17 Molecular Physiology of Toxin Production and Growth Regulation in Harmful Algae

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17.1 Introduction

Molecular physiology is a hybrid discipline whereby cellular metabolism and regulatory and functional interactions of metabolites within organisms are explored via technological tools and concepts of molecular biology. From this perspective, molecular physiology, and the corresponding field of metabolomics derived from genomics, is in a relative state of infancy as applied to marine protists (or eukaryotic microalgae). Yet recent access to genomic databases for a variety of lower eukaryotes, and targeted genome studies involving cDNA libraries offer enormous potential to unlock mysteries relating algal genomes to cellular and ecological function.

Among the more than 100 protistan taxa considered to be "harmful" and/or that produce harmful algal blooms (HABs), the phytoflagellates and especially free-living dinoflagellates are most prominent. A bloom can result from rapid proliferation of cells, i.e., high intrinsic growth rate, or the maintenance of a high cell concentration via behavioral adaptations (swimming, sinking), successful resource competition, life-history transitions or allelochemical interactions. These processes are not mutually exclusive, but all have a cell regulatory dimension reflecting gene function. Toxic microalgae, particularly bloom-forming taxa, are fruitful subjects for molecular physiological investigations. For example, compared to many co-existing diatoms or other flagellates, large toxic dinoflagellates tend to be rather slow-growing (typically $\mu<0.5\ d^{-1}$). Therefore, to understand how such species are capable of bloom formation and in some instances to dominate the phytoplankton, it is necessary to obtain molecular physiological insights into regulation of both growth and toxin synthesis.

Marine protists produce a bewildering array of secondary metabolites, and many of these compounds possess high biological activity. Among these bioactives, the phycotoxins feature prominently, but it would be erroneous to

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conclude that toxicity necessarily reflects their ecological and evolutionary significance. Phycotoxins are most often synthesized by marine phytoflagellates (and rarely by pennate diatoms); more than 80 % of the eukaryotic algal taxa that produce defined phycotoxins belong to the dinoflagellates (Cembella 2003). These bioactive secondary metabolites may play diverse roles (currently unknown) in intracellular regulation of cell growth and metabolism as well as in extracellular regulation of population growth via allelochemical interactions. The molecular physiological approach facilitates the generation of testable hypotheses regarding ecological niche differentiation defined by intrinsic as opposed to extrinsic factors (e.g., nutrient acquisition, turbulence, etc.).

Certain cyanobacteria (colloquially "blue-green algae") share with the dinoflagellates the capability of synthesizing saxitoxin and other toxic tetrahydropurines. The cyanobacteria also produce hepatotoxic cyclic peptides (microcystins, nodularins) and the potent neurotoxic anatoxins, which are included as "phycotoxins," but these are dealt with here only by analogy. Molecular genetics of cyclic peptide production and regulation in cyanobacteria are well advanced (comprehensively reviewed by Börner and Dittmann 2005). For example, a biosynthetic operon (*myc* gene) for microcystins has been sequenced from a number of cyanobacterial genera. Homologies and differences in molecular organization for such toxin genes among the cyanobacteria have been interpreted as an indication of a common ancestor. Subject to many caveats, such advanced work on cyanobacteria provides guidance for corresponding work on toxin genetics in eukaryotic algae with larger and less tractable genomes.

Complex transcriptional changes and genetic regulation of growth and bioactive secondary metabolite production occur via biotic interactions, life history transitions, and the mitotic cycle. Such gene expression and the consequent ecological responses can be described and quantified using tools of modern molecular biology. Because we have limited knowledge about the molecular physiology and gene regulation of harmful algae, we have biased this review to consider only the few highlights of recent findings, supplemented with emerging methodologies that are currently being applied or will be used in the near future.

17.2 Phycotoxin Biosynthesis

From a molecular physiological and chemical ecological perspective, toxin classification based upon structural homology presumably reflects shared elements of biosynthetic pathways (Wright and Cembella 1998). Biosynthetic evidence from stable isotope–labeling studies indicates that most if not all polyether phycotoxins are produced via polyketide pathways (Shimizu 1996,

cited in Cembella 1998), in which acetate units are added sequentially from acetyl-CoA within a pathway regulated by polyketide synthases (PKS). The classical approach is to provide an isotopic enrichment of low-molecular-weight putative precursors (e.g., ^{13}C-acetate, ^{15}N-glycine) and then to follow the incorporation pattern into the target phycotoxin by NMR spectroscopy. This approach has provided structural elucidation and plausible biosynthetic schemes for key phycotoxins, including saxitoxin and analogues from amino acid precursors in *Alexandrium tamarense* and the cyanobacterium *Aphanizomenon flos-aquae* (Shimizu 2000, cited in Cembella 2003), brevetoxins from *Karenia brevis* (Shimizu 1996, cited in Cembella 1998), sulphated dinophysistoxins from *Prorocentrum maculosum* (Macpherson et al. 2003), and domoic acid from *Pseudo-nitzschia pungens* (Douglas et al. 1992, cited in Bates 1998).

