# Biology of toxic algae: A study of species of the genus *Chrysochromulina* (Prymnesiophyceae) and *Alexandrium* (Dinophyceae)

Dissertation

zur

Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

- Dr. rer. nat. -

Am Fachbereich 2 (Biologie/Chemie)

der Universität Bremen

Vorgelegt von

Uwe John

Bremen, April 2002

# *1. General Introduction*

The world's oceans cover around 70% of the earth's surface. In marine ecosystems, algae assume the role of “plants” and most belong to the phytoplankton, both in terms of biomass and diversity. Among the microalgae, phytoplankton account for approximately 50% of global primary production (Falkowski et al. 1998). Given their effect on the global carbon cycle, microalgae have therefore been studied intensively during the last few decades (Raven and Falkowski 1999). In addition, microalgae are the subject of other intensive investigations because of their contribution to biodiversity, value as a gene pool in times of global biodiversity loss (Pimm et al. 1995), role in major evolutionary scenarios, and as a potential source of natural products (Shimizu 1996). Phytoplankton play an important role in the food web by serving as food for suspension-feeding bivalve molluscs (oyster, mussels, scallops, clams), for many species of micro- and macro-zooplankton, including the larvae of crustaceans and ichthyoplankton, and as food vectors for finfish and whales via copepods and krill (Hallegraeff 1993; Parsons et al.1984).

Microalgae can live in nearly every habitat on earth, but most commonly, they are found in aquatic systems, from small puddles to oceans. In the marine environment, micoalgae form part of the plankton suspended in the water column but also contribute to the microphytobenthos in sediments and attached to surfaces.

Microalgal nutrition is highly variable, and does not always depend upon obligatory photosynthesis, the feature commonly used to distinguish “plants” from “animals”. In fact, “microalgae” may be more properly described as protists – unicellular eukaryotic organisms- rather than “algae”, since many species are motile flagellates and lack photosynthetic apparatus. Whereas the majority of microalgae are unicellular, some do tend to form colonies (e.g., *Phaeocystis globosa*) or chains (e.g., *Skeletonema costatum*), or live endosymbiontically (such *Symbiodinium* as Zooxanthellae in corals).

Some microalgal species can live either as photoautotrophic or mixotrophic organisms, which means that they are capable of photosynthesis, as well as heterotrophic uptake of dissolved organic compounds and/or of phagotrophy, whereby they can ingest bacteria, other micoalgae or even microzooplankton. Within certain algal groups, such as the dinoflagellates, heterotrophic taxa may constitute up to 50% of the species. Such heterotrophic dinoflagellates may exhibit a parasitic life-style or live as predators upon bacteria, microalgae, microzooplankton or even fish.

### *1.1 Classification and Phylogeny*

The "algae" can be classified into groups based upon their cellular organization and on the different structural features of their chloroplasts, e.g., their origin and the number of surrounding membranes. According to one classification scheme (Lee 1999), the first group contains the prokaryotic Cyanobacteria, which phylogenetically belong to the Eubacteria. The second group consists of the Rhodophyta (red algae) and the Chlorophyta (green algae), where the chloroplasts are surrounded by only two membranes of the chloroplast envelope. The third evolutionary group contains the Euglenophyta (euglenoids) and the Dinophyta (dinoflagellates), for which the chloroplast is surrounded by one additional membrane of chloroplastic endoplasmatic reticulum. Finally, the fourth group of evolutionary lineage contains the Cryptophyta (cryptophytes), Chlorarachniophyta, Heterokontophyta, including the diatoms and the Phaeophyceae (brown algae), and the Haptophyta: all have chloroplasts surrounded by two additional membranes of chloroplastic endoplasmic reticulum. The last two groups result from secondary endosymbiosis, as can be deducted from the number of membranes surrounding their plastids (Margulis and Bermudes 1985; Medlin et al. 1997).

Until recently, chemical (e.g., pigments) and morphological characteristics may have been obvious and consistent enough to classify the algae into divisions, classes, orders, families and species on the basis of similarities between organisms. Unfortunately, morphological characteristics can be affected by environmental conditions, thus may vary among populations. Hence, differences in morphology, such as scale structures, do not always characterize a distinct taxon; in fact, they may merely indicate two stages in the life cycle of a single species (Larsen 1999). An impressive example of the flexibility in morphology and life form is the ichthyotoxic coastal dinoflagellate *Pfiesteria piscicida*, characterized by a triphasic life cycle of at least 19 different flagellate, amoeboid, and cyst stages (Burkholder et al. 1995; Lassus et al. 1995). In the case of the chlorophyte *Chlorella* spp., classical taxonomists classified the genus as a monophyletic group based on morphological characteristics within the Chlorococcales, but phylogenetic studies of ribosomal RNA sequences have shown it to be paraphyletic (Huss and Sogin 1990).

In summary, the comparison of morphometric characteristics can be misleading and may result in group-formation based only on convergent evolution. The introduction of molecular biological techniques, e.g., polymerase chain reaction (PCR) with subsequent sequencing, has led to the several phylogenetic revisions in the algae. In some cases even unexpected (hidden) biodiversity especially among prokaryotes and microalgae have been discovered (Lopez-Garcia et al. 2001). The discovery of a third kingdom of organisms, the

Archaebacteria, between the Eubacteria and the Eukaryota, is an example of a dramatic change in phylogeny and our perception of the living biodiversity as interpreted from molecular data (Woese et al. 1990). Ribosomal DNA (rDNA) sequences are the most commonly used sequence data used to reconstruct phylogenies. The rDNAs code for the rRNA occurring in ribosome; they are structured as a small subunit (SSU), two internal transcript spacers (ITS1 and ITS2) flanking both sides of the 5.8S rDNA, and the large subunit (LSU). Each of these molecular features has been used for phylogenetic investigations, but the SSU rDNA sequences are more conserved than the LSU genes or spacer regions, with the 5.8S being so conservative that it is almost unusable for phylogenetic reconstructions. An argument for using rDNA is its universality, i.e., all prokaryotic, eukaryotic nuclear, plastid and mitochondrial genomes encode the rDNA. The SSU rRNA is conserved in function and evolves at a moderate rate of change in most organisms. In addition, these subunits are unlikely to be exchanged by lateral gene transfer because of their essential function and profound effect on many intermolecular interactions (Woese 1987). Therefore, the SSU data have been used to reconstruct the phylogeny of phyla, classes, and orders and even down to interspecific relationships. Nevertheless, to clarify the phylogeny of a genus, of closely related species, or of one species and its populations, the more variable ITS, or the hyper variable D1/D2 region of 28S rDNA, have been used most successfully (Adachi et al. 1996; Scholin et al. 1994; Medlin et al. 1998).

Molecular biological methods frequently bring new insights to all levels of algal phylogeny. For example, rDNA sequence data for the Haptophyta showed that different morphotypes may only reflect different life cycle stages or generation types and not independent species, as in the cases of *Prymnesium parvum* and *P. patteliferum* (Larsen 1999) or *Chrysochromulina polylepis* α and β type (Edvardsen and Paasche 1992; Edvardsen and Vaulot 1996), and the heterococooliths and the holococcoliths (Sáez and Medlin, unpubl.).

