, and next generation sequencing (NGS) techniques]. Each molecular technique has its own strengths in detecting microalgae, but they may sometimes have limitations in terms of detection of other organisms. Among current technologies, qPCR may be considered the best method for molecular quantification of microalgae. Metagenomic microalgal diversity can easily be achieved by 454 pyrosequencing rather than by the clone library method. Current NGS, third and fourth generation technologies pave the way for the high-throughput detection and quantification of microalgal diversity, and have significant potential for future use in field monitoring.

**Keywords** Microalgae · Molecular techniques · Detection · Quantification · Diversity

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## Introduction

Microalgae are microscopic, unicellular species that exist solitarily or in chains and are typically found in aquatic systems. They play a vital role in primary production in the aquatic environments and contribute to global atmospheric carbon dioxide acquisition. The commercial value of microalgae is increasing day by day; for example, good nutritional values (Becker 2007), large utilization in aquacultures (Brown 2002), and a possible use for biofuel production (Chisti 2007). Moreover, some microalgae, such as dinoflagellates and diatoms, can form harmful algal blooms (HABs) and contain biotoxins that affect humans and many other organisms that consume these algae (e.g., bivalves, which can filter the toxic species). Microalgae are incredibly diverse, and their species are estimated to amount to ca. 200,000–800,000, of which only about 35,000 are described (Cheng and Ogden 2011). Microalgal species have traditionally been discriminated by morphological observations and pigment profiles. Molecular discrimination methods are sometimes very effective for their identification, especially for the pico-sized fractions that have very few morphological features that can be used for identification (Not et al. 2007). From a historical viewpoint, the advent of the use of molecular technology in phycology began in the 1970s. During this time, phycologists developed molecular techniques as indirect detection methods; they had been limited by the traditional methodologies for microalgal discrimination

and identification. These indirect discrimination methods include the detection of carbohydrates, nucleic acids, proteins, and toxins from microalgae. Since then, DNA-based approaches have been extensively developed, and these findings have greatly expanded our understanding of genetic diversity, molecular systematics, evolution, and even adaptive responses, from an ecological basis, for all organisms and not just the microalgae (Bott et al. 2010; Kudela et al. 2010; Medlin and Kooistra 2010).

Molecular techniques used for planktonic microalgae (or phytoplankton) have been developed or modified by biologists for a long time, and these methods have been reviewed over the last decade (Medlin et al. 2000, 2006, 2007; de Bruin et al. 2003). Recently, Bott et al. (2010) extensively compared routine DNA-based detection methods for the development of practical, specific, sensitive, and rapid diagnostics of marine pests, including harmful algae (HA). In addition, Kudela et al. (2010) provided a detailed outline of the molecular tools available for comparative harmful algal bloom programs in upwelling systems, focusing on cell enumeration and identification, molecular phylogenetics, and applications of high-throughput sequencing methods. Most of the molecular methods have been primarily developed for the discrimination of planktonic microalgae, particularly HA, and tested under laboratory conditions. The lack of rigorous field testing of the applicability of these molecular techniques often poses a problem. Factors that need to be addressed include the selection of suitable genetic markers for the identification, quantification, diversity analysis, and isolation of genomic DNA from environmental samples. Currently available methods do not really place an emphasis on the quantification of microalgal cells, but enumeration of cells plays a crucial role in field assessment. On the other hand, routine molecular techniques and more recent advanced technologies, such as next-generation sequencing (NGS) technologies, should be focused on molecular quantification, which is usually a tedious process and also on the large-scale microalgal detection from the environment.

In the present paper, we review molecular technologies, including recent advanced techniques [e.g., DNA chip, loop-mediated isothermal amplification (LAMP), and NGS technologies], for accurate, rapid detection and environmental monitoring, as well as for the quantification and large-scale diversity assessment of microalgae.

## Molecular Technologies for Microalgae Detection

In general, all kinds of detection techniques used in molecular biology can be applied to microalgal studies because the principle of molecular detection is to target universal biomolecules, such as nucleic acids and proteins. Table 1 provides an outline of various techniques used to identify and detect microalgae, especially the harmful bloom-forming species. Overall, these methods can be categorized into five groups of detection tools according to the target molecule: toxin, proteins, carbohydrates, RNA, and DNA. Toxin profiles are only used as discrimination markers for biotoxin-producing species (Cembella et al. 1987); these methods are thus rare and can vary even among strains of a single species. On the other hand, proteins and nucleic acids have frequently been the target molecules of choice. Protein-based methods include allozyme electrophoresis and immunoassay (or ELISA) with antibodies (Shapiro et al. 1989; Sako et al. 1990). Most methods are DNA-targeting technologies, including restriction fragment length polymorphism (RFLP), denaturing gradient gel electrophoresis (DGGE), single-stranded conformation polymorphism (SSCP), random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellites, and polymerase chain reaction (PCR), among others (see Table 1). DNA-based methods have been developed for the taxonomic identification of species or strains of microalgae and are often used for phylogenetic analyses. Microsatellites and internal short sequence repeat (ISSR) analysis are often used in population genetics of animals and higher plants and have recently been applied to microalgae (Nagai et al. 2007; Evans et al. 2009; Casteleyn et al. 2010; see later reviews). These studies have shown that the oceanic environment is highly fragmented and that microalgal populations are highly diverse, even on a local level.