In polyketide synthesis of the sulphate esters DTX5a and DTX5b, the amino acid in the sulfated side-chain originates from glycine, whereas oxygen insertion occurs after polyketide formation (Macpherson et al. 2003). Biosynthetic studies on *Alexandrium ostenfeldii* supplemented with [1,2-$^{13}C_2$]-acetate and [1,2-$^{13}C_2$, ^{15}N]-glycine demonstrated by NMR spectroscopy that 13-desmethyl spirolide C is also polyketide-derived (Cembella et al. 2004). A biosynthetic scheme for pinnatoxins, presumed to be of dinoflagellate origin as are other spiroimine toxins (Wright and Cembella 1998), proposes derivation from a single polyketide chain and bears resemblance to the spirolide pattern.

Molecular ecological studies on ichthyotoxins and/or haemolysins produced by eukaryotic microalgae, particularly by prymnesiophytes and raphidophytes, but also including some dinoflagellates, are hampered by poorly described links between toxicity and chemical structures. For example, Yasumoto et al. (1990, cited in Eschbach 2005) attributed haemolysis and ichthyotoxicity of the prymnesiophyte *Chrysochromulina polylepis* to galactolipids, 1-acyl-3-digalacto-glycerol and polyunsaturated fatty acids (PUFAs). Later evidence indicting no differences in lipid and PUFA profiles between toxic versus non-toxic strains has challenged this assumption (John et al. 2002, cited in Cembella 2003). Where toxin biosynthetic pathways and structures are unknown, bioassays may be used to target genetics of toxicity expression through the cell cycle and under varying environmental conditions.

17.3 Growth and Regulation of Toxin Production

Cellular growth rate of marine microalgae is a function of extrinsic abiotic factors – salinity, temperature, turbulence, light, nutrient concentrations, ratios and supply rates, and accumulation of allelochemical and auto-

inhibitory metabolites. Growth rate is typically a hyperbolic function of the limiting resource, with the rate increasing with increasing available resource up to saturation. A diminution of growth rate accompanies an increase in resource limitation or stress. Exogenous environmental variables can be altered under controlled conditions to yield differential growth rates and hence insights into growth-rate dependency of gene expression. The maximum growth rate for each strain or species is also regulated by intrinsic factors, e.g., synthesis and activation of bioenergetic metabolites (ATP, NADPH), nucleic acids, and cell cycle regulators.

Many biologically active secondary metabolites, such as antibiotics produced by bacteria and fungi, are subject to induction effects, or genetic "up-regulation," when cells are exposed to unfavorable environmental conditions. These metabolites are often presumed to function in cell defense; thus it makes evolutionary sense and is metabolically efficient to reserve genetic expression for when they are critically required.

With respect to effects of abiotic factors, the relationship between growth and toxin production has been extensively reviewed for the dinoflagellates that produce saxitoxin and analogues (Cembella 1998), polyether toxins (Wright and Cembella 1998), and diatoms that synthesize domoic acid (Bates 1998). Perhaps paradoxically, there are few cases of stress-dependent triggers in toxigenic eukaryotic microalgae, even for dinoflagellate polyethers derived from polyketide pathways that are analogous if not homologous to those in bacteria and fungi.

The most notable exceptional example of apparent stress metabolism is the induction of domoic acid synthesis in *Pseudo-nitzschia* spp. under phosphate- and silicate-limitation (Bates 1998). The gradual diminution and eventual loss of toxin production in "older" mitotically reproducing strains without sexual recombination in this toxic diatom (reviewed in Pan et al. 1998; Bates 1998) is not usually apparent in toxigenic strains of dinoflagellates, which are nominally haploid in the vegetative stage. Sexual recombination is not essential for maintenance of toxicity in flagellates (at least over several years), although loss of toxicity perhaps through random mutation and clonal selection in culture may rarely occur.