### *1.2. Molecular markers (RAPD, RFLP, AFLP, microsatellites)*

The use of sequence data in phytoplankton analyses has certain biases and resolution limits. For population level studies, rDNA, RuBisCO (ribulose 1,5-bisphosphate carboxylase/-oxygenase), and other genes do not contain sufficient base substitutions to investigate and characterize different populations. Within the evolution of molecular techniques, several “fingerprint” methods have been developed for population and genomic DNA analyses. A rather traditional method is the use of restriction fragment length

polymorphism (RFLP). In this method, DNA is digested using specific restriction enzymes and resulting DNA fragments are size-separated by agarose gel electrophoresis. However, the handling becomes more complicated when large genomes are to be investigated. The relative few polymorphisms detected by the RFLP method entail a considerable amount of time and require large quantities of DNA (Karp et al. 1996).

The random amplified polymorphic DNA (RAPD) method may be used even when genetic information and DNA are insufficient. The RAPD technique uses a single arbitrary primer in a PCR ("polymerase chain reaction"), resulting in the amplification of several discrete DNA products. However, the technique is very sensitive to slight differences in reaction conditions and lacks reliable reproducibility (Pérez et al. 1998).

A more recently developed method is amplified fragment length polymorphism (AFLP) (Vos et al. 1995), which combines the advantages of both techniques described above. The technique involves restriction digestion of very small amounts of genomic DNA, adapters ligated to the ends of the digested fragments, and PCR amplification with specific primers to amplify a subset of those fragments, which can be visualized by polyacrylamide gel electrophoresis. The latter method was primarily used for gene mapping and population studies in land plants (Barrett and Kidwell 1998), but was also introduced to zoology (Seki et al. 1999) and microbiology (Janssen et al. 1996). Recently AFLPs were used to study the population structure of the red alga *Chondrus chrispus* (Donaldson et al. 1998; 2000).

Microsatellites (simple sequence repeat, SSR) are alternative molecular markers, which are generally highly informative and co-dominant, unlike RAPDs and AFLPs. This PCR-based assay uses species-specific designed primers to amplify the nucleotide repeat unit and is therefore highly reproducible. Unfortunately, development of a sufficient number of microsatellite primers is very time consuming, requires knowledge of the genome (not necessary for AFLP), and the primers work only for one species. Species with the ability to survive in different habitats and under a wide range of environmental conditions require either extensive phenotypic acclimatisation or adaptative capabilities, presumably combined with high genetic variation within the species. At this level, sequence analysis often cannot yield resolution of population structure, but molecular markers, such as AFLPs, can be used to investigate the genetic diversity of isolates among a single species, e.g., during the formation of an algal bloom.

### *1.3. Historical Biogeography*

Besides the study of phylogenetic relationships among certain taxa, phylogenetic systematics can be combined with biogeographic data to understand evolutionary processes underpinning biodiversity. Fro example, molecular data have been used to elucidate population structure of a dinoflagellate (*Alexandrium*) in relation to the hypothesized geographic origin (Scholin et al. 1995). In such cases, molecular evidence can be used to reconstruct the historical biogeographic distribution of species and to provide explanations for their recent distribution, with respect to evolutionary processes (e.g., vicariance and dispersal) and geological processes (e.g., forming and closing of ocean basins, and the movement of the continents followed by changes in water circulation and climate) (Medlin et al. 1996; Kooistra et al. 1993). Both processes can contribute to species distribution. Vicariance is the term for the mechanism leading to separation of homogeneously distributed species after the formation of natural barriers, whereas dispersal is caused by movement of species from their origin to another region. However, if the reconstruction of the historical biographic distribution is to be accomplished with sequence data, then the data must be tested to ensure that all sequences used in the reconstruction are evolving at a approximately the same rate (Takezaki et al. 1995; Medlin et al. 1996). The assumption is that they conform to a stochastic, regular, clock-like but random process for the occurrence of base substitutions, a so-called neutral evolution. However, there are many reports in the literature that a universal molecular clock may not exist, e.g., because of genetic drift and selection, and that base substitution rates vary within lineages and genes (Pawlowski et al. 1997). Once a uniform rate of evolution as been established, then the divergence time of a species or population can be estimated. Dates for geological processes or the first appearance dates of fossils can be used to calibrate a so-called molecular clock, which can be used to extrapolate the time of divergence of the branches within a phylogenetic tree.

### *1.4. General Microalgal Blooms*

Among the phytoplankton, bloom-forming microalgae play a key role in marine ecosystems. Algal blooms can be difficult to define (Smayda 1997b). A significant increase in population numbers seems to be the most important criterion for an algal bloom. However, other criteria like water discoloration, toxic or other harmful effects, or generally high

phytoplankton biomass above certain level, have been used to characterize algal blooms as well. Most of our understanding of algal blooms stems from diatom-dominated upwelling systems and the typical cold temperate spring blooms (Smayda 1997b). Suitable meteorological conditions can be regarded as one of the most beneficial conditions for algal blooms. Wind driven upwelling events characterized by deep mixing are known to support algal bloom formation by introducing nutrient into the euphotic zone. On the other hand, stable, calm weather can be favorable to blooms for several reasons. They are associated with high radiation enhancing primary production and growth even in deeper water layers. An apparently important aspect, is the fact that periods of low winds lead to less turbulence in the water column and stratification of the water column permitting the accumulation of biomass (Donaghay and Osborn 1997; Smayda 1997a). Most algal blooms have been observed near the coast and/or in upwelling ecosystems (Smayda 2000), indicating the important role of nutrients in bloom-formation. Besides the natural, mostly seasonal, increases of nutrients through the winter or autumn storms, anthropogenic eutrophication also likely force bloom formation in certain cases.

In addition to diatom dominated blooms at upwelling ecosystems and at spring time, which are mainly forced through physical and chemical conditions, several other bloom phenomenon have been reported. To understand bloom phenomena, other than those in spring time and in upwelling systems, biological regulation factors influencing the growth dynamics within a bloom also must be examined. There are three types of growth that can be distinguished. First, there is cellular growth, for which intrinsic genetic factors set the maximal potential growth rate of the individual taxon or strain of a given species. Growth is restricted by tolerances to environmental variables, e.g., light, temperature, or nutrients. Second, population growth is basically dependant upon cellular growth but is also affected by loss processes, such as physical dispersal, sedimentation, grazing, and viral infection. Additionally, competition among algal taxa for light, nutrients, etc., perhaps by using allelochemical substances to outcompete other algae – could influence the population growth. The third type of growth is community growth, which is the result of combined cellular and population growth from several different species (Smayda 1997a). The third level of growth is affected by eutrophication, because eutrophication in coastal regions often changes the ratios of nitrate (N) to phosphate (P) and silicate (Si) in such a way that N and/or P may increase but not Si. Consequently, these changes may favor the growth of flagellates against Si-dependant diatoms. Changes in the P:N ratio may also affect phytoplankton composition,

because low N:P ratios tend to favor nitrogen-fixing cyanophytes (Sommer 1994), whereas P deficiency might lead to toxic flagellate blooms (Gjøsæter et al. 2000).

In contrast to the plants in terrestrial ecosystems, natural mortality of phytoplankton has only a minor impact in the oceans because grazing by protozoa or crustaceaens has a much more important effect on population size (Peters and Thomas 1996). Grazing is one of the key parameters regulating bloom formation and termination (Smayda 1997a, 1997b). The breakdown of an algal bloom is usually rather rapid - dense algal blooms can completely disappear within a few days (e.g. Lindahl 1983). Beside physical factors, such as mixing and dispersal during changes in wind velocity and direction or other meteorological events, or physiological and biological factors also influence the progress of an algal bloom. In dense blooms, cells may be exposed to high physiological stresses through nutrient deficiency, self-shading or possibly also through autoinhibitory substances or their own toxins (Maestrini and Graneli 1991; Brussaard et al. 1995). Recently, biological interactions in sudden population changes have been discussed. For example, the potential of lytic viruses and bacteria to control algal populations has been shown for certain marine phytoplankton, which may destroy blooms within days (Zingone 1995; Kim et al. 1998).

