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Using the molecular toolbox to compare harmful algal blooms in upwelling systems

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ABSTRACT

Harmful algal blooms (HABs) are now generally recognized as occurring over a wide range of habitats from oligotrophic to hypernutrified, and appear to be expanding globally. Unlike many other ecosystems impacted by HABs, upwelling systems worldwide share a common set of physical parameters and are likely to respond similarly, regardless of locale. The Core Research Project on HABs in Upwelling Systems, a component of the scientific programme on the Global Ecology and Oceanography of Harmful Algal Blooms (GEOHAB), promotes a comparative approach to identify the similarities and differences in the manifestation of HAB events in these systems. As applied to the goals of this programme, molecular techniques are a powerful suite of tools for HAB species identification, for determining genetic similarity within morphologically indistinguishable species, and ultimately, for assessing spatial and temporal patterns in ecophysiological responses in these upwelling systems. Knowledge of HAB organisms will be enhanced by comparing and contrasting the responses of these organisms in similar upwelling regions. Here, we provide an update on the availability of molecular and genetic tools for comparative HAB programmes in upwelling systems, focusing on four broad applications: cell enumeration and identification, molecular phylogenetics, functional/comparative genomics, and applications of high throughput sequencing methods. We highlight the rapid evolution, the promise, and the potential pitfalls, of the molecular toolbox, focusing on specific examples of how scientists and resource managers currently apply these methods. Specific examples are developed using relevant case studies from the California, Benguela and Iberian systems. We summarise by providing a synthesis of future research directions and goals that would be particularly relevant to advancing the comparative method for HAB genetics with an emphasis on upwelling systems.

1. Introduction

The Core Research Project (CRP) on HABs in Upwelling Systems, a component of the scientific programme on the Global Ecology and Oceanography of Harmful Algal Blooms (GEOHAB), has identified the use of molecular tools and development of comparative genomics as powerful approaches facilitating comparative studies between upwelling systems (GEOHAB, 2005). The comparative method can make use of genomic information in the context of naturally occurring temporal and spatial variations in existing conditions and phenomena, to infer or identify the underlying causal mechanisms of harmful algal bloom dynamics. Molecular techniques can be used for HAB species identification, toxin assessment and as indicators of the genetic and physiological similarity between morphologically and/or ecologically indistinguishable species in space and time.

Molecular methods are now widely used and the HAB community has access to commercially available as well as project-specific kits and methods; this wide availability, standardization and quality control has moved many of these techniques out of the hands of specialists and promoted broader application by the research, monitoring, and management communities (Grossman, 2005). We loosely categorize the "molecular toolbox" as the application of gene-based (focusing on specific genes, gene products, or processes), genomic (genetic analysis of an organism, rather than specific genes or products) and metagenomic (genetic information derived from a community of organisms, usually incomplete, that does not rely on laboratory growth and culturing) techniques, in this case to the study of harmful algae in upwelling systems (Fig. 1). It ranges from species identification (using a variety of approaches), toxin quantification, and functional genomic studies (i.e. identification of genes or gene products directly related to the ecophysiological functioning of harmful algae, such as growth, grazing, and toxin production genomic signatures), to complete sequencing of algal genomes and representative sequencing of

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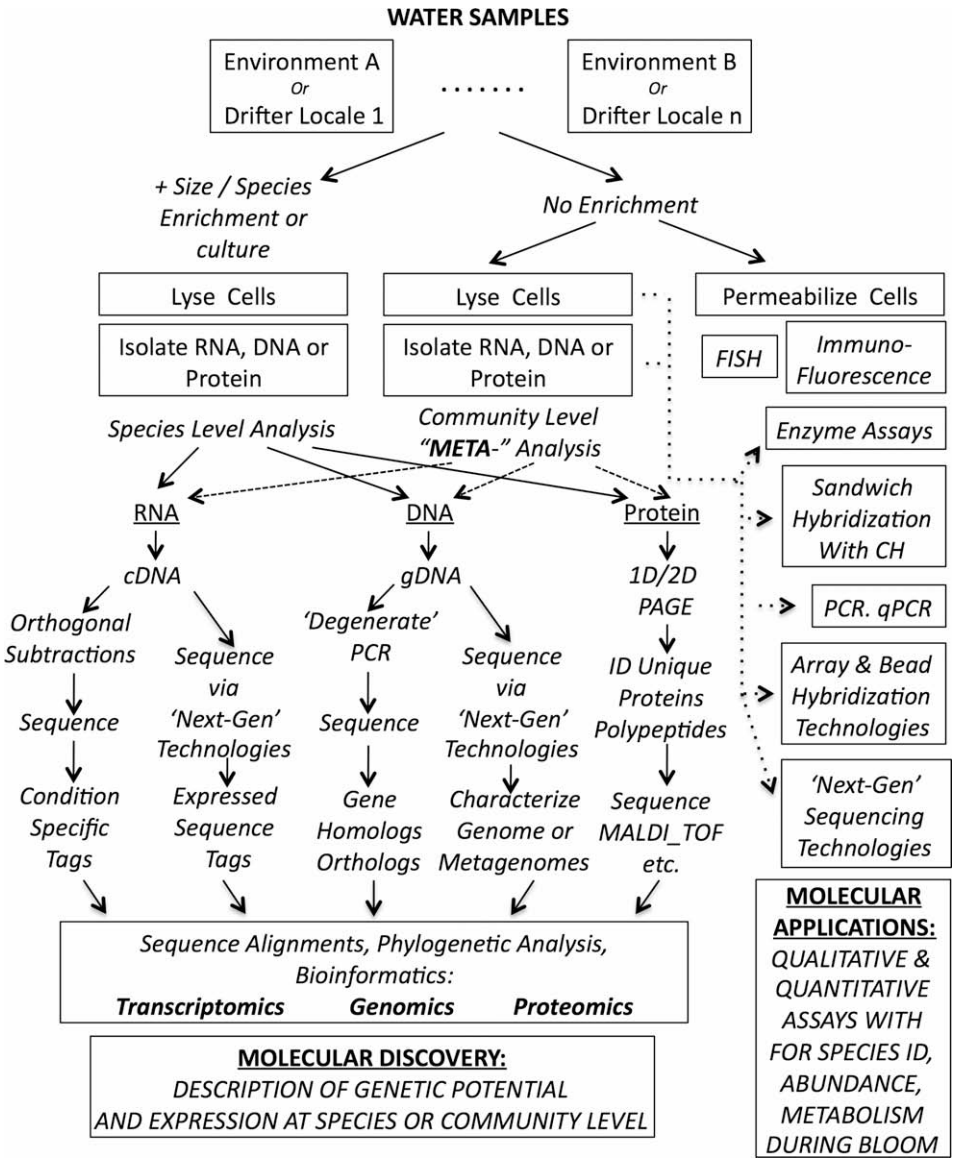

Fig. 1. Application of the molecular toolbox for comparative analysis within and between upwelling influenced ecosystems (or other comparative studies). A suite of technologies is available for analysis of molecular constituents (RNA, DNA, protein) for single species or assemblages, enabling description of genes and gene products, species, and populations within a given environmental regime. These approaches can be used to define and optimize specific assays targeting quantification of species dynamics and physiological function spatially and temporally using standard oceanographic or robotic sampling vehicles. Although not shown, temporal analysis of entire cellular metabolite pools (metabolomics) is equally compatible with this scheme of information flow. A comparative analysis would begin with collection of samples within or between systems (top), and would flow towards the "products" (bottom). Culture-based methods follow the left-most pathway and typically include additional steps to select for particular strains or species. See main text and references therein (particularly review articles) for explanations of specific methods.

entire communities. The toolbox comprises a set of overlapping technological approaches enabling rapid progression from fundamental descriptions or discovery of species and communities in disparate environments, to assays of species abundance or cellular function along environmental gradients such as those characterizing upwelling dominated environments (Fig. 1).