It is not reasonable to conclude that nutrient limitation "induces" toxin production, based only upon evidence of high cell toxin quota in growth-limited nutrient–deficient cells. An increase in toxin cell quota may result merely from a decrease in the *rate* of cell division relative to the *rate* of toxin synthesis (Cembella 1998). This would account for high cell toxicity in *Chrysochromulina polylepis* (Edvardsen et al. 1996, cited in Eschbach 2005) in stationary growth phase under P-limited conditions. Under non-limiting nutrient conditions, for dinoflagellates (Cembella 1998) and other phytoflagellates, e.g., *C. polylepis* (Eschbach et al. 2005), toxin content is typically highest in mid-exponential phase, when the growth rate is maximal.

17.4 Toxin Production Through the Cell Cycle

The eukaryotic cell cycle, divided into M (mitosis), G1 (Gap 1), S (DNA synthesis), and G2 (Gap 2) phases, is universal for microalgae, even for the dinoflagellates (Taroncher-Oldenburg et al. 1997; Pan and Cembella 1998, both cited in Eschbach et al. 2005), which have a unique mitosis. Reduction in growth rate is correlated to changes in the duration of the cell cycle, and is attributable to expansion of a single cell-cycle phase (G1).

Circadian rhythms among the phototrophic microalgae, e.g., in cell division, nutrient assimilation, bioluminescence, toxin production, onset of sexual reproduction, and vertical migration, are linked to both nutrients and light regime. The cell-division cycles are therefore phased or synchronized to photocycles. Mitotic division in the dark is typical, but there are exceptions, e.g., shade-adapted dinoflagellate species such as *Prorocentrum lima* (Pan and Cembella 1998, cited in Eschbach et al. 2005) and *Amphidinium operculatum* (Leighfield and Van Dolah 1999) may divide in the light. In the latter species, it is the time from dark-to-light transition that dictates occurrence of M-phase, not whether it is in the dark or light period.

Induction and regulation of toxin production is best studied by analysis of synchronized cells because biosynthesis then occurs essentially at the same time-point. Entrainment of microalgal cells towards synchronous division is often accomplished by the block/release method via deprivation of light and/or regulation of nutrient concentration and supply rate (Taroncher-Oldenburg et al. 1997; Pan et al. 1999, both cited in Eschbach et al. 2005), or use of metabolic inhibitors (Van Dolah et al. 1998, cited in Eschbach et al. 2005). Cells arrested in G1 phase (then termed G0 phase), when released from the block, proceed through the cell cycle as a homogeneous population. Alternatively, cells can be synchronized by exposure to a tightly defined light/dark cycle (Eschbach et al. 2005). In synchronized eukaryotic microalgae, toxin production is generally discontinuous, restricted to a defined period of the cell cycle, and is light-dependent, although not always confined to the light phase (Taroncher-Oldenburg et al. 1997; Pan et al. 1999, both cited in Eschbach et al. 2005).

Discriminating between toxin expression events that are truly cell-cycle dependent (e.g., linked to G1) and those which are merely light-dependent (entrained by photoperiod) can be very challenging, particularly when complete synchrony in cell division is not maintained. In *Alexandrium fundyense*, saxitoxin analogues are produced only during a restricted period in G1, with slight time shifts in synthesis of different derivatives (Taroncher-Oldenburg et al. 1997, cited in Eschbach et al. 2005). In *Prorocentrum lima*, DTX4 production is initiated early in the light period in G1 whereas other derivatives are synthesized during S and G2 phases (Pan et al. 1999, cited in Eschbach et al. 2005). Production of spirolides in *A. ostenfeldii*, occurs primarily during dark in G2+M phases (John et al. 2001, cited in Cembella 2003). These differences

suggest that not even polyketide toxins share a universal function in regulation and expression in the cell division cycle.

17.5 Molecular Approaches to Growth and Toxin Expression

Current data are insufficient to resolve the genetics of cell-cycle regulation in eukaryotic microalgae, but homologues of cell-cycle regulators have been found. Gene sequence data are available from the chlorophyte *Dunaliella tertiolecta* (Lin and Carpenter 1999) and cyclin B homologues have been discovered from genome projects on the apicomplexan *Cryptosporidium parvum* and the prasinophyte *Ostreococcus tauri*. Universal cell-cycle regulators, cyclin-dependent kinases (CDKs) and cyclin B-like proteins, have been reported from the dinoflagellate cell cycle (Van Dolah et al. 1998 cited in Eschbach et al. 2005, Barbier et al. 2003). A mitotic cyclin from the (often toxigenic) *Lingulodinium polyedrum* was isolated by functional complementation in yeast, then cloned and sequenced (Bertomeu and Morse 2004); expression levels through the cell cycle indicated a typical eukaryotic pattern.