### *1.5. Harmful algal blooms*

When algal blooms have a negative impact on the environment, human and animal health, or economic viability of resource harvesting, these phenomena are termed “harmful” (often synonomous with or overlapping the terms “toxic”, “noxious”, or “nuisance”) (Smayda 1997b). Blooms that are formed by such species are called harmful algal blooms (HABs). In most cases, HABs are considered from a human point of view. Humans are directly influenced by seafood-vectored poisoning or indirectly through socio-economic losses. The latter is the result of losses in aquaculture or wild harvest fisheries (i.e., closing of shellfish beds or loss of finfish due to mortality), or drawdowns in the tourist industry (i.e., bloom outbreaks washed onto beaches or other recreation areas). The magnitude of annual economic losses can be severe because 66 million tonnes of marine species (75% finfish and 25% shellfish) are harvested annually as food. This involves a total international trade in seafood products of up to $US 100 billion per year. For example, a loss of about $US 60 million was caused by a single bloom in a shellfish culture area (Shirota 1989), and $US 500 million was lost due to a bloom of *Chattonella antiqua* affecting caged yellowtail fish (Okaichi 1989). A third impact is the ecological effects of the bloom to the ecosystem.

Hallegraeff (1993) and Smayda (1997a) have discussed different types of harmful algal blooms:

1. Blooms from species that produce harmless water discolorations, which can cause losses in flora and fauna under exceptional conditions, such as anoxia due to intense *Ceratium* blooms, or ammonia toxicity resulting from *Noctiluca* blooms.
2. Blooms of species that produce phycotoxins, where the effect can occur upon exposure to secreted toxins through direct ingestion of a harmful species or from toxin vectoring through the food web up to top predators (Geraci et al. 1989).
3. Blooms of species that are non-toxic to humans but harmful to invertebrates and fish by mechanically damaging or clogging their gills. Blooms of *Chaetoceros convolutus*, for instance, lead to a mechanical damage and hereby to the death of crustaceaens or finfish (Hallegraeff 1993 and references therein).
4. Blooms of species affecting fish and invertebrates via their gills or external regulatory epithelium, such as prymnesiophytes *Chrysochromulina* and *Prymnesium*, producing chemical substances that affect the gill permeability and causing death due to a disturbed ion balance without accumulating in the food web.

The history of harmful algal blooms, according to the first written reference, began about 1000 years BC and was documented in the Bible: "..all the waters that were in the river were turned to blood. And the fish that was in the river died; and the river stank, and the Egyptians could not drink of the water of the river.." (Exodus 7:20-21). It is believed that in this case a non-toxic algae became so dominant that it generated anoxic conditions and subsequently killed flora and fauna of the river. Among the early references to human poisoning, the notes of Captain George Vancouver in 1793 are among the first. Besides mentioning the intoxication of humans after eating shellfish, he also noted that for local Indians it was taboo to eat shellfish during a certain time a year.

In recent times, HABs are considered to be a world-wide phenomenon, with an apparent global increase in their frequency, intensity and geographic distribution (Graneli et al. 1990; Hallegraeff 1993). Up to 2000 human cases of seafood poisoning associated with algal toxins (with 15% mortality) are reported each year. Accompanying an increase in aquaculture, the annual economic losses seem to increase as well (Hallegraeff 1993). Several reasons for the increases of the HAB phenomena have been discussed. On one hand, the increased scientific awareness of toxic species combined with mandatory monitoring for

HABs in several areas has likely led to more observations. On the other hand, however, climate changes reflected, for example, in more frequent El Niño events could favor HABs (Maclean 1989). But there is also evidence that through direct human activity the frequency and global distribution of HABs have increased. HAB species have been introduced via ballast water or shellfish stocks to regions where those toxic algae were previously unknown, e.g., in Australia where the toxic species *Gymnodinium catenatum*, *Alexandrium catenella* and *A. minutum* were apparently introduced within the last 10-20 years (Hallegraeff 1998). There are also impressive examples for the relationship between HABs and eutrophication of coastal waters, e.g., the increase of blooms in Hong-Kong Harbour as a function of human population and domestic and industrial pollution (Lam and Ho 1989).

### *1.6. Toxic species*

The diversity of microalgae is still not fully elucidated, but roughly 5000 phytoplankton species have been described thus far, whereas about 300 species have been reported to form blooms that discolour the water (Hallegraeff 1993), including diatoms, raphidophytes, silicoflagellates, prymnesiophytes, and dinoflagellates (Sournia et al. 1991; 1995). Only 60 to 80 species, however, tend to form HABs, of which dinoflagellates are by far the predominant representatives (Smayda 1997a). In addition to the common harmful species, new species and those formerly regarded as non-toxic are now found to be responsible for exceptional outbreaks of HABs and/or toxicity (e.g., *Chrysochromulina polylepis*; Gjøsæter et al. 2000).

Among the diatoms, several species regularly form blooms, but only a few members of this group tend to form HABs. Species of the pennate diatom genus *Pseudonitzschia* are responsible for amnesic shellfish poisoning of humans and die-offs of piscivorous birds, and seals (Bates et al. 1989; Work et al. 1993; Scholin et al. 2000).

An example of a toxic raphidophyte is *Chattonella antiqua*, which causes HABs world-wide (Anderson et al. 1998). Mass mortality of cultured yellowtails (Okaichi 1989) was reported in 1989 and were associated with blooms of raphidophytes.

Among the Haptophyta there are several important bloom-forming species, such as *Emiliania huxleyi*. Some species of the genera *Chrysochromulina*, *Prymnesium*, and *Phaeocystis* form HABs, often resulting in large fish kills and consequently great economic losses (Anderson et al. 1998; Anderson et al. 1998; Rosenberg et al. 1988).

As mentioned above, the majority of the globally distributed harmful algal species belong to the dinoflagellates and most of them are photosynthetic (*Pfiesteria piscicida* and *Noctiluca scintillans*, which often contains a photosynthetic endosymbiont, are exceptions). One of the first reports of HABs of dinoflagellates inside European waters was linking the presence of *A. ostenfeldii* (probably described erroneously as *Pyrodinium phoneus*; Woloszynska and Conrad 1939; Anderson et al. 1998) with symptoms of human intoxication resembling those of paralytic shellfish poisoning (PSP). The second outbreak of PSP in Eurpope occurred in 1968 along the English coast, with >80 humans poisoned (Adams et al. 1968). Annual reports of PSP poisoning and mussel bed closures are reported from the Orkney Islands. In the summer 1971, a HAB involving *Procentrum micans* occurred in the Netherlands and delayed the harvest of mussels for several weeks (Kat 1979).

### *1.7. Toxins and other bioactive substances causing HABs*

In recent years, interest in microalgal metabolites has steadily increased, as microalgae are recognized as a potential source of new types of drugs (Shimizu 1996). Among the secondary metabolites produced by microalgae, the chemically very heterogeneous phycotoxins, with their potential to poison humans, have been most intensively studied. Marine organisms, such as bivalve molluscs, gastropods, crustaceans and fish, can accumulate the toxins in the food chain and pose a threat to seafood consumers. The toxic syndromes that may occur after consumption of contaminated seafood designate their classification.