Despite the rapid development of molecular approaches for algal ecophysiology, much work remains to be done to fully integrate these methods, and accompanying knowledge, into studies of harmful algal bloom ecology. There are relatively few examples of truly comparative studies using molecular techniques within or between upwelling systems, and we have therefore broadened the topic herein to include an overview of the most common methods that could be applied to such comparative studies, expanding on the many excellent reviews relevant to molecular methods for HAB organisms (e.g. Plumley, 1997; Bates and Trainer, 2006; Cembella and John, 2006; Dyhrman, 2008; Hudson, 2008). We provide an overview of the molecular toolbox, starting with the most frequently used methods of species identification and molecular phylogenetics, and then progress to applications of full genome sequencing and functional genomic approaches. To frame the discussion, we provide case studies for how these techniques can advance the goals of comparing HABs in upwelling systems, focusing on three groups: the saxitoxin producers (*Alexandrium*), domoic acid producers (*Pseudo-nitzschia*), and yessotoxin producers (*Lingulodinium*, *Gonyaulax*, *Protoceratium*), which make up a large fraction of the HAB problems in upwelling systems (c.f. Trainer et al., this issue). In the final section, we provide an overview of future directions and recommendations specific to the GEOHAB Core Research Project on HABS in Upwelling Systems.

2. The molecular toolbox

2.1. Cell detection, identification and quantification

A fundamental requirement for the detection, enumeration and study of HAB organisms is identifying individual species amongst what is often a complex milieu of planktonic organisms. For morphologically distinct organisms, or in the somewhat rare case of monospecific blooms, this can be a straightforward task. More frequently, however, it is difficult or impossible to visually identify to species potentially harmful organisms. For ecological studies, it is often useful to fully characterize the associated algal assemblage, a time-consuming task for phytoplankton ecologists relying on microscopic enumeration. The task can be made substantially more difficult or impossible if the organisms are rare, "hidden" in zooplankton fecal material, or poorly preserved.

Perhaps the clearest example of the difficulty in positively identifying HAB organisms is exemplified by the diatom genus *Pseudo-nitzschia*. Since identification of *Pseudo-nitzschia* as a toxin producer in 1987 (Bates et al., 1998), 11–12 species of this genus have been confirmed as toxigenic (c.f. Bates and Trainer, 2006), in addition to the related *Nitzschia navis-varingica* (Kotaki et al., 2000). Although the potential for domoic acid toxicity is widespread in *Pseudo-nitzschia,* congeners have been identified that are apparently non-toxic, whereas documented isolate-specific variability within toxigenic species (Smith et al., 2001; Kudela et al., 2004; Bates and Trainer, 2006) presents further challenges for establishing robust monitoring programmes targeting toxigenic blooms. This is exacerbated by the fact that the production of domoic acid is not necessarily constitutive for those species that are toxigenic (e.g. Pan et al., 1996a–c; Garrison et al., 2002; Kudela et al., 2004), and the numerous reorganizations of this genus (Lundholm et al., 2006). In addition to the difficulties of working with intraspecific differences in toxin production, the primary challenge for monitoring individual *Pseudo-nitzschia* species of interest is that many morphologically similar species co-exist in the marine environments where they are found.

Discriminating toxin producers from non-toxic congeners is thus critical, but not simple. For instance, discerning the relatively non-toxic *Pseudo-nitzschia pungens* from the toxin producer *Pseudo-nitzschia multiseries* has traditionally required using electron microscopy on cleaned valves to reveal fine-scale morphological differences that are not visible under light microscopy. Similarly, the toxin producer *Pseudo-nitzschia australis* cannot be confidently differentiated from several relatively non-toxic coexisting species without using electron microscopy (e.g. Turrell et al., 2008). Thus, difficulties inherent in identifying species using laborious traditional morphology-based methods, while providing the gold standard for robust identification, make this approach both time and cost inefficient for monitoring programmes. These concerns, along with a dwindling supply of active expert taxonomists, have motivated a concerted effort to develop more rapid, simple and efficient methods for robust species identification amenable to standard field collection practices. Since the 1990s several methods have been developed, including gene sequence-based hybridization methods and immunological or algal cell surface lectin detection of species-specific antigens (Scholin et al., 2003; Sellner et al., 2003; Metfies et al., 2006; Turrell et al., 2008). We limit our discussion to genetic methods, because these have met with significant success and are in common use worldwide by a number of laboratory groups and regulatory agencies.

Molecular genetic approaches to species identification may be broadly grouped as either "whole cell" (WC) or "cell homogenate" (CH) based. Whole cell approaches treat cells in a manner such that they remain intact throughout sampling and processing, and indeed, must be intact to be detected, providing an additional level of quality assurance for the assay by enabling discrimination of false positives. Alternatively, for CH methods, cells are collected and disrupted to liberate cytoplasm, and the resulting cell homogenate is analyzed, typically with subsequent calibration of the signal back to cell number.

Whole cell probing, also known as fluorescent *in situ* hybridization (FISH), employs short *in vitro* synthesized "probes" with a covalently linked fluorescent dye ("reporter") and is complementary to a target sequence within the target cells. If designed properly, these probes are intra-specific, but not inter-specific. They most commonly utilize oligonucleotide sequences that bind to the nucleic acid pools in the target cell, but other probe modalities are available, including peptide nucleic acid probes (Litaker and Tester, 2006). For a given "reporter" molecule, detection signal will be proportional to the abundance of the cellular target. Typical sequence targets are the highly abundant ribosomal RNAs (rRNA), although probes can also be designed for hybridization to other lower abundance genomic targets, such as nuclear or organellar DNA, or even unprocessed RNA (preRNA) in the nucleus, although potential total detection signal will be reduced accordingly. Cells require permeabilization, often accomplished with preservation using ethanol, in order to promote access of the probes and for efficient removal of unbound probe during buffer washes. Control of hybridization temperature and buffer salt content are used to standardize the sequence specificity of these hybridizations. Epifluorescence microscopy can routinely be used to visualize, identify and enumerate labelled target cells, especially when the hybridization target is the abundant rRNA (DeLong et al., 1989; Miller and Scholin, 1996, 1998, 2000; Scholin et al., 1996). Good practice dictates that a positive control probe, complementary to sequences shared among taxa related to the target organism (e.g. all eukaryotic organisms), and a negative control to account for indiscriminate probe binding be run on splits of the same sample material to provide quality assurance for the conduct of both the hybridization and wash steps in FISH assays. As an alternative to microscopy, cells can be labeled as described, and subsequently analyzed on a flow cytometer fitted with a fluorescence detection system, or through the use of solid-phase cytometry, which images cells immobilized on a solid support with a laser (Metfies et al., 2006). This technique is particularly suited for analysis of large cells that can challenge the size constraints imposed by flow channels. Using these techniques, cell detection and enumeration is partially automated, while retaining the benefits of whole cell inspection.

The advantages to whole cell probing approaches are that they are quantitative and both less expensive and faster than electron microscopy. Additionally, since cells remain intact, they can be visually inspected to assess false positive rates and for morphological comparison using "traditional" methods. In addition, the co-occurring species assemblage is retained and is visible in the positive control, enabling characterization of the entire phytoplankton community. Disadvantages of whole cell probing include the relatively small volumes typically sampled (often 10–20 mL) required for efficient enumeration of the sample field and to reduce the amount of potentially interfering debris. Autofluorescence from residual chlorophyll, other pigments, accumulation bodies, mineralized structures and fixation artifacts common with samples from diverse algal communities can make it difficult for new users to quantitatively and accurately observe target cells. Whereas visual counting of samples via epifluorescence microscopy ultimately limits the speed at which samples can be processed, the effectiveness of automation through advents in image analysis software or solid-phase cytometry (Metfies et al., 2006) will depend on human assessment of the results. Labeling of target cells may be poor or

obscured if cells have been ingested by grazers, packaged in fecal pellets, or if cells are senescent, but this information, if documented, can also be leveraged to provide information of the fate or condition of the bloom over successive sampling periods.

Enumeration methods can also be based on preparations of cellular homogenates (CH) treated with molecular probes. Cells are lysed and the nucleic acid pool from the entire natural assemblage is available to react with the introduced probes. Cell homogenate methods include sandwich hybridization (Scholin et al., 1997, 1999), microarray hybridization (Gescher et al., 2008) and quantitative polymerase chain reaction (qPCR; Ausubel et al., 1995; Innis et al., 1995; Paul, 2001; Caron, 2005). These methods differ primarily in that sandwich hybridization is analogous to the FISH assays: a reporter molecule attaches to the intact rRNA of the target sequence within the cell lysate, and is quantified directly. Microarrays are also hybridization-based methods in which a single-stranded DNA copy (cDNA) of the RNA pool in the CH is labeled, hybridized to probes immobilized on the microarray, and quantified using the signal from the cDNA-probe binding. In contrast, PCR methods first amplify the nucleic acid pool in the CH by making repeated copies of a targeted gene sequence (optimally 2× per step), thus greatly enhancing the limit of detection. As a direct sequence amplification approach, PCR is advantageous for detection of low abundance targets. Such methods are not without drawbacks, including the potential to generate artifacts due to inhibitors present in environmental samples or poor primer design leading to spurious amplification of non-target DNA. If robust comparison of species abundance during bloom evolution is desired, all of these methods require careful calibration in order to account for potential variation in target sequence abundance with respect to species, geographic populations within species, and at a minimum, growth status, because variability can be introduced as a function of cell nutritional status, growth stage, and other factors that result in differential expression of rRNA on a per-cell basis (Anderson et al., 2005).