Other limited genomic approaches to gene expression in toxigenic flagellates, e.g., expressed-sequence-tag (EST) analyses of *A. tamarense*, *A. ostenfeldii*, *Karenia brevis*, *P. lima*, and *Chrysochromulina polylepis* have not revealed homologues of CDKs or cyclins. However, 18 ESTs for *C. polylepis* (John et al. 2004a), 6 ESTs for *A. ostenfeldii* (Cembella et al. 2004), and 16 ESTs from *K. brevis* (Lidie et al. 2005) are attributable to orthologous genes for cell division and DNA replication.

The molecular expression of the proliferating-cell-nuclear-antigen (PCNA) gene has been described from several microalgal species (Lin and Corstjens 2002, and references therein). This auxiliary protein for DNA polymerase is essential for nuclear DNA synthesis and thus can serve as a cell-cycle marker for S-phase.

Molecular techniques for toxic algae allow for the detection of genes that are "turned on" during toxin production, and not merely on the detection of the toxins themselves (Plumley 1997). Modified versions of subtractive-hybridization libraries, DNA microarrays, EST libraries, and real-time polymerase chain reaction (QPCR) permit identification of differentially expressed genes in organisms grown under different conditions. These methods are currently being applied to toxic eukaryotic microalgae, for example, to investigate expression differences between toxic versus non-toxic strains (John et al. 2004a).

There are only a few defined examples of genetic regulation of toxin production in marine protists. Differential display (DD) of mRNA was used to identify differential gene expression of synchronized batch cultures of *Alexandrium fundyense* during toxin production at time-points in G1 phase

versus time-points in G1, S and G2 + M phases where toxin production did not occur (Taroncher-Oldenburg and Anderson 2000). Three differentially expressed genes were identified – for S-adenosyl-homocysteine hydrolase, methionine aminopeptidase, and a histone-like protein – but none were clearly related to PSP toxin synthesis or regulation.

The limited genomic approach to gene expression, involving creation of a cDNA library for coding genes, followed by EST sequencing has proven to be extremely fruitful as a complement or alterative to whole genome sequencing. This is particularly true for dinoflagellates where the genome size can exceed 200 gbp; in contrast, the entire human genome is about 3.2 gbp! The genome size and technical constraints on sequencing dinoflagellates, perhaps the richest source of "toxin genes" among lower eukaryotes, restricts availability of whole genomic information.

Since many (perhaps all) polyether phycotoxins are derived via polyketide metabolism regulated by PKS, this enzyme complex merits particular attention in gene expression studies. Given the complex structures of the polyethers produced by eukaryotic microalgae (e.g., maitotoxin congeners originating from the dinoflagellate *Gambierdiscus toxicus* are >3,000 Da and contain 32 transfused ether rings), Type I modular PKS synthases are the most likely candidates for polyether synthesis (see models for structure and function of modular PKS in Börner and Dittmann 2005). In *Cryptosporidium parvum*, a close protistan relative of the dinoflagellates, a large modular PKS gene has been identified, with up to 40 kbp open reading frames (ORFs), and containing no introns in genomic DNA (Zhu et al. 2002). Putative PKS genes have been reported from the dinoflagellates *Gymnodinium catenatum*, *Amphidinium operculum*, *Prorocentrum lima*, *Karenia brevis* (Snyder et al. 2005, and references therein) and *Alexandrium ostenfeldii* (Cembella et al. 2004), and from the prymnesiophyte *C. polylepis* (John et al. 2004a). At least for *K. brevis*, the PKS genes have been confirmed to be of dinoflagellate as opposed to bacterial origin (Snyder et al. 2005). Nevertheless, no full-length PKS genes have been fully characterized and validated with published sequence data from toxic flagellates, and thus no definitive attribution can be made regarding their role in biosynthesis of polyketide toxins. We cannot rule out that an entirely new class of PKS may be involved in biosynthesis of the highly unusual polycyclic polyketides in dinoflagellates.