#### *1.7.1. Paralytic Shellfish Poisoning (PSP)*

Illness and death from PSP have been recorded at least since the 18$^{th}$ century (Dale and Yentsch 1978). Cases of PSP are increasing world wide and at least since 1968 have frequently been recorded from European waters with anecdotal references going back to the last century. The toxins that cause PSP syndrome include saxitoxin and approximately 20 derivatives. These neurotoxins are commonly found and widespread in coastal waters; PSP toxins are primarily produced by dinoflagellate species, but also by a few Cyanophyceae, certain red algae, and as recently discovered perhaps also by a few Eubacteria (reviewed in Cembella et al. 1998). Saxitoxin and its derivatives are water soluble and heat stable tetrahydropurines. Based on their molecular structure, PSP toxins can be classified into various groups: (1) the highly potent carbamate toxins, (2) the medium potency decarbamoyl

toxins and (3) the weakly toxic N-sulfocarbamoyl toxins. The differing toxic potential of the three PSP toxin groups depends on stereometrical effects and on different net-charges located one the molecules. The toxins block neuronal and muscular sodium-channels and thus block nerve signal transmission. Syndromes typical for PSP intoxification are muscle paralysis leading to asphyxiation (Watson 1998).

### *1.7.2. Diarrhetic Shellfish Poisoning (DSP)*

Outbreaks of gastrointestinal illness after consumption of shellfish exposed to dinoflagellates were first reported in 1961 from the Netherlands (Taylor and Seliger 1979). This episode was followed by documentation from Japan in 1976 of a similar illness associated with shellfish consumption, and this caused major problems for the scallop industry (Yasumoto et al. 1980). Species of the genera *Dinophysis* and *Prorocentrum* produce toxins associated with diarrhetic shellfish poisoning (DSP) (Falconer 1993). DSP is caused primarily by lipophilic, polyether compounds, specifically by okadaic acid and derivatives, such as dinophysistoxins-1 and dinophysistoxins-2. The toxicity is based on the effective inhibition of serine/threonine protein phosphatases, which regulate several metabolic processes in eukaryotic cells. Phosphorylated proteins accumulate and cause calcium influx and cyclic AMP or prostaglandin production followed by subsequent secretion of fluid in gut cells. The final result of DSP intoxication is diarrhoea and vomiting (Falconer 1993; Botana and MacGlashan 1993; Botana et al. 1996).

### *1.7.3. Neurotoxic Shellfish Poisoning (NSP)*

NSP occurrs mainly in North America, but in 1993, a large outbreak was recorded in New Zealand with almost 200 affected humans (Mackenzie et al. 1995). These toxins are produced by *Gymnodinium* spp. and can cause fish kills, but can also affect bathers. The toxins may cause dermatitis, conjunctivitis and respiratory problems (Scoging 1998). The causative toxin is brevetoxin and its numerous derivatives, which induce influx of sodium into voltage dependent sodium-channels and hence the release of synaptic neurotransmitters (Scoging 1998).

### *1.7.4. Ciguatera Fish Poisoning (CFP)*

CFP has been recorded for centuries as the most common fish poisoning syndrome with about 20,000 cases a year (Lipp and Rose 1997). There are a few hundred tropical reef-feeding fish species from areas within 35 degrees latitude north and south of the equator, which can potentially accumulate the causative ciguatoxins and cause CFP. Epiphytic and benthic dinoflagellates from the genera *Gambierdiscus*, *Ostrepsis*, *Prorocentrum*, *Amphidinium*, and *Coolia* are associated with CFP, but not all of these may produce congeners (gambieric acid, etc.) that are the precursors of ciguatoxins in fish. The ciguatoxins manipulate neurotransmission by opening sodium channels on neuromuscular junctions via competitive occupation of calcium receptor sites. Symptoms consist of variable combinations of gastrointestinal, neurological and cardiovascular ailments, presumably because several different algal toxins are involved (Scoging 1998).

### *1.7.5. Amnesic Shellfish Poisoning (ASP)*

The syndrome was first observed in 1987 from the east coast of Canada, where it caused three deaths and more than a hundred incidents of human poisoning after consumption of blue mussels (Bates et al. 1989). The toxin responsible for ASP is domoic acid, and as source of the toxin, species of pennate diatom genus *Pseudonitzschia* were identified (Bates et al. 1989). This was the first reported case of a diatom producing a neurotoxin. It is now reported from the Orkey Islands. Even though domoic acid is an amino acid, enzymes involved in its synthesis have not yet been indentified (Anderson et al. 1998).

These described phycotoxins are only examples for the most common shellfish poisoning syndromes. However, new toxins are still discovered frequently, such as the polyether spirolides, which were recently described by (Hu et al. 1995) and which are produced by the dinoflagellate *Alexandrium ostenfeldii* (Cembella et al. 2000). Besides the phycotoxins, there are compounds acting as allelochemicals and influencing chemically regulated interspecific competitions, or allelopathic substances, which influences grazer-prey relationships. These substances are distinctively different from phycotoxins because allelo-metabolites cannot usually be vectored through the food web. They are typically directly targeted and therefore they often have a high ecological impact for the aquatic community (Smayda 1997a).

### *1.8. Organisms investigated in this study*

*1.8.1* Alexandrium *spp***.**

Among the several dozen reported toxic species of phytoplankton, those belonging to the marine dinoflagellate genus *Alexandrium* spp. (Halim) Balech (1995) are perhaps the most thoroughly investigated for their toxic properties. The genus belongs to the family *Goniodomaceae* of the order *Gonyaulacales* (Fensome et al. 1993). In his monograph on the morphology of *Alexandrium* spp., Balech (1995) recognizes 29 species, nine of which have been frequently implicated as the cause of PSP in human consumers of contaminated seafood. The best-known species of the genus, *Alexandrium tamarense* (Lebour) Balech was first described as *Gonyaulax tamarensis* (Lebour 1925) from the Tamar estuary near Plymouth, UK. The occurrence of this species has been widely reported, from temperate to sub-Arctic regions and even tropical latitudes (Taylor 1984). *Alexandrium catenella* and *A. fundyense* are morphologically distinguished by minor differences from *A. tamarense*. Taxonomists identify these species based on their cell shape, the geometry of their apical pore complex (APC), the presence (in *A. tamarense*) or absence (in *A. catenella* and *A. fundyense*) of a ventral pore on the apical plate (1‘), and additionally by their tendency for chain-formation. Since the studies of Cembella et al. (1987; 1988) using morphological features combined with enzyme electrophoresis and toxin profiling, these variants have been suggested to be merely alternative morphotypes rather than distinct species, and have been termed the *A. tamarense* species-complex (Anderson et al. 1998; Scholin 1998). The *A. tamarense* species-complex was the focus of several studies. Phylogenetic studies based on the 18S rDNA (Scholin et al. 1994), the D1/D2 region of the 28S rDNA (Scholin et al. 1994; Medlin et al. 1998; Higman et al. 2001), and ITS sequences (Adachi et al. 1996) have shown that the phylogenetic relationship among strains of the species complex reflects geographic areas and not the different morphotypes of *A. tamarense*, *A. fundyense*, and *A. catenella*. Scholin et al. (1995) have described five different “ribotypes”, such as the North American, the western European, temperate Asian, Tasmanian, and the tropical Asian clade. The North American and the temperate Asian clade consist of toxic strains, whereas the three other ribotypes comprise non-toxic variants.