Recent advances in qPCR methods also allow multiple species to be assayed simultaneously, using techniques called multiplexing and multiprobing (Handy et al., 2006). These methods involve application of either multiple fluorescent markers with corresponding molecular probes (multiplex) or multiple markers with a common set of primers (multiprobe). This opens the possibility of rapidly and non-subjectively enumerating multiple organisms within the same sample, but introduces added complexity in the methods development (e.g. ensuring the primers and probes do not interfere and that the PCR signal increases at similar rates for all probes).

Advantages of cell homogenate approaches for bloom dynamics include amenability for automation. Unlike FISH probes, the intact cell is never visually identified; instead, a quantitative reporter signal, typically colorimetric, is measured, often using a 96-well microplate with an automated detection system. Being extract-based, these methods permit analysis of larger environmental sample volumes and thereby avoid random sampling errors inherent in the small sample size used for whole cell methods. The potential for environmentally derived assay interference also increases, but can be assessed by inclusion of internal controls in the sample-processing stream. A disadvantage of cell homogenate methods is that it is not possible to visually inspect positive samples to verify their representation of cells morphologically similar to the species of interest, rather than being the result of a cross-reaction to a non-target organism. Because cell homogenate methods detect liberated nucleic acids, all sources of target sequences can contribute to the signal including senescent cells or cells obscured in food vacuoles or fecal pellets, highlighting the need for careful inter-calibration when applied to comparative studies. Finally, the cell homogenate methods are generally most applicable to laboratory settings where specialized equipment and supplies are available, potentially limiting applicability to end-users in less developed regions and by monitoring and management agencies that have neither the budget nor expertise to replace traditional microscopy.

Despite the rapid adoption of molecular methods for cell detection, several pitfalls and caveats remain, particularly for application within a comparative systems approach. A primary concern is in the design of the molecular probe. Specificity (the ability to identify the target organism and discriminate from non-target organisms) is dependent on the availability of DNA sequence information from both the target and taxonomically related organisms in genome databases. Due to the high levels of genetic diversity present within certain species and because probes are frequently designed from sequences of isolates collected over a limited geographic area during a limited time period, it has often been problematic to apply the same probes elsewhere (Parsons et al., 1999; Orsini et al., 2002). For example, a recent study in Scottish coastal waters applied FISH probes designed for *Pseudo-nitzschia* species from the US west coast. While some worked well, others did not, in some cases due to a single-base pair change in the local (Scottish) species (Turrell et al., 2008). Hybridization between *Pseudo-nitzschia* species within a population has been documented and can give rise to mixed combinations of ribotypes and chloroplast genes. This has been shown for genetically distinct strains of *Pseudo-nitzschia pungens* from the North Pacific coast of the US (Casteleyn et al., 2009) and in populations of *Pseudo-nitzschia multistriata* from the Gulf of Naples (D'Alelio et al., 2009). Combined genotypes in progeny resulting from hybridization can confound species identification with molecular methods. Prior to application of molecular probes to a new region or system, it is prudent to isolate the target organism(s), sequence the target gene(s) and verify probe specificity. Similarly, it is as important to test cross-reactivity with non-target organisms to verify that application of the probes within the new system will not identify previously unrecognized cross-reactions with other species.

A second concern is that molecular methods are infrequently directly compared to other cell enumeration methods such as light microscopy or lectin binding, or even between methods (e.g. FISH vs. qPCR), making it difficult to ensure continuity with historical cell detection methods and/or comparability between studies. These concerns are widely recognized by molecular ecologists, and are being addressed through careful, simultaneous evaluation of various cell detection methods (Handy et al., 2006; Godhe et al., 2007; Turrell et al., 2008). Whereas the core technological approaches may be shared, each target species and even water type can present unique challenges and to date, relatively few intercomparison studies have been conducted, particularly across environmental regimes (ACT, 2008). Based on these few studies, molecular methods have been shown to provide increased sensitivity, decreased variability and enhanced capacity to distinguish morphologically similar organisms.

2.2. Molecular phylogenetics and species diversity

A closely related application of molecular cell detection methods involves the identification of molecular diversity within or between target species. The use of molecular phylogenetics in algae was initially demonstrated by Gallagher (1980) and Brand (1981), and is now widely used, in addition to traditional methods (such as morphology, ultrastructure, life cycles and the fossil record) to understand the evolutionary history of many organisms, including harmful algae (Daugbjerg et al., 2000; John et al., 2003; Lundholm et al., 2006). Ribosomal DNA (rDNA), the genome region that codes for rRNA, has been the target of many of these studies because ribosomes are universally present in living organisms and rDNA contains regions that are well conserved across

divergent taxa (Lenaers et al., 1991; Schlötterer, 1998; Fensome et al., 2000). The Large Subunit (LSU) consists of several variable domains (D1, D3 and D8) that are used to evaluate phylogenetic relationships of closely related species (Lenaers et al., 1991; Scholin et al., 1994; Miller and Scholin, 1996). The Internal Transcriber Spacer (ITS) regions (comprised of ITS1, 5.8S and ITS2) are also commonly used to analyze closely related and geographically different species (Schlötterer, 1998; Coyer et al., 2001; LaJeunesse, 2001; Saito et al., 2002; Shao et al., 2004; Litaker et al., 2007). Molecular sequence information has proved valuable for evaluation of relationships within genera and especially among morphologically indistinguishable species of various HAB organisms. This information can help to differentiate poorly described or difficult to identify species, and can be used to target functional assays for toxicity at the inter- and intra-species level, such as for the yessotoxin (YTX) producers.

There are three known producers of the emerging, globally distributed phycotoxin, YTX. These are the dinoflagellates *Protoceratium reticulatum*, *Lingulodinium polyedrum* and *Gonyaulax spinifera*. Production of YTX within and among each species is highly variable and toxic and non-toxic isolates have been identified, often from the same geographic location, from Spain, the United Kingdom and California (Riobo et al., 2002; Stobo et al., 2003; Paz et al., 2004; Armstrong and Kudela, 2006). Isolates of *P. reticulatum* have been identified as toxic in British Columbia, Canada (Cassis, 2005) and South Africa (Fawcett et al., 2007), whereas strains isolated from Washington and California have been reported as both toxic (Paz et al., 2004) and non-toxic (Paz et al., 2007). This has led to some difficulty in characterizing YTX production within and between these regions, with YTX production ascribed to a wide variety of dinoflagellates, including organisms not known to produce the compounds (e.g. *Prorocentrum*; Howard et al., 2009).

As described by Howard et al. (2009), phylogenetic analysis of molecular sequence information of known YTX-producing dinoflagellates, *P. reticulatum*, *L. polyedrum* and *G. spinifera,* were examined in order to determine if there are informative genetic differences among geographically distinct populations (defined by isolate metadata) or between toxic and non-toxic isolates within species. The identification of other potential, but yet unidentified, YTX-producing dinoflagellates was also evaluated as it can be difficult and labour intensive to conclusively determine the biological origin of toxin during events (c.f. Howard et al., 2008).

In all of the ITS and LSU analyses, the three confirmed YTX-producing species fell within the order Gonyaulacales, in agreement with morphological taxonomy (Fig. 2). At this observational level, toxic and non-toxic isolates of *L. polyedrum* were indistinguishable and robust markers of YTX toxicity for most genera within the Gonyaulacales order are not feasible. However, development of rDNA-targeted markers for toxic strains may be possible within the genus *Gonyaulax* (Howard et al., 2009; Riccardi et al., 2009).

Of relevance to the comparative approach for HABs in upwelling systems, we can conclude the following: future testing during YTX-contamination events should be prioritized on species in the order Gonyaulacales; the high degree of sequence similarity between geographically distinct populations suggests that molecular identification methods such as PCR and FISH would be widely (possibly globally) applicable to all members of this group, and would simplify technology transfer from one region to another; however, these rRNA approaches are limited for identifying physiological differences that lead to toxin production, and future research would benefit from whole genome approaches.