Molecular tools that have been developed for environmental samples are mostly designed to detect certain harmful microalgae (e.g., *Alexandrium*, *Heterosigma*, *Gyrodinium*, *Gymnodinium*, *Karenia*, *Pfiesteria*, *Pseudo-nitzschia*, etc.) rather than to quantify these cells. However, quantitative PCR (qPCR) has recently been applied to some species in environmental samples (e.g., Dyhrman et al. 2006, 2010). Of the available methods, fluorescently labeled lectins, antibodies (immunoassays), and oligonucleotide probes were first used to bind or hybridize molecules located on the cell's surface (Lindquist 1997; Simon et al. 2000), and internally to ribosomes (Hosoi-Tanabe and Sako 2005, 2006). Among the microalgae, ribosomal RNA (rRNA) probes have routinely been applied to several harmful algal species, such as *Alexandrium tamarense*, *Pseudo-nitzschia*, *Heterosigma akashiwo*, and *Fibrocapsa japonica* (Scholin and Anderson 1993; Scholin et al. 1994; Tyrrell et al. 2002), and also for the detection of some picoeukaryotoc microalgae, such as *Micromonas pusilla* and *Bathycoccus prasinos* (Not et al. 2004; Lepère et al. 2009; Shi et al. 2011). Whereas, in bacterial research, they have been widely used to characterize the entire community (Britschgi and Giovannoni 1991; Amann et al. 1995). Probe-labeled cells are observed by light microscopy and counted, but they are limited in their use because only one or two unique probes with different fluorochromes can be used at one time. Fluorescent in situ hybridization (FISH) using rRNA-targeted probes has been used as a method for

**Table 1** Molecular technologies involved in the detection of microalgae, their advantages, and limitations

| Techniques | Advantages | Limitations |
|---|---|---|
| ▪Toxin profile | -Detect toxic species | -Toxin difference among strain, difficult to analysis |
| ▪Fluorescent-conjugated lectin probe | -Characterization of closely related species | -Lectins bind non-covalently with polysaccharides on cell surfaces |
| ▪Allozyme (or isozyme) electrophoresis | -Easy to develop and cost-effective | -Underestimates the level of genetic variation |
| ▪Antibody probe | -Rapid quantification and identification | -Variation in cell protein caused by external factors, cross-reactivity with antigens |
| ▪Sandwich hybridization assay (SHA) | -Variation in surface protein can be characterized, minimize false negative signals | -Requires homogenization of cells, specificity, and sensitivity of the probe in field studies is a question |
| ▪Fluorescence in situ hybridization (FISH) | -Highly specific, rapid identification, analysis of natural samples | -Labor intensive, time consumption, expensive and requires technical expertise to manufacture species-specific probe |
| ▪Oligonucleotide array | -Rapid, sensitive, simultaneous detection and allows the enumeration of species | -Expensive, longer hybridization incubation time, non-flexible platform, expensive equipments |
| ▪Restriction fragment length polymorphism (RFLP) | -Discrimination of closely related species | -Requires large volume of pure, high molecular weight genomic DNA |
| ▪Random amplification of polymorphic DNA (RAPD) | -Discrimination of closely related species | -Low reproducibility rate in genotyping and sensitive to reaction conditions |
| ▪Amplified fragment length polymorphism (AFLP) | -Genetic variation between species, highly reproducible | -Expensive, requires more technical expertise |
| ▪Single-strand conformation polymorphism (SSCP) | -Identification of species in complex assemblages | -Time consuming |
| ▪DNA sequencing | -Most accurate comparison, easy to use | -Require comparable data |
| ▪Massively parallel signature sequencing (MPSS) | -Discriminate closely related species | -Species-specific sequence bias, loss of specific sequences |
| ▪Automated ribosomal intergenic spacer analysis (ARISA) | -Determining the presence of an organism form environmental samples | -Require comparable data; intra-species variation |
| ▪Isothermal nucleic acid sequence-based amplification (NASBA) | -Rapid and reliable identification of species in samples containing low concentration of cells | – |
| ▪Loop-mediated isothermal amplification method (LAMP) | -Simple operation, rapid reaction and ease of detection | – |
| ▪Single cell PCR | -Detection of non-culturable species, applicable to preserved samples rapid | -Labor intensive in single cell isolation |
| ▪Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) | -Differentiating similar strains, comparison of composition of natural population | -Non-quantitative detection, expensive equipment |
| ▪Melting curve analysis | -Easy to handle, cost-effective | -Low resolution for discrimination |
| ▪Inter simple sequence repeat (ISSR) | -Simple to use and inexpensive | -Few marker available, difficult marker developments from large number of isolates |
| ▪Microsatellites/single sequence repeats | -Largely used to study population structure and intra-specific genetic diversity | -Few marker available, difficult marker developments from large number of isolates |
| ▪Multiplex/semi-multiplex PCR | -Highly specific, sensitive, can determine multiple species | -Decreases the sensitivity |
| ▪PCR coupled dotblot/low density microarray hybridization | -Rapid and highly specific, requires less quantity of genome | -Unsuitable for field applications |
| ▪High throughput microarray | -Accurate, inexpensive maintenance, good sensitivity and specificity, rapid, easy to use, easy configuration of assay platform | -Unsuitable for field applications |
| ▪Terminal restriction fragment length polymorphism (T-RFLP) | -Highly reproducible results for repeated samples | -Possible appearance of false (pseudo) T-RFs |
| ▪PCR assay | -Simple to use, cost-effective | -Require specific primers |
| ▪Real-time PCR | -Highly specific, sensitive, cost-effective, and quantitative, applicable to preserved environmental samples | -Gives information one species or few target species |

easy identification of microalgal cells (Hosoi-Tanabe and Sako 2005). Oligonucletotide probe methods were later modified for application in sandwich hybridization assays and biosensors (e.g., DNA biosensor, sensor chip), in a cell-free format where many probes could be used simultaneously. Protein-targeting methods have been questioned, however, because the nature and abundance of cell surface proteins varies with environmental conditions, different stages of growth, and the physiological state of the cells (Anderson 1995).