*Alexandrium ostenfeldii* was first described (as *Goniodoma*) by Paulsen (1904, 1949) from Iceland and other locations in Scandinavia, but inadequacies in the plate tabulation warranted a redescription (Balech and Tangen 1985) from Norwegian specimens. Although *A. ostenfeldii* was suspected as a possible source of PSP toxicity in Norwegian shellfish (Balech

and Tangen 1985), this has not been definitively established because of a temporal overlap with the presence of *A. tamarense* blooms in locations such as Oslofjord. Certain cultured strains of *A. ostenfeldii* from Limfjord in Denmark produce low levels of PSP toxins (Hansen et al. 1992), whereas a few cultured isolates from New Zealand were found to be very toxic (Mackenzie et al. 1996). A novel group of marine toxins, macrocyclic imines known as spirolides, were isolated and characterized from shellfish (Hu et al. 1995, 1996) and later from plankton (Cembella et al. 1998, 1999) collected from coastal waters of Nova Scotia, Canada. The causative organism of spirolide toxicity in shellfish was recently identified as *A. ostenfeldii* (Paulsen) Balech & Tangen (Cembella et al. 2000), and certain isolates of this species can produce a wide diversity of spirolides in unialgal batch culture (Hu et al. 2001).

### *1.8.2.* Chrysochromulina polylepis

The genus *Chrysochromulina* (Prymnesiophyceae) belongs to the division Haptophyta (Green and Leadbeater 1994). The defining character for haptophytes is the haptonema (Parke et al. 1955), a flagellar-like organelle which is unique. The haptonema differs structurally from flagella, and functions as an attachment or food gathering organelle (Green and Leadbeater 1994). The genus *Chrysochromulina* was first described by Lackey (1939) and consists of around 75 species. *Chrysochromulina polylepis* was described from the North Sea in the mid- 1950s (Manton and Parke 1962). Although this species was previously believed to be non-toxic to fish (Manton and Parke 1962), an extraordinary bloom of *C. polylepis* in the Kattegat/Skagerrak area and off the Norwegian Coast in 1988 caused extensive fish kills. Since then, a number of blooms of different *Chrysochromulina* species have been reported, some of which have caused fish mortality (reviewed by Edvardsen and Paasche et al., 1998). The 1988 bloom extended over an area of approximately 75,000 $km^2$ (Granéli et al. 1993), and was characterized by pronounced toxic effects to various organisms. These included farmed and wild fish populations, mussels, echinoderms, polychaetes, ascidians, cnidarians, sponges, red and brown algae (Rosenberg et al. 1988), as well as bacteria, protozoans, copepods (Nielsen et al. 1990) and other microalgae (Dahl et al. 1989; Graneli et al. 1990). The factors and mechanism that led to the bloom have been intensively reviewed and discussed (Maestrini and Graneli 1991; Gjøsæter et al. 2000). In addition to unusual physical and chemical conditions in 1988, adverse effects of *C. polylepis* to planktonic grazers may have played an important role in the development of the almost monospecific bloom, as reduced or inhibited grazing is generally believed to be an important factor in HAB dynamics (Smayda 1997b).

Paasche et al. (1990) have discovered a second cell type of *C. polylepis*, which they named the "alternate" cell type. The two cell types exhibit similar length of their haptonema, flagellar length, and cell form but differ slightly in cell size and body scale morphology. Whereas the "authentic" has been described to be more toxic and tolerant to environmental factors, such as light, temperature and salinity, it is exclusively haploid and the "alternate" cell type could be haploid or diploid (Edvardsen and Vaulot 1996). Edvardsen *et al*. (1996) therefore suggested that the cell types corresponding to stages of a haplo-diploid life cycle and may function as gametes.

Generally, toxins produced by *C. polylepis* are non-selective, interfering mainly with membrane function, and thus may affect organisms ranging from protozoa to fish. Their chemical structures are not fully elucidated, although Granéli et al. (1990) described them as lipids and/or fatty acids. Toxicity of *Chrysochromulina* was demonstrated to be highly variable even within the same species (Edvardsen and Paasche et al. 1998 and references therein), however, little is known of the factors triggering toxicity. Phosphate deficiency (Edvardsen et al. 1990; Edvardsen 1993), cellular N:P ratio (Johansson and Graneli 1999), as well as growth phase and pH (Schmidt and Hansen 2001) are known to influence toxicity of *C. polylepis*.

### *1.9. Aim of the thesis*

The importance of the mentioned toxic phytoplankton species *Alexandrium tamarense* and *Chrysochromulina polylepis* with respect to ecological and economic factors, and the lack of knowledge about a number of aspects of the expression of their toxicity, warranted a closer investigation of these topics. The objectives of the present study were to provide insights into toxicity and toxin production regulation of the species *Chrysochromulina polylepis* and *Alexandrium* spp. Molecular techniques were applied to improve our understanding of the biogeographic distribution and population dynamics of the toxic algae of the genus *Alexandrium*. Molecular probes were developed to facilitate monitoring programs for harmful algal blooms under *in situ* field conditions and/or on board ship.

## *1.10. Outline of this thesis*

### *1.10.1 Toxin Function*

To understand the processes initiating algal toxicity requires some knowledge about the biochemistry of the involved toxins and their effects on other organisms. Phycotoxins have often a complex chemical structure and their biosynthesis seem to be rather energy intensive (Shimizu et al. 1989; 1990). Thus, the ecological impact of phycotoxins has been examined in several studies (Anderson et al. 1998). As mentioned before, PSP and its causative organism in the genus *Alexandrium* are among the best studied subjects in harmful algal research (Cembella, 1998; Scholin 1998). The specific significance of saxitoxin and its more than 20 naturally occurring derivatives (Shimizu 1996) as compounds of cellular metabolism is not fully understood. Contradictory observations have led to many speculations about PSP toxin function (Cembella, 1998). Early suggestions indicated that PSP toxins may be linked to purine biosynthesis, but experiments using radioactively labelled precursors make this hypothesis highly unlikely (Shimizu 1996). Alternatively, it has been suggested that PSP toxins might serve as N-reserve, but <10% of the cellular-N is bound in those toxins and the chemical structure points to a relatively high energy consuming biosynthetic pathway, whereas free amino acids, urea or small peptides would be more efficient storage compounds (Cembella 1998). A third hypothesis, by Wyatt and Jenkinson (1997), suggested that PSP toxins may serve as pheromones, but there is no proof of this interesting idea.

It has been commonly hypothesized that these toxins act as a chemical defence against grazing activity. Shaw et al. (1997) have shown that a mixture of pure PSP toxins dissolved in seawater behaved as a feeding deterrent without lethal effects to the copepod *Tigriopus californicus*. Inconsistent results have been obtained with other copepods. For example, Teegarden (1999) suggested that cells containing PSP toxins can be discerned by copepod grazers prior to ingestion and thus can be rejected without mortal damage. Earlier observations, however, indicated that PSP-toxicity of food items was apparently not a factor in food selection for the copepod species *Acartia tonsa* and *Eurytemora herdmani* (Teegarden and Cembella 1996).

Various *Alexandrium* strains affected the heterotrophic dinoflagellate *Polykrikos kofoidii*, from growth-support to causing cell death via cell lysis. The observed results proved to be independent of the specific strain and whether it produced PSP toxins or not (Matsuoka et al. 2000; Cho and Matsuoka 2000).

In any case, phycotoxins are vectored through the food chain, e.g., the affected organisms are often at the end of the food chain, such as fish, seals, whales or other marine mammals, which are not directly microalgal predators. Smayda (1997b) has emphasized that there is no clear field evidence that HAB outbreaks depend upon phycotoxins or other allelochemicals, because most bloom forming species are non-harmful. To clarify the role of PSP toxins or potential allelochemicals in *Alexandrium*/protozoa interactions, the effects of different *Alexandrium* spp. on heterotrophic dinoflagellates were investigated (**Publication I**), including those with and without toxin producing capability.