Size polymorphisms in microsatellite sequences generally comprised of 2–4 base pair sequence motifs occurring as tandem repeats in nuclear and organelle DNA (Fig. 1B) are another method for resolving intra-specific diversity and population structure. These repeating sequences are evolutionarily neutral; they provide a unique molecular marker indicative of inter- and intra-species variability. Improvements in protocols for efficiently isolating microsatellite regions specific to target organisms have recently enabled algal researchers to characterize the biogeography and local population dynamics of phytoplankton species assemblages with a resolution generally not accessible using traditional phylogenetic approaches. These methods are best applied to regional studies due to the highly variable nature of the microsatellites and the difficulty of adequately sampling the population for biogeographic analysis at larger spatial scales.

Pioneering efforts in this field were conducted by Rynearson and Armbrust (2000, 2004), who demonstrated that the diatom *Ditylum brightwellii* exhibited genetically and physiologically distinct subpopulations within spatially interconnected estuaries, presumably responding to subtle differences in environmental forcing; these distinct subpopulations were stable for at least two years (Rynearson and Armbrust, 2004). Evans et al. (2004) compared 25 distinct isolates of *P. multiseries* from eastern Canadian, European and Russian waters, of which 22 genetically distinct subpopulations were identified, with only two clonal isolates (obtained within 15 km of each other). In a much broader study, Evans et al. (2005) examined the genetic diversity of *P. pungens* in the North Sea, using 1050 clonal isolates collected over 18 months and approximately 100 km. Of the initial pool, 45% were successfully analyzed, and demonstrated a high (98%) level of subspecies diversity but weak genetic structure (Evans et al., 2005).

At least as important as the ecophysiological characterization and evolution of harmful species, the molecular toolbox provides a powerful method for inferring the introduction of non-indigenous organisms from one system to another by enabling identification of genetic markers distinguishing subpopulations or strains associated with distinct biogeographic regions. The best example of this approach comes from the analysis of what is referred to as the *Alexandrium tamarense/catenella* species complex. Relatively soon after the introduction of molecular phylogenetics to the study of harmful algae, the suite of species belonging to the general grouping of "*Alexandrium tamarense/catenella*" were elegantly described based on globally distinct ribotypes. Strains of these species are organized into distinct regional genetic groups that likely evolved due to isolation of subpopulations by spatial isolation (Scholin et al., 1995; Scholin, 1998; John et al., 2003). Against this natural background of range expansion and speciation, it has been suggested that members of this complex have "appeared" in locations such as Australasia, in the absence of obvious transport mechanisms other than ballast-water transport (Bolch and de Salas, 2007).

Phylogenetic analyses have also been applied to *A. catenella*, *Alexandrium tamayavanichi* and *Alexandrium minutum* from South African waters (Ruiz Sebastián et al., 2005; Pitcher et al., 2007). These analyses suggest that *A. catenella* and *A. tamayavanichi* are unlikely to have been introduced via ballast water. In contrast, phylogenetic analysis supports (but does not confirm) the possible introduction of *A. minutum* to South Africa. Whereas the molecular toolbox can provide evidence in support of ballast-water transport as one mechanism leading to the "global spreading hypothesis" (the general concept of a global expansion of HABs; Smayda, 2007), molecular information alone cannot provide definitive proof for ballast-water transport. As summarized by Bolch and de Salas (2007) and Smayda (2007), multiple supporting lines of evidence must include molecular data, toxin profiles, biogeographical information, and evidence for a strain's availability for and capacity to survive ballast-water transport.

2.3. Functional and comparative genomics

Harmful algae are promising targets for the application of 'omic' or whole system investigations of their ecophysiology (e.g.

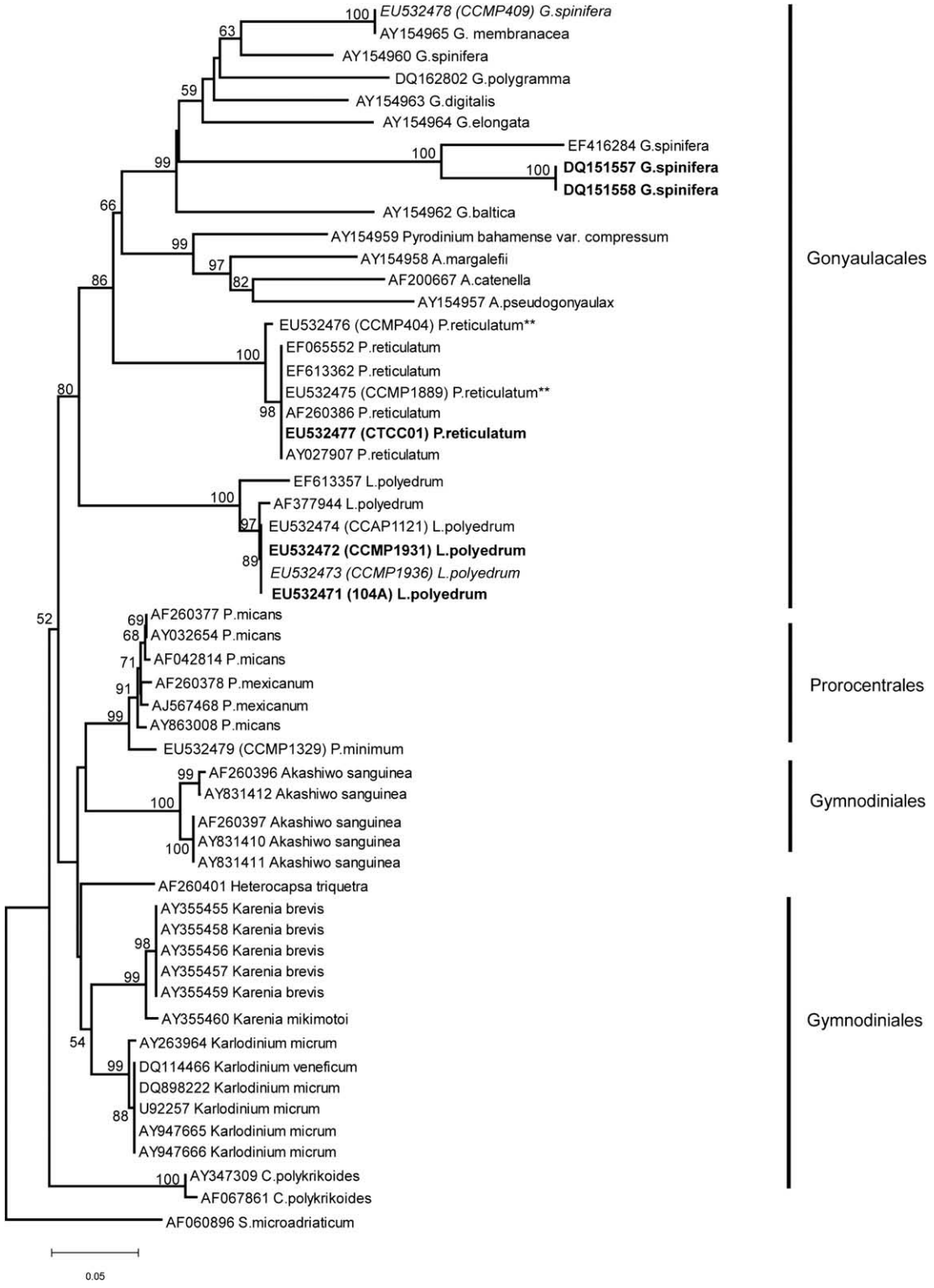

Fig. 2. Neighbour-joining analysis of the LSU ribosomal DNA. Bootstrap values (1000 replicates) are listed as a percentage of 100 and only values >50% are shown. *Symbiodinium microadriaticum* (GenBank accession number AF060896) was the outgroup. Cultures of *Lingulodinium polyedrum, Protoceratium reticulatum* and *Gonyaulax spinifera* in bold are toxic and cultures of *L. polyedrum* and *G. spinifera* in italics are non-toxic. Cultures of *P. reticulatum* for which there are conflicting studies on toxicity are indicated with an asterisk (**) after the name. The toxicity is unknown for all of the YTX-producing species in regular font.

molecular genomic, proteomic, and transcriptomic or metabolomic studies of HAB organisms). A primary goal of molecular genomics as applied to harmful algae is to describe the gene(s) or gene products associated with toxin production that could subsequently be used as markers of toxigenic blooms (Plumley, 1997), and more generically, to identify the genetic expression signatures associated with ecophysiological responses to known conditions in the natural environment. For phycotoxins, at least, these compounds are likely secondary metabolites, whose biosynthesis is often controlled by multiple gene products and interaction of multiple

metabolic pathways, making identification of gene targets more challenging (Plumley, 1997). New technological advances enable the HAB community to move away from methods for taxon identification and description based on single sequences, towards comparison of strains and species (comparative genomics) and identification of key genes and gene products influencing the physiological performance of the organism (functional genomics).