In *A. ostenfeldii*, a normalized cDNA library was used to generate ESTs (approximately 5,300), representing 2,400 unique sequences (updated from Cembella et al. 2004). Only 9 % of the total sequences were homologues to known genes. Nevertheless, annotation revealed at least three putative PKS genes with sequences consistent with Type 1 modular PKS. Putative modular PKS genes also correlate in their expression profile with toxicity in synchronized *C. polylepis* (John et al. 2004a).

The low degree of sequence homology in *A. ostenfeldii* and perhaps other dinoflagellates presents a challenge for determining the expression and regu-

latory function of biosynthetic genes for dinoflagellate secondary metabolites. Developments in understanding the structure and function of operons for PKS and NRPS (non-ribosomal protein synthase) in toxic cyanobacteria (Börner and Dittmann 2005, and references therein) indicate that it may be difficult to identify "toxin genes" for polyketides in eukaryotic flagellates, even with high overall sequence homology.

Proteome analysis can elucidate which proteins are expressed in different growth and cell division phases under various growth conditions, during toxin synthesis and allelochemical interactions. Further information is provided on how these proteins and processes are post-translationally modified and how variation is expressed due to epigenetic phenomena.

Application of proteomics to harmful algae is thus far very limited and has been primarily targeted towards "molecular fingerprinting" of bloom populations. Recent characterization of microalgal populations, including *Prorocentrum* and other dinoflagellates (Chan et al. 2004) involved 2D-GE profiles for species identification and as potential protein biomarkers for bloom description and prediction. Existing protein databases have enough sequence data that a few distinctive proteins for HAB species can be identified from 2D-GE and/or peptide profiles, but the genomic databases necessary for protein profile screening of key toxic species are lacking.

Cryptic genetic diversity may be an important feature in the selection and viability of particular strains responsible for the formation of natural bloom populations. Molecular methods, such as rDNA sequencing (Scholin et al. 1994), amplified-fragment-length polymorphism (AFLP) (John et al. 2004b) and micro-satellite analysis (Evans et al. 2005), are being used for genotyping toxic microalgae, to differentiate genetically distinct sub-populations within species. Determination of genetic heterogeneity is an essential preliminary step in establishing the expected degree of variability in gene expression among populations and species.

In the *Alexandrium tamarense* species complex, the best-studied group of toxic dinoflagellates, the global expression of toxin phenotype is diverse, characterized by the production of more than two dozen saxitoxin analogues. The toxin profile of *Alexandrium* strains is relatively stable under favorable culture conditions but can differ markedly among strains. Unknown allelopathic compounds with biological activity against a wide range of planktonic organisms are also produced by members of the *A. tamarense* complex and by *A. ostenfeldii*, but these appear to be unrelated to the potent saxitoxin analogues or spirolides, respectively, produced by the dinoflagellates (Tillmann and John 2002, cited in Cembella 2003).

Toxin expression studies on *A. tamarense* populations from eastern Canada (Cembella and Destombe 1996, cited in Cembella 1998) and the Scottish east coast (Alpermann et al. 2004) indicated that multiple isolates (clones) from the same geographical area can be highly heterogeneous in their toxin profiles. Analysis of other phenotypic properties, including the expression of

fatty acid profiles, has also revealed a high level of cryptic genetic diversity within populations. Studies of genetic structure based upon genetic markers such as AFLP analysis for the *Alexandrium tamarense* species complex (John et al. 2004b) and microsatellite DNA from *Pseudo-nitzschia pungens* from the North Sea (Evans et al. 2005) indicated high levels of genetic diversity, but low genetic and morphological differentiation. Preliminary genetic analysis of North Sea populations of *A. tamarense* using both microsatellites and AFLP as genetic markers, and toxin profiles as phenotypic markers, demonstrated similarly high genetic variation among isolates from discrete populations, but no correlation of genotypic markers with toxin expression patterns (Alpermann et al. 2004). A correlation between genetic markers and toxin expression or growth rate is not necessarily expected, but such studies indicate that dependence upon single or few clones in autecological investigations may lead to erroneous conclusions regarding regulation of growth and toxin biosynthesis.

17.6 Current and Future Perspectives

The way forward in molecular ecological studies on growth regulation and toxin production of harmful algae is to unite classical approaches to structural elucidation and metabolic pathway determination with genomic and proteomic technologies for gene expression. Analytical and physical-chemical methods such as tandem mass-spectrometry with liquid-chromatographic separation (LC-MS/MS) and NMR can resolve structures and provide basic biosynthetic pathways to phycotoxins, but provide little direct evidence on specific biosynthetic enzymes and regulatory functions. A combined approach linked to genomics and proteomics is required to fully describe toxin biosynthesis and regulation.