The role of secondary metabolites (allelochemicals and phycotoxins) in HAB dynamics remain unclear. There is strong evidence that HAB species show negative effects towards microbial, zooplanktonic, and benthic taxa and they are involved in ichthyotoxic blooms, such as *Karinia brevis* (= *Gymnodinium breve*) blooms and its massive fish kills in the Gulf of Mexico and *C. polylepis* in Scandinavian waters (Gjøsæter et al. 2000). In order to find out whether or not phycotoxins or allelochemicals might have forced formation of monospecific blooms, several experiments about grazer activity on HAB-species have been conducted. When different algal species are used in comparative studies for feeding experiments, it is difficult to link differences in growth and grazing activity causatively to algal toxicity. Cell properties other than toxin content, namely size, cell shape or swimming speed show significant influence on observable grazing efficiency (e.g., Hansen et al. 1992; Buskey 1997; Tillmann and Reckermann, 2002).

An elegant way to overcome these problems is to compare grazing on toxic clones versus grazing on non-toxic clones of the same algal species, which than can be assumed to be virtually identical in all other aspects except toxicity (Teegarden 1999). In **Publication II**, grazing experiments with two different clones of *C. polylepis* that differed in toxicity (as defined by their toxic effects on the brine shrimp *Artemia franciscana*) have been conducted, using the heterotrophic dinoflagellate *Oxyrrhis marina* as a grazer. To understand the mode of toxic action, detailed knowledge on the chemical identity of the reactive compounds is a prerequisite. Therefore, lipid fractions and fatty acids of both algal clones were analyzed and compared in order to follow the hypothesis that toxicity of *C. polylepis* is caused by liposaccharide, lipids, or fatty acids (Yasumoto et al. 1990; Simonsen and Moestrup 1997a).

### *1.10.2. Toxin Regulation/Production*

The potential of a species or strain to be toxic might be genetically determined, but the toxin cell quota ($Q_t$) can vary significantly within a given species or strain. The $Q_t$ results from interactions between the rate of anabolism and catabolism, leakage/excretion, cell growth and cell division (Cembella et al. 1998). Changes in environmental variables, such as light, salinity (Parkhill and Cembella 1999), temperature, macronutrients, (Anderson 1990; Anderson et al. 1990), and turbulence (Berdalet and Estrada 1993), influence the $Q_t$ of toxin, either by direct effect or via a feedback interaction with cell growth rate. As the cell divides, the toxin is partitioned between the daughter cells (Cembella et al. 1998; Taroncher-Oldenburg et al., 1997). The synthesis of PSP toxins in *Alexandrium* takes place during vegetative growth in the G1 phase of the cell cycle (Taroncher-Oldenburg et al. 1997), thus any prolongation of the G1 phase (decrease in growth rate [μ]) may result in higher $Q_t$ even if the rate of toxin synthesis is constant.

In photoautotrophic microalgae, the photoperiod influences many diurnal physiological processes, including cell division, nutrient assimilation, vertical migration, bioluminescence rhythms and toxin production (see introduction and Taroncher-Oldenburg et al., 1997; Pan and Cembella, 1998; Pan et al., 1999). The direct dependence of cellular processes on light/dark (L/D) cycles can be utilized to phase or synchronize the cell division cycle. Therefore distinct L/D cycles were used to synchronize cultures of *C. polylepis* (**Publication III**) and *A. ostenfeldii* (**Publication IV**) to study the effects of photoperiod and cell division on the production and accumulation of ichtyotoxic allelochemicals and spirolides, respectively.

As described before, the phenomenon of harmful algal blooms is increasing not only in intensity, but also in geographic distribution (Hallegraeff 1993). In addition to the common harmful species and their toxic effects, several new species, such as *C. polylepis* (reviewed in Gjøsæter et al. 2000) have been involved in toxic incidents even though they were formerly known as non-toxic. New toxins, such as spirolides (Hu et al. 1995), have been discovered during the last few years. In order to understand the increase of these different aspects, e.g., number of bloom events, species diversity, and causative toxins, the phylogenetic relationship of the toxic species versus the non-toxic species should be investigated. The human impact upon the global increase of HABs could be indirectly mediated by climate changes (see above), and directly through active distribution of toxic species, e.g., via ballast water and shellfish stock transfer.

### *1.10.3 Phylogeny and Biogeography*

Molecular data can be used to elucidate the biogeographic history of species and might help to understand the recent global distribution. Cembella et al. (1988) opened the discussion about the differentiation between *A. tamarense* and *A. catanella* and since then much effort has been applied to understand the geographic and genetic distribution of the *A. tamarense* species complex. Today, our knowledge of the molecular biology of this species complex is primarily the result of the work of Scholin (as summarized in Scholin, 1998). As mentioned above, phylogenetic studies of the *Alexandrium* species complex reflects geographic areas rather than the three morphotypes *A. tamarense*, *A. catenella*, and *A. fundyense* (Scholin et al. 1994; Adachi et al. 1996; Medlin et al. 1998; Higman et al. 2001). Based on these workers studies of the D1/D2 region of the LSU rRNA, five ribotypes have been described thus far, each of which contains exclusively either toxic or non-toxic strains. The worldwide occurrence of the species complex led to suggestions that there may exist more than the five ribotypes. Several new species of the genus *Alexandrium* and strains of the *A. tamarense* species complex were analyzed with respect to their phylogenetic relationship. Few scenarios for the recent distribution of the species complex have been proposed, mostly based on the geological events, such as the opening of the Bering strait (3 MA) without any calculation of possible divergence times of the ribotypes (Scholin et al. 1995; Medlin et al. 1998). Therefore, a molecular clock was generated as a hypothetical tool based on the evolution rates of the 18S rDNA gene and calibrated through fossil records (**Publication V**). The resulting possible divergence time of the species complex was then combined with geological processes to hypothesize on the evolution and the biogeographic distribution of the *A. tamarense* species complex.

Various harmful algal species consist of toxic and non-toxic strains, which either show minor structural differences or are morphological indistinguishable. In the case of *Chrysochromulina polylepis,* the non toxic strain B11, (authentic type), can only be distinguished from the toxic strain (alternate type) by the morphology of the body scale - only visible using electron microscopy (Paasche et al. 1990; Edvardsen and Paasche 1992). However, the *Alexandrium tamarense* species-complex consists of the above described three morphotypes, in which all known isolates of the morphotypes *A. catenella* and *A. fundyense* are toxic. In the *A. tamarense* type either toxic or non-toxic strains have been described (Scholin et al. 1998). Phylogenetic studies have shown that only two ribotypes, the North American and the temperate Asian are toxic despite containing all three morphotypes. When an analysis of strains of the species complex showed a ribotype different from that expected

for the area where the strain was isolated, introduction caused by human activity has been invoked as a likely cause (Scholin et al. 1995; Higman et al. 2001).

In order to study the global distribution of the *A. tamarense* species complex and to follow possible introduction due to human activity, molecular probes specific for the different ribotypes of the species complex were developed, rather than to rely on the expensive and time-consuming sequencing of D1/D2 regions of the LSU for strain identification (**Publication VI**). Because there are only few base substitutions among the 28S rDNA sequences within one ribotype, a detailed examination of the geographic clades was not possible through sequence analysis. A more detailed characterization of strains with respect to their geographic origin could be helpful to follow recent distribution due to dispersal or human activity. In **Publication VI**, a relatively recently developed molecular marker type called Amplified Fragment Length Polymorphisms (AFLPs) (Vos et al. 1995) were applied to investigate several strains from different ribotypes and isolates of one HAB to give information about the genetic diversity within one species during a bloom.