Because of the instability of RNA (particularly mRNA) and protein, most detection tools used for microalgae rely on detecting DNA (Fig. 1). DNA-based methods can be categorized into three groups: (1) restriction patterns of genomic DNA (e.g., RFLP), (2) PCR-based methods [e.g., RAPD, AFLP, SSCP, isothermal nucleic acid sequence-based amplification (NASBA), LAMP, DGGE, temperature gradient gel electrophoresis (TGGE), single-cell PCR, real-time PCR, inter simple sequence repeat, and PCR], and (3) DNA sequencing [e.g., massively parallel signature sequencing], including PCR + sequencing. Advantages of DNA-based methods include the large number of DNA sequences available in public databases (e.g., DDBJ, EMBL, and NCBI) and the user-friendly applications available for the rapid and easy discrimination, quantification, and phylogenetic affiliation determination of various microalgae. Another significant advantage is that DNA can be isolated from frozen, fresh, or preserved samples because the genome is relatively stable (Bott et al. 2010). DNA can now be routinely extracted and PCR amplified from archived global plankton samples collected by continuous plankton recorder and stored for decades in formalin (Ripley et al. 2008). The use of DNA-based methods in field applications and for quantification purposes is slowly advancing (Sellner et al. 2003; Dyhrman et al. 2006; Ki and Han 2006). Moreover, these methods are continuously being modified and involved in microalgae related studies; for instance, the DNA sequences obtained from rRNA, *actin*, α- and β-*tubulins*, mitochondrial cytochrome oxidase subunit I, and the chloroplast large subunit of ribulose bisphosphate

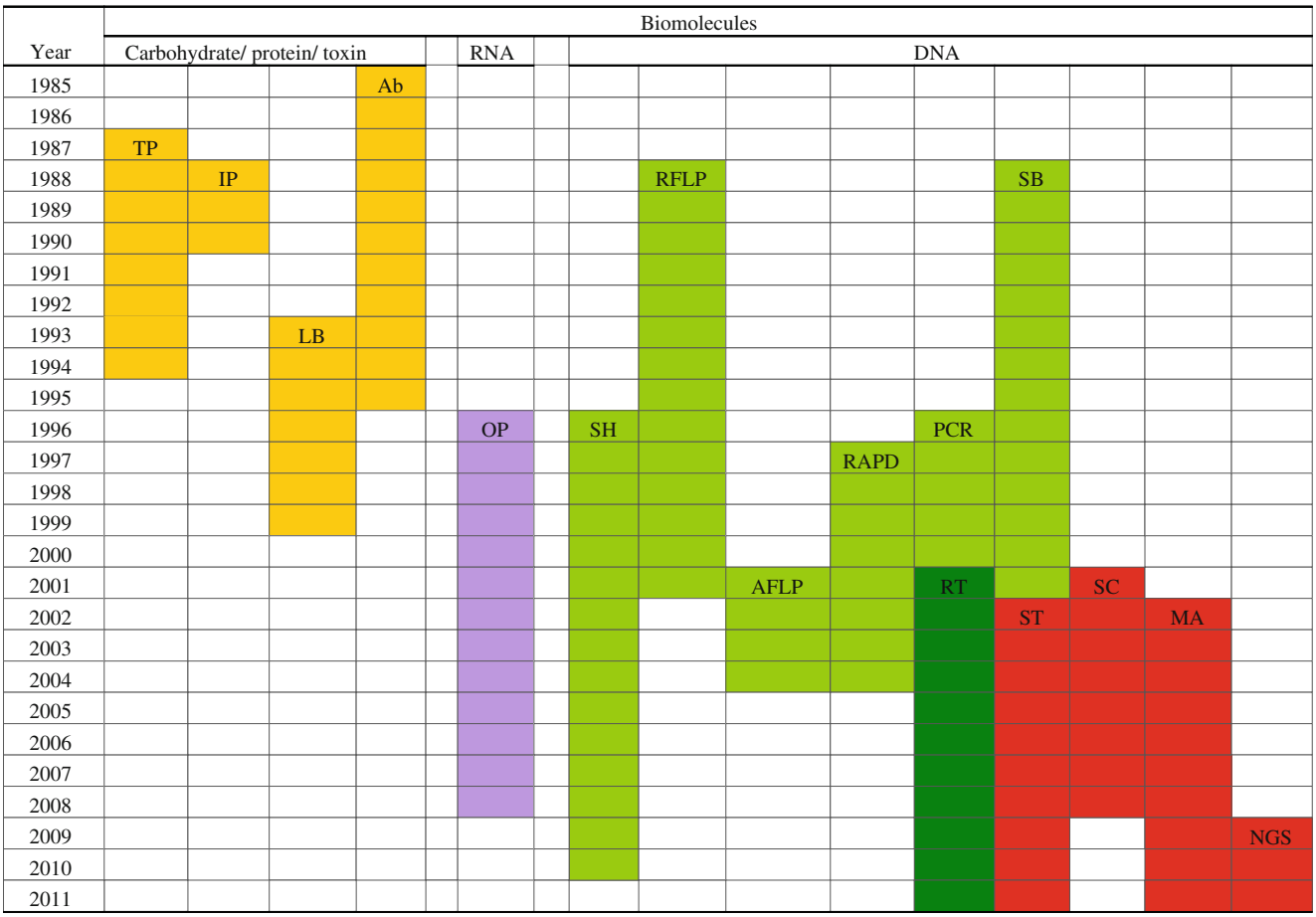


**Fig. 1** Indirect methods for the detection, quantification, and diversity of microalgae based on the published papers (different time scale). *Colored cells in each column* represent individual technologies based on corresponding years of publications between first and the latest. *TP* toxin profile, *IP* isozyme pattern, *LB* lectin binding, *Ab* antibody, *OP* oligonucleotide probe, including FISH, *SH* sandwich hybridization, *RFLP* restriction fragment length polymorphism, *AFLP* amplified fragment length polymorphism, *RAPD* random amplification of polymorphic DNA, *PCR* polymerase chain reaction, *RT* real-time PCR, *SB* sequence-based discrimination, *ST* sequencing typing, *SC* single-cell PCR, *MA* microarray, *NGS* next generation sequencing

carboxylase genes are being used for detection and phylogenetic studies in microalgae.