Many analytical technologies (e.g., LC-MS) have already been applied on board ship for field measurements and toxin profiling in near real-time in natural populations (Scholin et al. 2005). Furthermore, species-level taxonomic probes are available for field use even on deployable buoy systems in automated mode. As hypotheses or laboratory techniques are developed, these must be rapidly applied to field populations to evaluate the predictability, variability and role of environmental cues on phenotypic expression. Traditional autecological approaches to studies of gene expression, growth and toxin production have often relied on individual geographically "representative" clones of uncertain provenance that are maintained in long-term culture on artificially enriched seawater. As a first step, a large number of isolates from various geographical populations must be cultured under rigidly defined conditions and subjected to genotyping (DNA probing) and phenotyping (toxin characterization).

Total genome sequencing is desirable for gene-expression studies. The rapid advent of novel genomic technologies and associated databases for a host of prokaryotes and eukaryotes offers straightforward alternatives for sensitive and stringent gene screening. Whole-genome sequencing of a few eukaryotic microalgae (*Thalassiosira pseudonana*, *Phaeodactylum tricornutum, Ostreococcus tauri*) has been completed, is in progress (*Emiliana huxleyi*) or will soon be underway (e.g., toxigenic *Pseudo-nitzschia multiseries*). Nevertheless, no complete sequences are yet available for toxic eukaryotic algal species.

The DNA microarray approach is also a promising strategy to screen for genes that are induced or repressed as a result of intra- and inter-specific interactions and response to other environmental triggers in marine protists (Taroncher-Oldenburg et al. 2003; Lidie et al. 2005). DNA microarrays have been used to identify differentially expressed genes during toxin synthesis in the fungus *Aspergillus* (O'Brian et al. 2003). The basis for microarray development is availability of a substantial sequence data-base for a given species. From sequence data, gene-specific oligonucleotides can be designed or cDNA fragments can be generated using PCR. DNA microarrays loaded with many putative genes for cell cycle regulation and toxin synthesis can then screen for expression of toxin-specific genes in toxic versus non-toxic strains within a species or across a wide spectrum of taxa from field populations and laboratory cultures.

For full-length sequences of large gene candidates, such as modular PKS genes, as well as information on the 5' and 3' untranslated regions (UTRs), a cosmid library, or a bacterial artificial chromosome (BAC) library, must be constructed. Cosmids are useful for cloning large segments of foreign DNA (up to 50 kbp). For large genomes and for large target genes, BAC libraries of vectors containing inserts of high molecular-weight DNA from 75–150 kbp can be produced. Given the huge genome size of dinoflagellates, 7.5×10^6 clones may be required to achieve reasonable coverage of the genome with a mean insert size of ~150 kbp.

Identification of genes or primary gene products (enzymes) involved in cell growth and/or toxin synthesis is facilitated by comparison with strains of the same taxon, which lack the capability to express the particular trait. For some toxigenic species, e.g., *A. tamarense*, non-toxic analogues of the morphospecies are available. Mutations induced in a toxic strain by exposure to UV radiation or chemical mutagens also provide an alternative for comparison. Chemical mutagens can induce point mutations resulting in base mispairing and substitution or even deletions. Small deletions are desirable because they are more likely to abolish gene function but also to affect only a single gene, thus avoiding generation of complex phenotypes.

Proteomic approaches involving large-scale systematic analysis of proteins of harmful algae and their post-translational modifications can be accomplished by combining two-dimensional gel electrophoresis (2D-GE) and pro-

tein microanalysis with bioinformatics. Two-dimensional difference gel electrophoresis (2D-DIGE) allows co-separation of different CyDye DIGE fluorlabelled protein samples from toxic and non-toxic strains on the same gel. Protein spots of interest can be selected and excised from the gels for further characterization by matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) with post-source decay, or by Edman sequencing. After accurate determination of peptide masses, databases and EST libraries of investigated taxa must be searched for identification of original proteins.

Once cell-based molecular diagnostics (microarray analyses, Northern blots, QPCR) have been established, the next stage is to explore functional genetics of allelochemistry and toxin expression on target organisms, such as predators and putative competitors of toxic microalgae. The hypothesis that these bioactive substances may serve as quorum-sensing compounds should be evaluated with respect to the formation, maintenance and senescence of HABs of eukaryotes and cyanobacteria.