### *1.10.4. Molecular Probes for Ecological Monitoring*

Toxic and non toxic species often co-occur in natural algal assemblages and in some cases they can only be distinguished using electron microscopy (Hasle et al. 1996), such as for *Pseudonitzschia* spp. *Pseudonitzschia multiseries* is toxic, whereas *P. pungens* is not. Nevertheless, both species were considered as one species, until recently (Hasle 1995). Moreover, in some areas different toxic species may occur simultaneously. Often time consuming techniques like microscopic observation, which is routinely performed in monitoring programs for harmful algae must be applied.

Within the genus *Alexandrium*, cells of *A. ostenfeldii* are difficult to discriminate reliably from those of *A. tamarense.* The vegetative cells of the former species are typically larger and more 'globose' than those of the latter (Balech 1995; Cembella et al. 2000), but the key diagnostic features, such as the size and shape of the ventral pore at the margin of the first apical (1') thecal plate, must be examined individually for each specimen, which can be a tedious procedure. **Publication VII** reports the development and the application of taxon-specific probes to discriminate between cells of *A. ostenfeldii* and *A. tamarense* in field plankton samples collected close to the Firth of Forth along the east coast of Scotland, where blooms of toxic *Alexandrium* spp. recur on annual basis (Medlin et al. 1998; Higman et al. 2001).

# 2. Publications

## *2.1. List of Publications*

This doctoral thesis is based upon the following publications:

I. Urban Tillmann and Uwe John, Toxic effects of *Alexandrium* spp. on heterotrophic dinoflagellates: an allelochemical defence mechanism independent of PSP-toxin content, Marine Ecology Progress Series, 230: 47-58.

II. Uwe John, Urban Tillmann and Linda K. Medlin, A comparative approach to study inhibition of grazing and lipid composition of a toxic and non-toxic clone of *Chrysochromulina polylepis* (Prymnesiophyceae), Harmful Algae, 1:45-57.

III. Erik Eschbach, Uwe John, Markus Reckermann, Bente Edvardsen and Linda K. Medlin, Cell cycle dependent toxin production of the ichthyotoxic prymnesiophyte *Chrysochromulina polylepis*, Journal of Experimental Marine Biology and Ecology, to be submitted.

IV. Uwe John, Michael A. Quilliam, Linda Medlin and Allan Cembella, Spirolide production and photoperiod-dependent growth of the marine dinoflagellate *Alexandrium ostenfeldii*, Proceedings of the IXth International Conference on Harmful Microalgae, G.M. Hallegraeff, S.I. Blackburn, C.J. Bolch & R.J. Lewis, eds., IOC-UNESCO, Paris, pp. 299-302.

V. Uwe John, Robert A. Fensome and Linda K. Medlin, The application of a molecular clock based on molecular sequences and fossil record to explain the biogeographic distribution within the *Alexandrium tamarense* 'species complex' (Dinophyceae). Molecular Biology and Evolution, in press.

VI. Uwe John, Linda K. Medlin and René Groben, Development of specific rRNA probes and the application of Amplified Fragment Length Polymorphisms (AFLP) to analyse clades within the *Alexandrium tamarense* species complex , Journal of Phycology, submitted.

VII. Uwe John, Allan Cembella, Christian Hummert, Malte Elbrächter, René Groben and Linda K. Medlin, Discrimination of the toxigenic dinoflagellates *Alexandrium tamarense* and *Alexandrium ostenfeldii* in co-occurring natural populations from Scottish coastal waters. European Journal of Phycology, 38: 25-40.

## *2.2. Publication I:* Toxic effects of *Alexandrium* spp. on heterotrophic dinoflagellates: an allelochemical defence mechanism independent of PSP-toxin content

Urban Tillmann[1] & Uwe John

Alfred Wegener-Institute for Polar and Marine Research
Am Handelshafen 12, D-27570 Bremerhaven, Germany

[1] e-mail: utillmann@awi-bremerhaven.de

***Abstract***

Sixteen strains of the red tide dinoflagellate *Alexandrium* spp. were tested for their short-term effects on the heterotrophic dinoflagellates *Oblea rotunda* and *Oxyrrhis marina*. Some *Alexandrium* strains, but not others, caused loss of motility and cell lysis of the heterotrophic dinoflagellates. A live counting procedure using *O. marina* was developed to quantify these toxic effects, which were compared with HPLC estimates of PSP toxin content. Within 5 strains, for which PSP toxins could be verified, both non-effective as well as effective strains were present and the same holds true for the other strains without detectable PSP toxins. This clearly indicates that the toxic effects are not due to PSP toxins. The observed effects are caused by extracellular substances, because *Oxyrrhis* did not ingest *Alexandrium* and lytic effects are also found in cell-free culture medium. The immobilisation effect was strongly dependent on the *Alexandrium* cell concentration. $EC_{50}$ concentration (*Alexandrium* cell concentration which caused 50 % immobilisation after 1 h exposure), as estimated for 5 effective strains, ranged from $2.1 \cdot 10^3$ cells $ml^{-1}$ down to $0.6 \cdot 10^3$ cells $ml^{-1}$. A quantitative comparison experiment showed that both heterotrophic dinoflagellate species are immobilised with the thecate species (*O. rotunda*) being even more affected compared to the athecate *O. marina*.

Key Words: *Alexandrium*, heterotrophic dinoflagellates, allelochemicals, lytic activity, PSP toxins

***Introduction***

Dinoflagellates of the genus *Alexandrium* are responsible for the occurrence of paralytic shellfish poisoning (PSP), a neurological affliction that has caused human illness for centuries (Prakash et al. 1971). As a consequence, there have been a large number of investigations spanning morphology, biochemistry, toxicity, genetics, bloom dynamics and evolution, making the genus *Alexandrium* arguably the best characterised harmful algal species known (Cembella 1998, Scholin 1998). Toxicity research has focused on the phycotoxin saxitoxin and its more than 20 naturally occurring derivates (Shimizu 1996). Their specific significance as compounds of cellular metabolism, however, is poorly understood. The frequent co-occurrences of HAB blooms and shellfish-borne toxicity or fish kills have fostered the notion that such blooms develop because the bloom species are phycotoxic. In this regard, the idea that PSP toxin production in *Alexandrium* spp. might be an adaptation for grazer defence has received great attention, as reduced or inhibited grazing is generally believed to be an important factor in harmful bloom dynamics (Fiedler 1982, Smayda 1997). Indeed, Shaw et al. (1997) recently showed that a mixture of pure PSP toxins dissolved in seawater behaved as a feeding deterrent without lethal effects to the copepod *Tigriopus californicus*. Recent results of Teegarden (1999) suggest that cells containing PSP toxins can be discerned by copepod grazers prior to ingestion and thus can be rejected without mortal damage. This is, however, in contrast to earlier observations, which showed that PSP-toxicity of food items was apparently not a factor in food selection for the copepod species *Acartia tonsa* and *Eurytemora herdmani* (Teegarden & Cembella 1996). Huntley et al. (1986) found that some dinoflagellate species caused inhibition of grazing in the copepods *Calanus pacificus* and *Paracalanus parvus*. Three of the rejected dinoflagellate species produced known neurotoxins. However, other species not known to be toxic also were rejected and some species, which produce PSP-toxins, were not rejected as food. Based on that evidence, they hypothesized that substances not associated with PSP toxins were inhibiting grazing. From experiments using filtrates of the rejected algal species they further concluded that potential inhibitory substances were extracellular exudates. Other studies strengthened the view that grazing interaction between *Alexandrium* spp. and copepods are highly variable and can vary greatly among zooplankton species. PSP-toxic strains of *Alexandrium* were rejected (Turriff et al. 1995) or ingested at lower rates in response to increasing toxicity (Ives 1985, Ives 1987). Enhanced mortality upon exposure to *Alexandrium* (Bagoien et al. 1996), reasonable high ingestion rates of toxic *Alexandrium* spp. with no apparent physiological effects (Teegarden & Cembella 1996) as well as negative long term effects such as reduction

of fecundity or lower hatching success (Dutz 1998, Frangopulos et al. 2000) have been observed.