### Small- and Medium-Scale (Depending on Species or Sample Size) Detection Techniques

DNA-based detection methods for microalgae are generally conducted by PCR amplification of a target gene or DNA markers. PCR methods can amplify minute amounts of template DNA and multiply even a single copy of a given DNA sequence by a factor of $10^{12}$; its high specificity makes this tool highly effective for species and strain identification over a wide range of organisms (Bott et al. 2010). The relatively low cost of the equipment and reagents makes PCR accessible to small laboratories. In microalgal studies, PCR-based assays have largely been used for the identification and characterization of HAB species with the use of species-specific PCR primers and genetic markers (Godhe et al. 2008; Penna and Galluzzi 2008; Wang et al. 2008).

Multiple species detection is achieved by a complex PCR with a mixture of many primers or run, in parallel or series, as a large number of individual PCRs (Anthony et al. 2000). In addition, a multiplex PCR that uses species-specific primers may detect several species and has been tested for detection of harmful microalgae (Oldach et al. 2000; Rublee et al. 2001). Multiple primer sets within a single PCR mixture produce amplicons of varying sizes that are specific to different DNA sequences. This method can detect several target species; however, multiple primers used in the same multiplex PCR reaction decrease the sensitivity and increase the chance of two unrelated primers producing spurious products (Anthony et al. 2000). Annealing temperatures for each of the specific primer sets must be optimized to work correctly within a single reaction, and amplicon sizes should be sufficiently different to form distinct bands when visualized by gel electrophoresis. For these reasons, the method has several limitations when detecting more species in natural assemblages.

In addition, conventional PCR methods have been used to detect specific single cells by single-cell PCR. Single-cell PCR makes it possible to amplify DNA fragments by PCR from 1 or a few cells of an organism. It has enabled the detection and determination of DNA sequences from non-culturable microalgae (Edvardsen et al. 2003; Ki et al. 2004; Ki and Han 2005; Takano and Horiguchi 2006) and is applicable to samples that are frozen or preserved in solvents, such as formalin (Bertozzini et al. 2005; Richlen and Barber 2005). In spite of the fact that the single-cell PCR assay is very useful for determination of DNA sequences from uncultured microalgae collected from the environment, it is still labor intensive to isolate these target cells from natural microalgal assemblages. In addition, pico-size cells cannot be isolated by capillary methods for single-cell isolations prior to cell extraction and subsequent PCR amplification. For this reason, initially it was a very useful tool to isolate and detect comparatively large cells, such as armored dinoflagellates because they were easy to isolate by capillary tubes using the inverted light microscope. Recently, pico-size eukaryotic cells in environmental samples can be isolated by flow cytometry, followed by whole genome amplification or clone library (Man-Aharonovich et al. 2010; Shi et al. 2011). This technological progress promises that single-cell PCR detection can be applied for various size microalgae.

### Large-Scale (High-Throughput) Detection Techniques

Conventional PCR detection with species-specific primers may only detect a single species, rather than the multiple microalgal species present in a given body of water. The use of species-specific PCR is therefore impractical for the routine analysis of field samples that may contain many different microalgal species. Multiple microalgal species detections are essential for monitoring harmful algal species in coastal waters. In the present study, we tentatively assigned some molecular methods that can simultaneously detect more than 50 species to the large-scale (or high-throughput) detection categories. Several advanced techniques have been developed for high-speed and high-throughput detection and monitoring of environmental microbes. These are based on cell-free systems and include terminal restriction length polymorphism (T-RFLP) or sometimes referred to as TRFLP, real-time PCR assay, and low-density microarray (DNA chip or phylochip). The T-RFLP method is a molecular detection technique for profiling microbial communities based on the position of a restriction site closest to a labeled end of an amplified gene. This method has been widely used in studies of bacterial diversity (Liu et al. 1997), but few studies have been attempted for microalgal detection (Countway et al. 2005; Joo et al. 2010). Recently, automated ribosomal intergenic spacer analysis, which is based on length variation in the internal transcribed spacer region (ITS) of rRNA, has been applied to microalgal detection (Hubbard et al. 2008).

Real-time PCR or qPCR is used to amplify and simultaneously quantify target DNA molecules. The procedure follows the same principle as standard PCR technique, but the key distinction is that the amplified DNA is detected as the reaction progresses in real time; therefore, it is more advantageous than conventional PCR because of its linearity, sensitivity, specificity, and the speed at which a large number of samples can be processed. This method has been applied to the detection and quantification of certain microalgal species (Galluzzi et al. 2004, 2010; Zhu et al. 2005; Godhe et al. 2008; Diaz et al. 2010). However, DNA contamination can lead to false positives and negative signals when using PCR-based assays.

Microarray (or DNA chip) technology has emerged as a method that allows parallel analysis of large numbers of biomolecules, such as DNA. The method was initially designed for gene expression analysis (Guo et al. 1994; Yershov et al. 1996); however, recently, it has been applied to the detection of bacteria and DNA-based typing of specific pathogenic bacterial strains for clinical diagnostics (Anthony et al. 2000; Wu et al. 2003; Mitterer et al. 2004). Microarrays with species-specific oligonucleotide probes have been used to simultaneously detect several harmful microalgae (Ki and Han 2006). In addition, Ellison and Burton (2005) have used a bead array technology for the simultaneous identification and quantification of many taxa in phytoplankton communities. Metfies et al. (2005, 2006), Metfies and Medlin (2008), and Gescher et al. (2008) used low-density microarrays to detect marine phytoplankton by using a hierarchical probe approach. These studies illustrate that the microarray technique constitutes a significant breakthrough for the high-throughput detection of microalgal species in complex samples. Fiber-optic microarray and bead array technology are multiplexed microarray technologies that have been developed for the detection of microalgae. With these methods, hundreds of species can be detected using a single optical fiber or bead-based assay (Ahn et al. 2006; Scorzetti et al. 2009; Diaz et al. 2010). The advantages of this method are a high sensor-packing density, smaller sample volumes, increased reuse of arrays, flexible assay design, and a reduction in false signals.