Cyanobacteria offer an advanced model for application of molecular techniques for toxin gene detection (cited in Börner and Dittmann 2005). Unfortunately, for eukaryotic microalgae, no definitive candidates for toxin genes are characterized, and transfection methods and expression systems are described for only a few non-toxic eukaryotes (such as *Ostreococcus* and *Phaeodactylum*). For cyanobacteria, a QPCR approach that is specific for the *myc* gene has been used to evaluate the relative contribution of toxic and non-toxic *Microcystis* in natural blooms, as well as to identify the microcystin producers among several potentially toxic species. Biosynthetic promoters for microcystins in cyanobacteria have also been fused to *lux* genes to evaluate environmental effects on toxin production. The cyanobacterial literature suggests that molecular tools should be taxon- rather than toxin-specific, but this model cannot be simply extrapolated to the eukaryotes. Genome organization in cyanobacteria and fungi is quite different from that of eukaryotic microalgae (especially dinoflagellates). Toxic cyclic peptides in cyanobacteria are not derived from pathways similar to those of the dinoflagellate polyketides. The vast diversity of eukaryotic toxins would defy diagnosis by a single genetic marker. Nevertheless, phylogenetic analysis suggests that focus on polyketide toxins is not unwarranted.

Further insight into the role of allelochemicals in the "watery arms race" sensu Smetacek is required to understand the capability of these species to form persistent, recurrent, dense, and on occasion nearly monospecific blooms. Knowledge of the cues and mechanisms that induce, regulate, and/or stimulate production and exudation of specific toxins, and yet-undefined extracellular lytic compounds, will significantly contribute to understanding the ecological significance of these allelochemicals in dynamics of key species and consequent effects on marine trophic webs.

References

Alpermann T, John U, Tillmann U, Evans K, Cembella A (2004) Genotypic and phenotypic variability in allelochemical potential within populations of *Alexandrium tamarense*. XI Inter Conf on Harmful Algal Blooms, Cape Town, South Africa (Abstract), p 57

Barbier M, Leighfield TA, Soyer-Gobillard M-O, Van Dolah FM (2003) Expression of a cyclin B homologue in the cell cycle of the dinoflagellate, *Karenia brevis*. J Eukaryot Microbiol 50:123–131

Bates SS (1998) Ecophysiology and metabolism of ASP toxin production. In: Anderson DM, Cembella AD, Hallegraeff GM (eds) Physiological ecology of harmful algal blooms. NATO ASI Series 41. Springer, Berlin Heidelberg New York, pp 405–426

Bertomeu T, Morse D (2004) Isolation of a dinoflagellate mitotic cyclin by functional complementation in yeast. Biochem Biophys Res Comm 323:1172–1183

Börner T, Dittmann E (2005) Molecular biology of cyanobacterial toxins. In: Huisman J, Matthijs HCP, Visser PM (eds) Harmful cyanobacteria. Springer, Berlin Heidelberg New York, pp 25–40

Cembella AD (1998) Ecophysiology and metabolism of paralytic shellfish toxins in marine microalgae. In: Anderson DM, Cembella AD, Hallegraeff GM (eds) Physiological ecology of harmful algal blooms. NATO ASI Series 41. Springer, Berlin Heidelberg New York, pp 381–403

Cembella AD (2003) Chemical ecology of eukaryotic microalgae in marine ecosystems. Phycologia 42:420–447

Cembella AD, John U, Singh R, Walter J, MacKinnon S (2004) Molecular genetic analysis of spirolide biosynthesis in the toxigenic dinoflagellate *Alexandrium ostenfeldii*. XI Inter Conf on Harmful Algal Blooms, Cape Town, South Africa (Abstract), p 82

Chan LL, Hodgkiss IJ, Lu S, Lo SC-L (2004) Use of two-dimensional gel electrophoresis proteome reference maps of dinoflagellates for species recognition of causative agents of harmful algal blooms. Proteomics 4:180–192

Eschbach E, John U, Reckermann M, Cembella AD, Edvardsen B, Medlin LK (2005). Cell cycle dependent expression of toxicity by the ichthyotoxic prymnesiophyte *Chrysochromulina polylepis*. Aquat Microb Ecol 39:85–95