The genome of an organism is its blueprint for its metabolic potential. Analysis of a complete genome can show the processes an organism has the potential to express and can indicate the metabolic potential an organism lacks. For example, comparison of the complete genome sequences from the non-HAB diatom species *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* show a higher number of species-specific gene families in *P. tricornutum* (Bowler et al., 2008) and genes regulated by Fe (Allen et al., 2008) that are missing in the *T. pseudonana* genome. Currently, the Joint Genome Institute is sequencing the genome of the domoic-acid producing diatom *Pseudo-nitzschia multiseries*. Some HAB species have extremely large genomes; dinoflagellates in particular have genome sizes that can be as large as 100 times the size of the human genome (Rizzo, 1987), and this presents a costly problem for both sequencing and assembly, even with current technologies.

Functional genomics generally involves the analysis of gene regulation (up- and down-regulation, referring to whether the gene is expressed or suppressed under a given set of environmental conditions) for specific metabolic processes, and can include analysis of multiple gene interactions. Marchetti et al. (2009) used this approach to demonstrate that members of the genus *Pseudo-nitzschia* utilize ferritin to sequester iron. The authors identified an expressed sequence tag (EST) for a novel ferritin-like protein for which transcript abundance titrated upward in a buffer-like pattern when iron-stressed cells were resupplied with iron.

With the identification of genes involved in nutrient transport, it is possible to combine more traditional physiological experiments with molecular methods to validate their predicted function, and elucidate the associated biochemical pathways and regulation. As an example, recent work has focused on the development of a "molecular *f*-ratio" (estimate of the relative proportions of new vs. regenerated nutrient utilization; Dugdale and Goering, 1967) by examining the genes encoding for nitrate, nitrite and ammonium assimilation directly. Hildebrand and Dahlin (2000) showed that NAT genes are strongly induced by N starvation, but repressed in the presence of NH_4 (even NH_4NO_3); in contrast, the AMT genes are less regulated by the presence or absence of N, but are always active (Hildebrand, 2005). Further along in the biochemical pathway, Takabayashi et al. (2005) have shown that the gene *glnII* isolated from the diatom *Skeletonema costatum*, which encodes glutamine synthetase (GS), the isoenzyme linking C and N metabolism in the cell, is up regulated in response to external NO_3, but not NH_4. These results provide intriguing evidence for both the molecular basis for the apparent preference of NH_4 versus NO_3, as well as functional assays for "new" vs. "regenerated" production (*sensu* Dugdale and Goering, 1967).

For organisms for which complete genome sequencing is not yet feasible due to cost or technical challenges, sequencing the suite of genes actively expressed under defined conditions provides an alternative way to compare genomes. Sequencing methods are directed at sequencing copies of mRNA molecules (cDNA, complementary nucleic acid produced from fully transcribed nuclear mRNA unique to expressed genes) or cDNA ends known as expressed sequence tags (ESTs) and can include subtractive hybridization libraries, DNA microarrays and variants on PCR (c.f. Cembella and John, 2006; Litaker and Tester, 2006). These methods are especially useful for dinoflagellate HAB species that have very large genomes (Hackett et al., 2005). Dinoflagellate EST sequence libraries have been analyzed for the toxin producers *L. polyedrum* (formerly, *Gonyaulax polyedra*) (Bachvaroff et al., 2004), *Amphidinium carterae* (Bachvaroff et al., 2004), *Karenia brevis* (Lidie et al., 2005), *A. tamarense* (Hackett et al., 2005), and *Alexandrium catenella* (Uribe et al., 2008).

Expressed sequence tag libraries can also be subjected to subtractive hybridization, which permits the identification of genes that are differentially expressed in organisms grown under different conditions; for example, those that do and do not induce toxin synthesis. The elegance of this technique is that it can enable the identification of genes involved in toxin production in the absence of detailed knowledge of underlying mechanisms of toxin synthesis, similar to the differential display approach reviewed by Plumley (1997). Regardless of whether cells are maintained under toxin-inducing or non-inducing conditions, the vast majority of genes expressed under either circumstance will be identical housekeeping genes necessary for cell viability. The key step is to "subtract out" all the housekeeping genes from analysis so that only the synthesis genes are left. This approach has been used to identify diatom genes required for silica (Hildebrand et al., 1993, 1997) and nitrate (Hildebrand and Dahlin, 2000) uptake and copper stress response (Davis et al., 2006). These techniques can also be used to identify genes associated with any process that is either up- or down-regulated under defined conditions. For example, Armbrust (1999) used a PCR-based subtractive cloning method to identify diatom genes expressed specifically during sexual reproduction. Diatom genes specific to cell cycle regulation, gene regulation, morphological changes and gamete recognition were also identified (Armbrust, 1999; von Dassow et al., 2006).

Genes for specific processes (functional genes) can also be targeted in species currently lacking genome data by designing gene-specific PCR primers based on conserved amino acid domains in homologous proteins described in other, preferably related, organisms. PCR primers can be designed to account for the nucleotide combinations encoding the conserved domains (degenerate primers) and used to amplify functional genes from multiple species. The degenerate PCR method has been used to characterize nutrient metabolism and transport in a range of phytoplankton species. Transporters are of particular interest as they are typically highly regulated, interact with specific nutrients and are keys to the environmental response of phytoplankton in the natural environment. Using diatoms as an example, in addition to the silica class of transporters (SITs; Hildebrand et al., 1997, 1998; Thamatrakoln et al., 2006), Hildebrand and Dahlin (2000) have isolated a nitrate transporter (NAT), and later, Hildebrand (2005) cloned and isolated a class of ammonium transporters (AMT).

With the availability of complete genomes (e.g. Armbrust et al., 2004), this work can rapidly be extended, particularly into the realm of functional and comparative genomics. Elegant analysis of *T. pseudonana* gene expression in response to environmental manipulation of light, temperature and nitrogen source by Parker and Armbrust (2005), identified transcriptional changes (up- and down-regulation) in pathways for the Calvin cycle, nitrate utilization and photorespiration. The corresponding model developed by those authors links the gene expression changes to specific physiological adaptations previously recognized from laboratory and field studies of whole cell and community physiology (e.g. Lomas and Glibert, 1999). Similarly, a recent comparative genomic analysis identified several gene families controlling cell signaling, transcription, cytoskeletal structure, cell cycle and stress responses in *T. pseudonana* (Montsant et al., 2007). These initial efforts put the scientific community well on its way to developing a suite of target genes whose expressional influences on algal ecophysiology can be assessed in both laboratory and field populations.

Diatoms as a group have proven amenable (i.e. culturability, reliable nucleic acid extraction, large physiological database) to a

range of genomic methods. However, differential expression analyses, while promising, are so far inconclusive in searches for "toxin" genes in this group. As reported by Bates and Trainer (2006), for example, use of cDNA subtraction libraries for the diatoms *P. multiseries* and *P. australis* generated 5372 and 2552 unique sequences, respectively; none of these sequences, as annotated by database comparisons, could be directly related to toxin metabolism. A more tractable approach may be to compare oceanic and coastal members of the *Pseudo-nitzschia* genus. Marchetti et al. (2008) identified (using ITS sequences) multiple species during the SERIES iron fertilization experiment in the northeast Pacific. In contrast to more coastal species (e.g. *P. multiseries* and *P. australis*), none of the oceanic *Pseudo-nitzschia* produced measureable domoic acid, under either iron-deplete or iron-replete conditions. Coastal species have been reported to up-regulate domoic acid production in response to iron stress (Wells et al., 2005), suggesting that there may be fundamental differences between the coastal and oceanic members of the genus.