Other high-throughput detection techniques, including NASBA and LAMP, are DNA amplification technologies that are recently gaining importance in microalgal research. LAMP amplifies the target sequence at a constant temperature of 60–65°C using several primers and *Bst* DNA polymerase, which has high strand-displacement activity (Notomi et al. 2000). The amount of DNA produced in LAMP is considerably higher than that produced in PCR-based amplification. Moreover, amplification products can be detected by photometry for the turbidity caused by the increasing quantity of magnesium pyrophosphate in solution (Mori et al. 2001), or even by the naked eye with the addition of SYBR Green. To date, LAMP is widely studied for detecting infectious diseases, such as tuberculosis, malaria, and sleeping sickness; it has yet to be extensively validated for the detection of microalgae. Recent work on the application of NASBA and LAMP for microalgal detection (Casper et al. 2007; Wang et al. 2008; Ulrich et al. 2010) shows the potential for its use in the large-scale detection of microalgae. These techniques can also be used for the estimation of microalgal diversity and species composition.

## Microalgae Diversity by Molecular Techniques

Microalgae have enormous diversity in aquatic ecosystems, and some species (e.g., diatoms and dinoflagellates) have been used as bioindicator species to assess water quality and environmental changes (Vaulot et al. 2008). In general, microscopic observation and analysis of the diversity of microalgae in environmental samples is considered the gold standard; however, this method is only applicable for nano-size and larger plankton (5~200 μm in body length), because of the microscope resolution. Other smaller microalgae (pico-size cells), however, have remained elusive because they lack morphological features for identification; sometimes, they are even ignored in cell counting by microscopy. Alternatively, metagenomic analysis have been applied to study the microalgal diversity, by comparing the 18S rRNA sequences as DNA taxonomic markers, because there is significant sequence data in public databases, such as GenBank. In addition, the 18S rRNA is universally present in living organisms and contains regions that are well conserved within a species and generally different between species. Indeed, microeukaryote metagenomics have shown a remarkably high diversity of microalgae and other protists from environmental samples (Díez et al. 2001; Countway et al. 2005; Amaral-Zettler et al. 2009; Burki et al. 2010). In addition, large numbers of undescribed microalgae in natural environments have been identified (Moreira and López-García 2002; Cheung et al. 2010). However, not all microalgae have been sequenced, but despite the obvious bias that recovered sequences may represent known species, the magnitude of novel sequences, even in well-known lineages, has revealed many cryptic species (Sarno et al. 2005). Molecular technologies have greatly expanded our understanding of the diversity of microalgae that are not detected by microscopy.

Table 2 lists some molecular tools that have been applied for studies on microalgal diversity. Comparative diversity analysis in a larger number of samples has been achieved with DNA fingerprinting methods, such as DGGE, TGGE, AFLP, and T-RFLP (Widmer et al. 2006; Kumari et al. 2009; Alpermann et al. 2010; Joo et al. 2010). Distinction of individuals below the species level can be obtained by using highly variable molecular markers (e.g., ITS sequences and microsatellites). Microsatellite sequences generally comprise 2–4 bp sequences occurring as tandem repeats in nuclear and organelle DNA. They are used as the method of choice for resolving intra-specific diversity because they provide a unique marker indicative of intra-species variability (Nagai et al. 2007; Cho et al. 2009; Evans et al. 2009). A detailed review of the methods available to estimate diversity in marine protists was published recently (Medlin and Kooistra 2010).

Clone libraries are well established to provide information for both the phylogenetic identity and, to some extent, the relative abundance of community operational taxonomic units (OTUs)—a term used to describe the diversity, or species richness, of a sample (Stackebrandt 2006). This is achieved by DNA sequencing of a clone library constructed

**Table 2** Microalgal diversity studies with molecular technologies

| Techniques | Descriptions | Target genes studied |
|---|---|---|
| ▪Clone library | -Discrimination of closely related species, highly reproducible, allows detection of non-culturable species, recognizes genetic individuality, rapid identification in field and laboratory | -18S rRNA |
| ▪DGGE/TGGE | -Easy discrimination in natural samples, high reproducibility, and reliability rates | -18S rRNA, rDNA ITS |
| ▪T-RFLP/Flu-RFLP | -Provides excellent resolution | -18S rRNA, rDNA ITS |
| ▪qPCR/in situ hybridization | -Sensitive and rapid | -18S rRNA |
| ▪High throughput sequencing | -Rapid, simultaneous sequencing, Reliable and cost-effective | -18S rRNA, *COI* |
| ▪NGS techniques | -Large-scale sequencing, high sequence reads, eliminates the requirement to clone DNA fragments avoiding cloning bias | -18S rRNA, *COI* |

from PCR amplicons of environmental DNA. This method was initially developed for bacterial diversity studies (Lane et al. 1985; Britschgi and Giovannoni 1991; Amann et al. 1995), and it also has been widely applied for diversity studies on microalgae (Countway et al. 2005; Medlin et al. 2006; Potvin and Lovejoy 2009; Shi et al. 2011). These metagenomic studies have shown that the vast majority of microalgal diversity was not detected by conventional microscopic methods, particularly the nano- and pico-size microalgae (Delong and Pace 1991; Stoeck and Epstein 2003). In the clone library approach, minor species or populations may be ignored if too few clones are selected for sequence analysis. One reason is that this method is labor intensive and the possibility of less number of clones being sampled. In addition, a PCR bias exists because primers preferentially bind to the dominant template in a sample, thus masking minor species and rare organisms. Moreover, this analysis is expensive, which makes it difficult to analyze multiple samples with replicates and high frequency. Recent pyrosequencing technologies (e.g., 454 Life Sciences) are rapidly replacing clone library methods because high-throughput reads do not require cloning. However, it requires extensive computer analyses for a large data process, and precise phylogenetic affinities are not always possible. It is discussed in detail in "NGS techniques for diversity of microalgae."