Evans KM, Kühn SF, Hayes PK (2005) High levels of genetic diversity and low levels of genetic differentiation in North Sea *Pseudo-nitzschia pungens* (Bacillariophyceae) populations. J Phycol 41:506–514

John U, Groben R, Beszteri B, Medlin L (2004b) Utility of amplified fragment length polymorphisms (AFLP) to analyse genetic structures within the *Alexandrium tamarense* species complex. Protist 155:169–179

John U, Tillmann U, Cembella A, Medlin L (2004a) Expression of polyketide synthases (PKS) during induction of toxicity in the ichthyotoxic prymnesiophyte *Chrysochromulina polylepis*. XI Inter Conf on Harmful Algal Blooms, Cape Town, South Africa (Abstract), p 147

Leighfield TA, Van Dolah FM (1999) Cell cycle regulation in a dinoflagellate, *Amphidinium operculatum*: identification of the diel entraining cue and a possible role of cyclic AMP. J Exp Mar Biol Ecol 262:177–197

Lidie KB, Ryan JC, Barbier M, Van Dolah, FM (2005) Gene expression in Florida red tide dinoflagellate *Karenia brevis*: analysis of an expressed sequence tag library and development of DNA microarray. Mar Biotechnol Oct 2005, p 46

Lin S, Carpenter EJ (1999) A PSTTLRE-form of *cdc*2-like gene in the marine microalga *Dunaliella tertiolecta*. Gene 239:39–48

Lin S, Corstjens PLAM (2002) Molecular cloning and expression of the proliferating cell nuclear antigen from the coccolithophorid *Pleurochrysis carterae* (Haptophyceae). J Phycol 38:164–173

Macpherson GR, Burton IW, LeBlanc P, Walter JA, Wright JLC (2003) Studies of the biosynthesis of DTX-5a and DTX-5b by the dinoflagellate *Prorocentrum maculosum*: regiospecificity of the putative Baeyer-Villigerase and insertion of a single amino acid in a polyketide chain. J Org Chem 68:1659–1664

O'Brian GR, Fakhoury AM, Payne GA (2003) Identification of genes differentially expressed during aflatoxin biosynthesis in *Aspergillus flavus* and *Aspergillus parasiticus*. Fungal Genet Biol 39:118–127

Pan Y, Bates S, Cembella AD (1998) Environmental stress and domoic acid production by *Pseudo-nitzschia*: a physiological perspective. Nat Tox 6:127–135

Plumley FJ (1997) Marine algal toxins: biochemistry, genetics and molecular biology. Limnol Oceanogr 42:1252–1264

Scholin CA, Doucette GJ, Cembella AD (2006) Prospects for developing automated systems for in situ detection of harmful algae and their toxins. In: Babin M, Roesler C, Cullen J (eds) HABwatch: monographs on oceanographic methodology. UNESCO, Paris, (in press)

Scholin CA, Herzog M, Sogin M, Anderson DM (1994) Identification of group-and strain-specific genetic markers for globally distributed *Alexandrium* (Dinophyceae). 2. Sequence analysis of a fragment of the LSU rRNA gene. J Phycol 30:999–1011

Snyder VR, Guerrero MA, Sinigalliano CD, Winshell J, Perez R, Lopez JV, Rein KS (2005) Localization of polyketide synthase encoding genes to the toxic dinoflagellate *Karenia brevis*. Phytochemistry 66:1767–1780

Taroncher-Oldenburg G, Anderson DM (2000) Identification and characterization of three differentially expressed genes, encoding S-adenosylhomocysteine hydrolase, methionine aminopeptidase, and a histone-like protein, in the toxic dinoflagellate *Alexandrium fundyense*. Appl Environ Microbiol 66:2105–2112

Taroncher-Oldenburg G, Griner EM, Francis CA, Ward BB (2003) Oligonucleotide microarray for the study of functional gene diversity in the nitrogen cycle of the environment. Appl Environ Microbiol 69:1159–1171

Wright JLC, Cembella AD (1998) Ecophysiology and biosynthesis of polyether marine biotoxins. In: Anderson DM, Cembella AD, Hallegraeff GM (eds) Physiological ecology of harmful algal blooms. NATO ASI Series 41. Springer, Berlin Heidelberg New York, pp 427–451

Zhu G, LaGier MJ, Stejskal F, Millership JJ, Cai X, Keithly JS (2002) *Cryptosporidium parvum*: the first protist known to encode a putative polyketide synthase. Gene 298:79–89