Several groups have proposed that the toxigenic diatom *Pseudo-nitzschia*, common to all eastern boundary current systems, is an ideal target for functional genomic approaches (GEOHAB, 2005). In addition to the apparent differences between oceanic and coastal *Pseudo-nitzschia* species, there is also considerable intra-species variability. For example, domoic acid has been associated with *P.* cf. *pseudodelicatissima* in the Juan de Fuca eddy region off Washington State (Trainer et al., 2007), but it is not known to produce toxin in Monterey Bay, California; *P. australis* is a common bloom-forming species in three major eastern boundary current upwelling systems (California Current, Canary Current, Benguela Current), and has been reported in Chile (Álvarez et al., 2009). Until recently, *Pseudo-nitzschia* was thought to be non-toxic in the Benguela system (Fawcett et al., 2007), and the source of domoic acid had not been unequivocally identified in Chile (Álvarez et al., 2009). In both the Benguela and northern Chile systems, a combination of microscopy and sequence analysis was used to positively identify the toxigenic species *P. australis* and *P. calliantha* (Álvarez et al., 2009; Kudela, Smith, and Miller, unpublished data), with *P. australis* identified in both cases as the dominant toxin producer. *P. australis* is persistently toxic in the California Current system, and more variable in the Canary Current system (Kudela et al., 2005).

Unlike most other toxin-producing algae, toxicity in domoic acid producers can be induced by a variety of stressors (cf. reviews by Bates, 1988, 1998; Bates, 2000; Bates and Trainer, 2006; Cembella and John, 2006). This opens up the possibility of using cDNA subtraction libraries to identify the presumably small set of genes up- or down-regulated during toxin production. Other methods, such as restriction fragment length polymorphisms (RFLP) or microsatellite analyses, could also be used to differentiate genetically distinct subpopulations (i.e. toxic vs. non-toxic) within a species. Preliminary results from various investigators have, perhaps not surprisingly, failed to identify specific gene markers for domoic acid production, despite the identification of almost 8000 ESTs using differential expression under toxin-promoting and suppressing conditions (Boissonneault, 2004; Smith et al., 2005; Bates and Trainer, 2006). Despite these setbacks, functional genomic approaches have led to promising early results, potentially leading to the eventual development of field-deployable assays for assessing health and toxicity of *Pseudo-nitzschia*.

We developed and analyzed over 2000 ESTs from three cDNA subtraction experiments using *P. australis* (Jenkins, B.D., Lin, E.O., Armbrust, E.V., and Kudela, R.M., unpublished). Some of the genes were targeted with transporter function and potential function in stress response to compare their expression in different nutrient stressors, stages of cell growth and in cells producing different amounts of domoic acid. A 20–90-fold induction of a gene related to glycerol-3-phosphate transporters in silica-limited cultures of *P. australis* was identified when cell growth slows (Fig. 3), providing a possible functional marker for growth status in *P. australis*. Despite the inconclusive identification of genes related to toxin production gleaned from annotation of the subtraction libraries, unique gene products were expressed in culture conditions where toxin levels were higher (late stationary growth).

As an alternative to subtractive methods, biochemical pathway analysis was also employed as a search heuristic for potential toxin synthesis pathways. We targeted a second gene product that has provided a species-specific genetic marker for toxigenic strains of *P. australis* (Smith et al., 2005). Previous physiological studies revealed that domoic acid accumulation was associated with unique free amino acid profiles in several *Pseudo-nitzschia* species, characteristically maintaining large ornithine (ORN), arginine (ARG) and

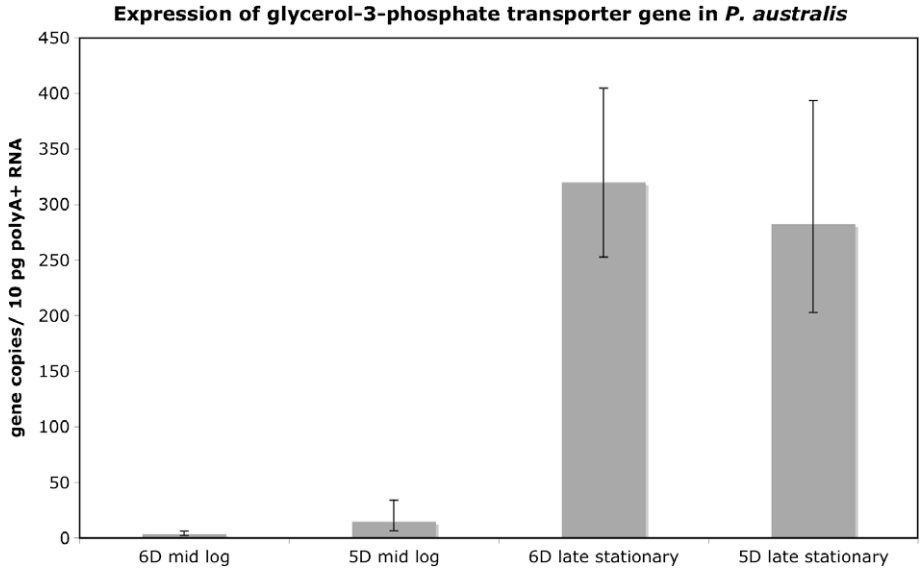

Fig. 3. Quantitative PCR analysis of expression of a gene related to glycerol-3-phosphate transporters in higher plants identified in *Pseudo-nitzschia australis* cDNA subtraction libraries enriched for RNA expressed under conditions of low growth rate, silica limitation and domoic acid production. This gene is induced 20–90-fold in silica-limited cultures with two different clones (6D and 5D) when cells reach late stationary phase. Poly A + RNA was isolated from total RNA. cDNA synthesis and quantitative PCR was conducted in triplicate.

low proline (PRO) pools relative to glutamate (GLU, Smith et al., 2001). Enzymatic assay screens also revealed higher activity along the ORN-dependent pathway for proline biosynthesis in toxic *P. australis* and *P. multiseries*, supporting the hypothesis that high ORN-cycle activity may be indicative of domoic acid production capacity. One of the up-regulated ESTs identified in the cDNA subtractions exhibited strong homology to a putative acetyl-ornithine aminotransferase (acOAT or argD) gene in *Thalassiosira pseudonana* and cyanobacteria. This gene product plays a central role in the ORN and ARG biosynthetic pathways and was targeted as a potential functional marker for toxin production.

PCR assays of cDNA (RT-PCR) and genomic DNA (gPCR) provide estimates of the relative abundance of the gene transcript pool (gene expression) and corresponding gene abundance (gene dose) within a given sample (Fig. 4). When normalized to either beta-actin (β-Act) or acOAT gene dose, the acOAT_L transcript (gene