## Quantification of Microalgae Using Molecular Techniques

Molecular quantification, or enumeration, of algal biomass and other important species regularly in an aquatic environment is a relevant parameter for the assessment of water quality and monitoring for possible algal bloom incidents. Most molecular tools have focused on the detection and discrimination of microalgae but are not commonly used in the field for quantification. Of the many techniques, those methods that are rapid and simple are preferred as replacements for the traditional methods (e.g., direct cell counting or chlorophyll estimation). Table 3 describes the molecular techniques available for the quantification of microalgae from environmental samples. Among current technologies (see Table 1), qPCR may be considered the best method for the molecular quantification of some targeting microalgal species. qPCR employs two different methods: (1) sequence-specific oligonucleotide probes that are labeled with a fluorescent reporter, such as TaqMan, and (2) non-specific fluorescent dyes (e.g., SYBR Green) that intercalate with any double-stranded DNA. Data are collected over the entire series of PCR cycles by using fluorescent markers that are incorporated into the amplicon product during amplification and directly in the exponential phase where PCR is precise and linear. In order to quantify cells, the parameters of qPCR are optimized using different standard curves (plasmid dilution or pure algal cultures). qPCR generates a standard curve of cycle thresholds (Ct) with known concentrations, and thus cell density can be compared. By comparison with known standards, cell density is calculated from the Ct value in qPCR. TaqMan-based qPCR assay, the principle of which is described elsewhere (Liu et al. 2006), has been tested for the quantification of harmful microalgae (Park et al. 2007; Handy et al. 2008). SYBR Green-based qPCR, commonly considered a relatively easy and cost-effective method, has been attempted for the detection and quantification of harmful microalgae, such as *Pfiesteria* sp., *Chattonella subsala*, *Pseudo-nitzchia* sp., *Ostreopsis* sp., and *Aureococcus* sp., (Galluzzi et al. 2010, 2011; Godhe et al. 2008; Andree et al. 2011; Perini et al. 2011). TaqMan-based qPCR is considered more accurate than intercalating dye methods. The former requires additional dye-labeled probes; however, double strand DNA (dsDNA) dyes, such as SYBR Green, will bind to all dsDNA PCR products, including non-specific PCR products like primer dimers. This can potentially interfere with, or prevent, accurate quantification of the intended target sequence. By applying this method to environmental samples, autofluorescence caused by microalgal pigments can interfere with an accurate quantification in SYBR Green-based qPCR. EvaGreen is a new DNA-binding dye that shows both a

**Table 3** Molecular tools for the quantification of microalgae

| Tools | Species tested | Field applicability |
|---|---|---|
| ▪qRT-PCR assay | -*Alexandrium catenella*, *A. fundyense*, *A. minutum*, *A. tamarense*, *Aureococcus* sp., *Chattonella subsalsa*, *Coscinodiscus* sp., *Gymnodinium* sp., *Heterosigma akashiwo*, *Osteropsis* sp., *Pfiesteria piscicida*, *Protoceratium* sp., *Prymenesium parvum*, *Thalassiosira pseudonana* | -High accuracy and sensitivity, reduction in material used, applicable to field samples |
| ▪Fluorescence in situ hybridization (FISH) | -*Symbiodinium*, *A. tamarense*, *A. catenella*, *Lingulodinium polyedrum* | -More precise and sensitive |
| ▪DNA chip (microarray) | -*Alexandrium* sp., *Dinophysis heterocapsa* sp., *Karenia* sp., *Micromonas* sp., *Prochlorococcus*, *Protocentrum* sp., *Synechococcus* | -Potential to generate data rapidly, cost-effective, adaptable, and comprehensive |
| ▪Locked nucleic acid (LNA) probes | -*A. miminum*, *A. ostenfieldii*, *Karenia brevis*, *K. mikimotoi*, *Prorocentrum* sp. | -LNA probes increases thermal duplex stability, extremely reliable |
| ▪High throughput sequecing | -*Amphidinium*, *Gymnodinium*, *Phaeodactylum* sp., *Peridinium*, *Prorocentrum*, *Prochlorococcus*, *Thalassiosira pseudonana* | -Linear quantification over a wide dynamic range no post PCR handling |

relatively low PCR inhibition and a relatively low tendency for non-specific amplicon interaction (Mao et al. 2007). Erdner et al. (2010) used EvaGreen dye for the quantification of plasmids and the enumeration of cysts in *Alexandrium* sp. Similarly, SYTO9 another intercalating dye used in qPCR was also used for the detection and quantification of the toxic dinoflagellate *Karlodinium veneficum* (Park et al. 2009). The main advantage of using qPCR is that it is highly sensitive, specific, accurate, and cost-effective and can be applied to preserved environmental samples (Galluzzi et al. 2004; Toyoda et al. 2010).

The rRNA molecules, particularly the non-coding ITS regions, are quite useful for developing specific primers for PCR amplification, but the eukaryotic rRNA gene has a high copy number—up to $10^4$ copies—and are tandemly organized (Schlötterer 1998). When a single-copy gene is detected by PCR, it represents a single cell. Similarly, multiple-copy genes within a cell are detected by the same manner, they may represent multiple cells, and there is a possibility for overestimation of real cell numbers. Recent qPCR techniques use real cells as standards, and these results are thus comparable with direct cell counts using microscopy, provided this technique can detect undetectable and fragile cells accurately (Park BS, personal communication).

As noted previously, microarray (DNA chip) methods are very effective for the simultaneous large-scale detection of microalgae (Gescher et al. 2008; Anderson and Walt 2009). Furthermore, this method is recommended as one of the high-throughput molecular quantification techniques. This technique detects labeled rRNA or labeled DNA amplicons from target regions, such as rRNA from genomic DNA. In this assay the fluorescence labeled DNA or RNA molecules are quantified and is proportional to cell numbers (Anderson et al. 2006) Hybridization of the labeled products is performed by exposing the microarray to signal probes, and the hybridization signal is recorded (Metfies and Medlin 2008). For quantification, the arrays have been hybridized using different concentrations of target cells, and the cells were subsequently enumerated from the signals generated (Ahn et al. 2006; Anderson et al. 2006; Scorzetti et al. 2009; Diaz et al. 2010). The microarray-based method has several advantages, such as low detection limit, shorter analysis time, and minimal false positive signals. The microarray has been tested in pilot studies to quantify absolutely certain specific algal species; it has great potential to quantify microalgal cells and alter the standard procedures used for microalgae monitoring programs. However, problems regarding signal acquisition needs to be well addressed.

FISH uses short fluorescently labeled, synthesized probes that are complementary to a target sequence within the target cells. FISH usually utilizes oligonucleotide sequences that bind to the ribosomes in target cells, although peptide nucleic acid probes are sometimes used (Litaker and Tester 2006). The target molecules in the cells will fluoresce, and in the case of ribosomes, the entire cell can be made fluorescent, making them easy to enumerate (Kudela et al. 2010). The advantages of FISH are that it is inexpensive, more rapid than electron microscopy, and reduces false positives and allows the characterization of the entire phytoplankton population, although only with 1 probe (1 fluorochrome) at a time. The use of signal amplification tools like tyramide signal amplification and catalyzed reported deposition have also been used along with FISH for the easy discrimination of microalgal species (Biegala et al. 2002; Töbe et al. 2006).

Non-molecular techniques, such as flow cytometry and advanced microscopy, can be combined with DNA-based

probes and dyes (nucleic acid-specific dyes, specific oligonucleotide probes, etc.) for the quantification of species in environmental samples. It has been observed that with the use of DNA-specific dyes, such as SYBR Green and EvaGreen, microalgal cells can be enumerated using fluorescence microscopy, and the results are comparable to those obtained by hemocytometer-based counting (Soto et al. 2005). This technique is more advantageous than normal microscope-based enumeration because it can specifically distinguish cells based on their shape and other morphological parameters and is less time-consuming. FISH has been combined with solid-phase cytometry for the enumeration of microalgae (Töbe et al. 2006); this method is rapid and has a lower detection limit, approximately one cell per filter.

## Next-Generation Sequencing Technologies for Diversity of Microalgae

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 (Metzker 2010; Nowrousian 2010). Table 4 summarizes the NGS technologies available and their major features. In field sample studies, NGS technologies are facilitating the gathering of DNA data from both environmental DNA and PCR products amplified from environmental DNA. These NGS applications differ from the clone library method because they do not require the cloning of template DNA into bacterial vectors; alternatively, DNA templates are bound to substrates and amplified by PCR to generate clonal representatives, and hence no cloning bias is imposed for metagenomics (e.g., Shendure and Ji 2008; Metzker 2010). In addition, the number of sequence reads by the NGS methods have been extremely high, revealing a high diversity of microbes that were not detected from clone library methods (Stoeck et al. 2010).

The development of NGS has made it possible to directly sequence a huge number of genomic fragments extracted from environmental samples (Rothberg and Leamon 2008), hence making NGS a potential tool for the identification and detection of microbes from environmental samples (Medinger et al. 2010). In 2006, Edwards et al. (2006) published, for the first time, sequences of environmental samples generated with the chip-based pyrosequencing developed by 454 Life Sciences. NGS techniques have enabled the discovery of novel genes from environmental samples for the massive characterization of functional genes and enabled study of the metagenomic diversity of unculturable bacteria and archaea in various

**Table 4** Next generation sequencing (NGS) technologies and their features

| NGS Technology (Company/platform) | Technology | Amplification approach | Reads (million) | Length (bp) | Throughput | Advantages |
|---|---|---|---|---|---|---|
| ▪Roche/GS-FLX (454)[a] | -Pyrosequencing | -Emulsion PCR | 1.0 | 600 bp | 600 Mb | -High quality reads, long read lengths, and capacity to handle repetitive regions, identification, and quantification of significant genes |
| ▪Illumina/Genome Analyzer *IIx* (GAIIx) | -Polymerase-based sequencing-by-synthesis | -Bridge amplification | 640 | -Single and paired end, 36–500 bp | 95 Gb | -A platform for genomic discovery and validation |
| ▪Illumina/HiSeq2000 | -Sequencing by synthesis (SBS) | -Bridge amplification | 2,000 | -Single and paired end 50–100 bp | 200 Gb | -Most accurate data for a broad range of applications |
| ▪ABI/SOLiD v.4 | -Ligation-based sequencing | -Emulsion PCR | 1,400 | -Fragment and paired end and mate pair 35–50 bp | 100 Gb | -Provides better sequencing fidelity |
| ▪Pacific Biosciences/ PacBio RS | -Phospholinked fluorescent nucleotides, real-time sequencing | -Single molecule templates | 800–1,000 | – | – | -Has the greatest potential for reads exceeding 1 kb |

[a] The GS-FLX had recently been upgraded to GS-FLX+ systems (performance: read length, up to 1,000 bp; mode read length, 700 bp; typical throughput, 700 Mb; reads per run, 1,000,000, respectively)

environmental samples (Roesch et al. 2007; Shi et al. 2009). NGS techniques have recently been applied for diversity evaluation and phylogenetic studies in protists and microalgae, such as diatoms, dinoflagellates, and haptophytes (Amaral-Zettler et al. 2009; Burki et al. 2010; Shalchian-Tabrizi et al. 2011).