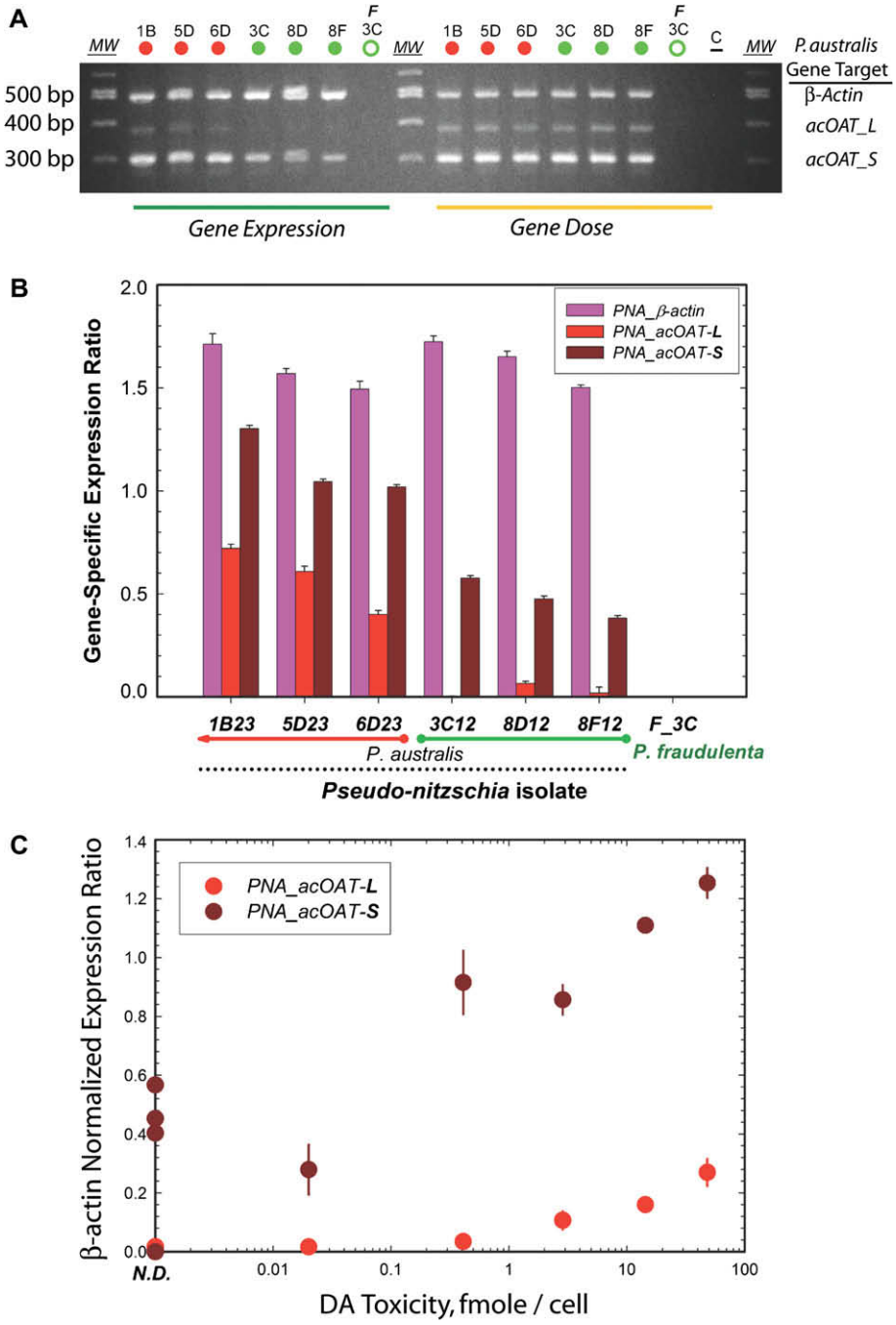

Fig. 4. An example of a PCR-based assay for the presence of domoic acid toxicity in isolates of *Pseudo-nitzschia australis*. Total nucleic acids are extracted from cell samples to screen DNA for presence of specific genes (Gene Dose) and RNA, after conversion to cDNA to screen for expression of specific gene products (Gene Expression). (a) Multiplex PCR assays for gene dose (right side) and expression (left side) of three gene targets in six *P. australis* (filled circles) and one *P. fraudulenta* isolate (open circle). The structural protein, *β-actin*, serves as an additional internal control. Target genes are present only in isolates of *P. australis*, demonstrating assay specificity, with the abundance of the enzyme acetyl ornithinine amino transferase, (*acOAT_L* and *acOAT_S)* transcripts highest in domoic acid accumulating isolates (red symbols). (b) Assessment of relative abundance of expression products relative to their specific gene dose by simple densitometry. Error bars represent standard deviation of multiple assay runs. (c) Application of this multiplex assay to a range of *Pseudo-nitzschia* cultures reveals a significant enhancement (⩾2-fold) of *acOAT* gene specific transcript abundance in cultures with significant domoic acid toxicity (⩾1 fmole cell^{-1}) (From Smith et al., 2005).

expression) was not detectable in log-phase cultures but highly expressed in stationary cultures. Expression patterns of the acOAT_L transcript are consistently higher (ca. 8×) in domoic acid accumulating *P. australis* isolates from Monterey Bay and Bodega Bay than in isolates that lack detectable domoic acid levels (Fig. 4B). In fact, acOAT_L and acOAT_S scale with cellular toxicity, providing a correlative assay for domoic acid accumulation in *P. australis* (Fig. 4C). This molecular genetic approach has proven to be species-specific and robust, providing an assay for growth status, active domoic acid toxicity, and species identification even in heterogeneous field samples (Smith et al., 2005). Intriguingly, other genes associated with proline metabolism have also been described by continuing annotation of the subtraction library clones (Parker et al., 2006).

Although still preliminary, these data demonstrate how functional genomics can be used in comparative ecosystem studies. Once specific gene products are identified and confirmed to track growth status or even toxin production, their gene expression can be followed in experiments with new isolates, specified growth conditions and time series sampling of natural environments in laboratory isolates or field populations using procedures comparable to those described for cell enumeration and detection. Given the widespread occurrence of *P. australis* and confirmed toxicity in all eastern boundary upwelling systems, this particular method provides an excellent candidate for a comparative analysis across systems.

2.4. Applications of high throughput sequencing methods

In the past few years, DNA sequencing technology has changed at breakneck speed (Bowler et al., 2009). New chemical and microfluidic methods have been developed that increase the throughput and dramatically decrease the cost of sequencing compared to the Sanger sequencing technology used since the 1970s. The throughput from these "next generation" methods is extremely high, however the length of individual reads is shorter than the Sanger method and is more difficult to assemble and interpret due to shorter sequencing reads. These methods have been most useful for sequencing genomes from individuals in species already represented by complete genomes (resequencing) and for extremely high throughput analysis of gene expression data (c.f. Hudson, 2008).

Gene expression analysis methods are being developed that utilize the newest generation of DNA sequencing methods to obtain quantitative data for large numbers of expressed genes and are sensitive enough to detect genes with low expression levels. Using a proprietary method called Massively Parallel Signature Sequencing (MPSS) Erdner and Anderson (2006), examined differential expression of transcribed genes (the transcriptome) in *Alexandrium fundyense* under N-limited conditions with lower saxitoxin production and P-limited conditions with higher saxitoxin production. The authors did not find "toxin genes" *per se*, but concluded that dinoflagellates exhibit complexity on par with multicellular eukaryotes, have multiple gene copies, and are likely to regulate cellular processes using both transcriptional and post-transcriptional processes.

Advances have also enabled metagenomic approaches in harmful algal research, because researchers are less frequently limited by the availability of direct sequence information. Whereas earlier studies directly assessed environmental samples to identify the presence or absence of particular organisms (e.g. RT-PCR quantification of *K. brevis*; Gray et al., 2003), higher throughput sequencing allows for richer community analysis. Hubbard et al. (2008) describe the development of PCR primers for about half the known *Pseudo-nitzschia* species (enabling the comparative analysis on the SERIES experiment employed by Marchetti et al., 2008), borrowing methods developed for bacterial genome analysis using automated ribosomal intergenic spacer analysis (ARISA). These new methods allowed identification of 13 organized taxonomic units (OTUs) off Vancouver Island, Canada, where microscopic analysis had identified only seven species in a previous study. Orsini et al. (2004) reported five distinct clades within 70 isolates of *P. delicatissima* from the Gulf of Naples (Mediterranean Sea) also using PCR methods on the ITS1 and ITS2 region, and reported greater intra-species variability during pre-bloom conditions compared to bloom periods; a first step towards comparative analysis within and between regions. Using a slightly different method, McDonald et al. (2007b) identified 10 of 14 known genotypes of *Pseudo-nitzschia* in the Gulf of Naples via LSU sequencing, in comparison to five species identified by microscopy, with three new genotypes also identified. Extending beyond the genus-level, McDonald et al. (2007a) used a metagenomic approach employing 16S rRNA gene probes and clone libraries to characterize the community assemblage (primarily at the class level) in the Gulf of Naples over a 15-month period.

Although some of these methods still rely on laboratory culturing to provide clone libraries, culture-independent methods such as 454 pyrosequencing (Sogin et al., 2006) and single-cell PCR methods (Godhe et al., 2002; Ki et al., 2004; Kai et al., 2006) will undoubtedly allow further characterization of inter- and intra-species variability, as well as community dynamics, more rapidly and at less expense than is currently possible. New sequencing methods have been used to analyze the sequence composition of expressed genes from naturally occurring phytoplankton communities (John et al., 2009), and these methods hold promise for correlating gene expression profiles from HAB communities to *in situ* dynamics of upwelling systems such as nutrient concentrations. Comparative studies in upwelling systems will benefit tremendously as these new methods replace PCR sequencing, allowing for the detection of the whole spectrum of genetic diversity at the intra-specific and intrageneric level directly from environmental samples, in combination with functional genomics of targeted processes (e.g. growth, toxin production), further enabled by advances in field instrumentation.

2.5. Field technology development

Particularly notable advances have been made in the application of molecular methods to field-based sampling (c.f. Sellner et al., 2003). Many of these new methods combine standard molecular techniques with new modes of deployment. Scorzetti et al. (2009) report the potential qualitative identification of up to 100 species simultaneously, or quantitative identification of fewer targets; for example, through the use of PCR combined with microsphere-probes (beads) assayed with flow cytometry. Going one step further, the Environmental Sample Processor (ESP) is essentially an *in situ* laboratory, capable of automatic analysis using both FISH and SH assays for any target organism for which probes are available, although in its current configuration FISH samples are archived but not processed onboard (Greenfield et al., 2006). While still of limited availability, the ESP could provide a robust and generic platform for multiple techniques beyond those already employed, including real-time simultaneous toxin detection (Fig. 5) and inclusion of a PCR module. Similar in concept is the Autonomous Microbial Genosensor (AMG; Paul et al., 2007). Unlike the ESP, the AMG is initially configured to target functional genes, rather than using FISH probes for cell enumeration (although both sensor systems could be modified to target anything with a genomic signature). The AMG provides an automated, *in situ* system capable of converting mRNA to cDNA, with subsequent detection using PCR of functional genetic markers; the first assay targets mRNA for rbcL in the dinoflagellate *K. brevis*. This is a particularly attractive target since mRNA is only expressed in active cells (i.e.