With the rapid progress in NGS technologies in recent years, various NGS platforms are now available, such as 454 pyrosequencing, HiSeq2000, GAIIx, and SOLiD, PacBio RS (see Table 4); however, their application to environmental diversity studies is restricted by sequence length per individual read. Sequence length of each read is usually less than 150 bp, though NGS tools generate a huge amount of sequence data with billions of sequencing reactions in a single run. However, the 454 pyrosequencing easily produces reads of 500 bp, and recently this technology has been upgraded as the GS FLX+ system, which enhances the read length up to 1,000 bp in optimum conditions with new reagents (http://www.my454.com/products/gs-flx-system/index.asp). In metagenomic diversity studies, individual sequences without assembly of sequence reads are subjected to phylogenetic analysis by comparison with well-defined DNA sequences as taxonomic markers or signatures. NGS-based metagenomics mostly targets the 18S rRNA molecules, as DNA taxonomic markers (e.g., Amaral-Zettler et al. 2009; Bråte et al. 2010), because of highly conserved sequences within a species, but generally different between species (Ki 2011), and comparably large dataset available in GenBank. Because of the relatively long reads (up to 1,000 bp) in 454 pyrosequencing, the Genome Sequencer FLX System is usually employed in metagenomic diversity studies using PCR amplicons (Amaral-Zettler et al. 2009; Medinger et al. 2010; Burki et al. 2010; Edgcomb et al. 2011; Mccliment et al. 2011; Shalchian-Tabrizi et al. 2011; Tai et al. 2011). The NGS system therefore enables a more comprehensive view into the diversity of various environmental habitats. Techniques, such as 454 pyrosequencing, Illumina, and SOLiD, are largely used to identify and detect malfunctioning cells and microbiota of the human body (Petrosino et al. 2009; Roesch et al. 2009). There is great potential for their use in diversity studies on microbes, such as bacteria, archaea, and microeukaryotes, including microalgae. In addition, these techniques can be used to count environmental gene tags, or PCR amplicons, to analyze the relative abundance of microalgal species under varying environmental conditions, although the NGS-based quantification is not completely proven at this stage, because of some possible biases (e.g., PCR-amplification bias, NGS reads, etc.).

## Conclusions and Remarks

Microalgae are major components of the aquatic ecosystem, and their diversity is strongly modified by rapid and accelerating environmental changes (Elmqvist et al. 2003). They are unicellular eukaryotes [excluding blue-green algae (cyanobacteria)] with distinct features, such as morphology, pigments, and photosynthetic activity, and can be extremely small in size (e.g., nano- and pico-size plankton). Hence, it is necessary for the correct identification, continuous monitoring, and enumeration of these microorganisms. Beyond the traditional microscopic methods, many molecular techniques have been developed as alternative methods to discriminate microalgal species. Even molecular methods developed by other molecular biologists can be used for microalgal studies because the target biomolecules (DNA, RNA, and protein) are universal. Each molecular technique has its own particular strengths in detecting microalgae but may have limitations when applied to other species. For example, single-cell PCR is considered a good molecular tool for studying uncultured microalgal cells from environments but is difficult to apply to pico-size cells; but by involving flow cytometry, this can also be made possible. To date, there are several reviews of molecular methods that consider different aspects, applications, and organisms. This paper highlights the practical molecular tools available for species detection, quantification, and diversity analysis of microalgae.

Despite rapid advances and many examples highlighting the application of molecular methods to a broad spectrum of applications, there are very few methodologies for quantification and diversity studies in microalgae. Molecular technologies ranging from automated Sanger sequencing to NGS technologies have opened doors for the easy and rapid detection and quantification of cells. Among the currently available methods, qPCR offers the most cost-effective, sensitive, and rapid analysis for the detection and quantification of microalgae in both laboratory and field situations. Isothermal DNA amplification techniques, such as LAMP and NASBA, are relatively new methods, which, because of their simplicity, ruggedness, and low cost, could provide major advantages. These methods therefore have the potential to be used as large-scale, simple screening assays for the detection and diversity estimation of microalgae. In addition, NGS techniques have been applied to various genomic research studies and have significant potential for future use in both discrimination and quantification of microalgae worldwide. At present, the operational cost and data analysis tools associated with NGS technology support the wide-range use in molecular monitoring and quantification of environmental microalgae. In the case of 454 pyrosequencing, costs can be greatly reduced by employing Multiplex Identifier-containing adaptors, which allow users to have greater multiplexing capabilities with the GS FLX Titanium sequencing chemistry. Moreover, the coming third- and fourth-generation technologies that are using techniques like single molecule sequencing (Pacific Biosciences Inc.) and single molecule electrical detection (Genia Technologies,

Inc.) will increase throughput and decrease the cost and time of acquiring results. This shows that NGS tools offer great potential to alter the manner in which researchers monitor microalgae.

Molecular metagenomic techniques have greatly expanded our understanding of the diversity of microalgae in environmental samples. In particular, NGS-based metagenomics show a remarkably high diversity of microalgae; however, a large portion of sequence data can be unassigned to molecular operational taxonomic units, because of an insufficient DNA taxonomic database (e.g., DNA barcoding, DNA reference, or signature sequences). Most data available in public databases have been derived from cultured strains of microalgae. In the future, we have to construct well-defined DNA databases of microalgae in order to achieve a comprehensive understanding of the molecular diversity of environmental microalgae. In this regard, the newest technology of single-cell genome analysis, in which a single cell can be isolated from the environment by flow cytometry and its genome can be amplified and sequenced, may be the only way that we can achieve such a goal (Yoon et al. 2011).

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