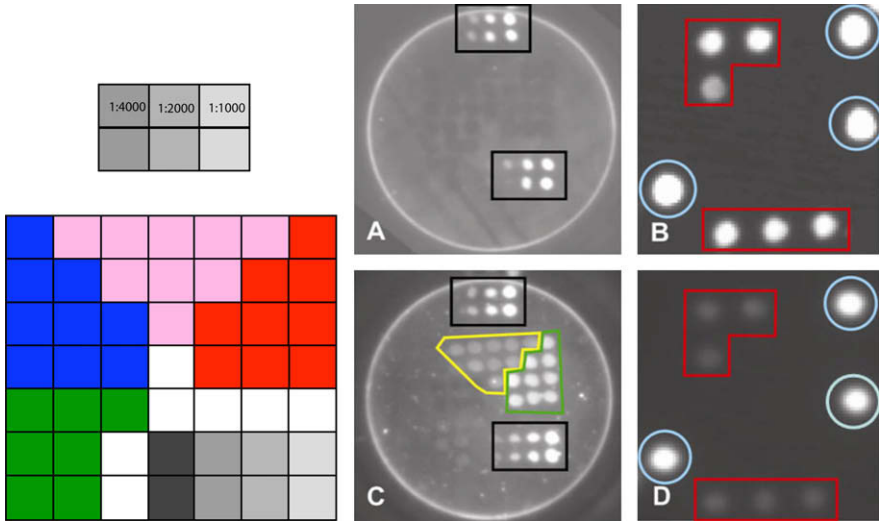

Fig. 5. Example of results from the Environmental Sample Processor (ESP) using a 25 mm diameter ESP array during an April 2006 deployment printed with HAB species probes: map includes *Pseudo-nitzzschia australis* (pink), *Pseudo-nitzschia multiseries/pseudodelicatissima* (red), *Alexandrium catenella/tamarense* (blue) and *Heterosigma akashiwo* (green). The top 6 and bottom 8 squares are a dilution series of control probes (independent of sample) that verify assay chemistry and orient the image. Array results are depicted according to date (rows) and array type (columns) showing negative results for April 10 (a) DNA probe array (no probe spots are illuminated) and (b) domoic acid array (assay is a competitive ELISA; negative spots are illuminated) compared to positive results on April 21 for the (c) DNA probe array (spots become visible) and (d) domoic acid array (spots dim). Blue ringed spots are IgG controls confirming consistency of detection chemistry. Black rectangles (a and c) are controls, green/yellow outlined spots are *P. multiseries/delicatissima* and *P. australis* respectively. Spots in red rectangles (b and d) are DA conjugate. Data and images are copyright MBARI, provided by C. Scholin and D. Greenfield.

those *K. brevis* cells fixing carbon). SH assays have also been coupled to fibre-optic microarrays using probes for multiple HAB organisms simultaneously (Ahn et al., 2006; Anderson et al., 2006). This technique (Fig. 6) provides rapid (ca. 45 min) and sensitive (as few as five cells) detection in both laboratory and field samples. A particularly attractive aspect of this approach is the ability to add more species-specific probes without having to deal with multiplexed signals, because each probe is associated with its own fibre-optic bundle (Fig. 6), similar to the microsphere-probe methodology employed by Scorzetti et al. (2009) but without the need for a flow cytometer. A third example involves the development of an electrochemically based, hand-held system that also utilizes SH probes (Metfies et al., 2006). This so-called DNA-biosensor differs from the other technologies primarily in that the detection signal is an electrical current proportional to the abundance of rRNA target molecules (detected with SH probes), providing a simple, robust readout system amenable to use by non-specialists.

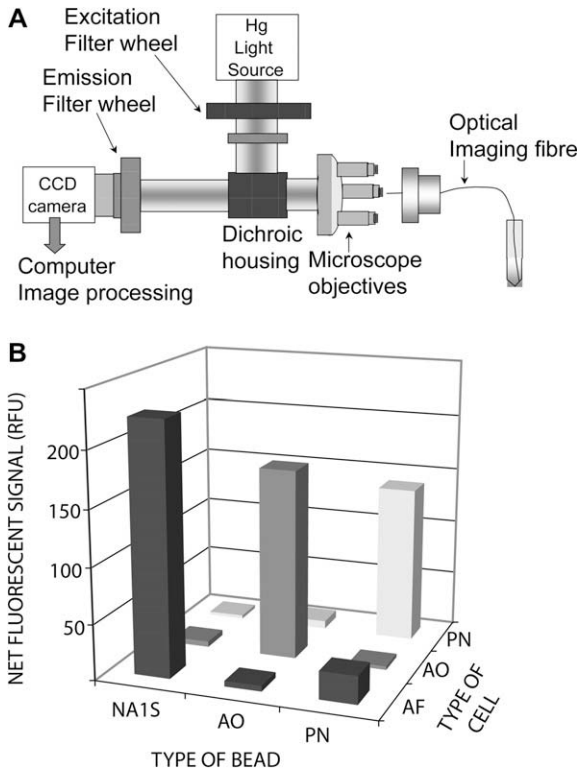

Fig. 6. Schematic of the fibre-optic instrumentation described by Ahn et al. (2006) and Anderson et al. (2006) in panel (a), and results showing net fluorescence signals using a multiplexed array using species-specific probes targeting cell lysates from *Alexandrium fundyense* (NA1S), *A. ostenfeldii* (AO) and *Pseudo-nitzschia australis* (PN) are presented in panel (b). Figures reprinted with permission from Anderson et al. (2006).

3. Summary and future directions

Here, we have highlighted some of the common tools in the molecular toolbox that may facilitate comparative approaches, and have provided some case studies. Despite the rapid advances and many examples highlighting application of molecular methods within specific systems, there are very few examples of the comparative approach applied specifically to HABs in upwelling systems. Three themes related to future directions emerge. First, molecular methods continue to develop rapidly, making it ever easier to apply these tools to laboratory- and field-based studies. Second, molecular tools are no more or less likely to "work as advertised" without extensive validation in each new system than any other method. Third, despite the tremendous advances in molecular techniques, we still have insufficient knowledge of the molecular underpinnings of the ecophysiological responses that are most directly of interest to oceanographers and resource managers, in part because of the limited (but growing) nature of the genetic databases available to researchers. Certainly, a range of approaches is available in the current molecular toolbox to discover and describe the drivers of physiological response and ecosystem function in natural phytoplankton communities.

We have a relatively poor framework for interpreting how the growing molecular database can best be utilized in comparative ecosystem studies, and efforts must be supported to integrate emerging "omic" descriptions of algal cell physiology within ecosystem functions. Given the physiological plasticity exhibited at the strain level for various HAB organisms (e.g. Kudela et al., 2004; Burkholder and Glibert, 2006) and the documented intraspecific genetic variability in natural assemblages, there is also a clear need for coordinated, national and international repositories for both HAB culture collections and genomic sequence information. These facilities are already in place (e.g. GenBank and the various culture archives), but the community should be encouraged to both support these facilities, and to deposit new strains and sequence information. Without these efforts, the ~50% "unknown" gene functions commonly observed, and the unintended cross-reactivity (or failure to probe putatively identical species) when using molecular probes, will be a significant barrier to the functional inclusion of the molecular toolbox as a core asset for comparative approach to investigating the dynamics of HABs in upwelling systems. The techniques reviewed here can provide better descriptions of the molecular underpinnings for the processes driving species and community bloom dynamics. As summarized by Bowler et al. (2009), "model microbial systems and laboratory-based experimentation combined with observation will give us a holistic perspective, moving beyond reductionist science to link descriptive and functional observations". The comparative method championed by GEOHAB can facilitate this for HABs in upwelling systems. Despite potential issues, the technology and methods necessary to facilitate this comparative analysis within and between upwelling systems exists today, and we encourage the community to move forward with the requisite targeted studies.

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