Although there are many papers on copepod grazing studies, there are only a few dealing with grazing interactions of protozoa and *Alexandrium*. Ciliates (Prakash 1963, Watras et al. 1985) and heterotrophic dinoflagellates (Carreto et al. 1986, Sampayo 1998, Matsuyama et al. 1999) can be found at high concentrations during field blooms of PSP-producing dinoflagellates. Subsequent laboratory growth experiments using protozoan cultures yielded mixed results; the tintinnid *Favella ehrenbergii* is able to grow on *A. tamarense* at low concentrations (Stoecker et al. 1981), but the growth response of *F. ehrenbergii* on *A. tamarense* is clone-specific (Hansen 1989). At higher *Alexandrium* concentrations, however, toxic effects became apparent, which, based on indirect evidence, were attributed to PSP-toxins (Hansen et al. 1992). Matsuoka et al. (2000) conducted laboratory experiments using the heterotrophic dinoflagellate *Polykrikos kofoidii* and noted widely varying feeding and growth responses to various strains of *Alexandrium*: some strains, including both PSP-toxic and non-toxic strains, supported rapid growth, whereas others rapidly caused cell death of the heterotrophic dinoflagellate. In a subsequent paper, ingestion of PSP-toxic *Alexandrium* cells was thought to be the cause of *P. kofoidii* cell lysis (Cho & Matsuoka 2000).

In order to clarify the role of PSP toxins or potentially of other substances in *Alexandrium*/protozoa interactions, the present paper analyses the effects of *Alexandrium* spp. on heterotrophic dinoflagellates using a wide range of different *Alexandrium* strains, for which PSP toxins were simultaneously analysed.

***Material & Methods***

*Alexandrium cultures*

Tab. 1 gives an overview of the different strains of *Alexandrium* tested in the present study. All strains were grown non-axenically with IMR 1/2 medium (Eppley et al. 1967), supplemented with selenite (Dahl et al. 1989), or K-medium (Keller et al. 1987)(see tab. 1) under controlled conditions at 15 °C with artificial light at 100 µE $m^{-2}$ $s^{-1}$ and a light-dark cycle of 16:8 h. Growth of algal cultures was followed by cell counts using the Utermöhl-technique to ensure that cells taken for experiments or for filtration for toxin analysis were in exponential growth phase. The corresponding doubling times of all strains are listed in table 1.

*Cultures of heterotrophic dinoflagellates*

The heterotrophic dinoflagellate *Oblea rotunda* was isolated by capillary isolation from a brackish pond near Büsum (Germany) in 1993. Stock cultures held in multiwell plates or 100 ml flask were fed with the raphidophyte *Fibrocapsa japonica*, which recently was shown to sustain rapid growth of *O. rotunda* (Tillmann & Reckermann 2002). Cultures were transferred about once a week to fresh medium containing late exponential *F. japonica* cells. *Oxyrrhis marina* (Göttingen culture collection, strain B21.89) was grown with *Dunaliella sp.* as food algae. Stock cultures of both heterotrophic dinoflagellates were maintained at 20 ° C and natural light. Cultures of heterotrophic dinoflagellates used in the experiments were grown to high densities until they become almost deprived of food.

*PSP toxin analysis*:

For toxin analysis, 30 – 50 ml of exponentially growing *Alexandrium* culture were gently filtered through 0.2 µm polycarbonate membrane filters (Nuclepore). The sample preparation protocol by Hummert et al. (1997) was applied for extraction. Briefly, 1 ml of acetic acid (0.03 N) was added to the algal filter, which was homogenized for 2 min using a Sonopuls HD 70 ultrasonic probe (Bandelin, Berlin, Germany), and centrifuged for 10 min (2980 g). The supernatant was passed through a 0.45 µm, 25 mm diameter PTFE filter (No. H250.1, Carl Roth, Karlsruhe, Germany) and subsequently injected into the LC equipped with a fluorescence detector. For determination of N-sulfocarbamoyltoxins 150 µl of the acetic acid extract were mixed with 37 µl of 1.0 N hydrochloric acid and heated for 15 min at 90 °C. After cooling down to room temperature the mixture was neutralized with 75 µl of 1.0 N sodium acetate. N-sulfocarbamoyltoxins concentrations were calculated by the difference

(increase) of the peak areas to those obtained by acetic acid extract. The toxin analyses were performed by automated HPLC applying ion-pair chromatographic separation, post-column oxidation with periodic acid, and fluorescence detection, based on the method of Thielert et al. (1991) with modifications as described detailed in Hummert et al. (1997) and Yu et al. (1998). LC was performed with a SIL-10A intelligent autosampler, an LC-10ATvp intelligent pump, an SCL-10Avp system controller, a 1 ml CRX400 post-column reaction unit (Pickering Laboratories, Mountain View, CA, USA), two LC-9A pumps for delivery of post-column reaction solutions, and an RF-10Axl fluorescence detector (all Shimadzu, Duisburg, Germany). Data were analyzed with Class-vp 5.3 software from Shimadzu. Saxitoxin (STX), neosaxitoxin (NEO) and gonyautoxins (GTXs) as PSP toxin standards were purchased from the National Research Council, Marine Analytical Chemistry Standards Program (NRC-PSP-1B), Halifax, Nova Scotia, Canada. The standard solutions of GTX2 and GTX3 contained dcGTX2 and dcGTX3 as minor components, but the exact content of these toxins was not given. dcSTX was provided by the European Commission (BCR, The Community Bureau of Reference, Brussels), for use as a standard during an intercalibration exercise from 1995 to 1996. All chemicals used were analytical grade.

*Effects of* Alexandrium *spp.:*

Initial experiments were carried out in order to qualitatively monitor the effects of different strains of *Alexandrium* on *O. rotunda*. *Oblea rotunda* mixed with *Alexandrium* spp. (3000 cells $ml^{-1}$ final concentration) were inspected under a stereomicroscope after 1 and 24 h of exposure. This visual inspection, however, was unsatisfactory since most *Alexandrium* strains and *O. rotunda* are very similar in size and swimming behaviour, making a clear differentiation between both cell types extremely difficult. In order to quantitatively study the effects of different strain of *Alexandrium*, the following experimental procedure was developed. We used the heterotrophic dinoflagellate *Oxyrrhis marina*, which can be easily distinguished from *Alexandrium* by size, cell shape, and swimming pattern, even at low magnification under a dark-field stereomicroscope. Aliquots of 0.5 ml of a dense *Oxyrrhis* culture (3000 – 6000 cells $ml^{-1}$) were mixed with 1 ml of sample. After a defined exposure time at ambient light and room temperature, the number of moving *Oxyrrhis* cells was estimated using a droplet-counting procedure: 100 µl of cell suspension was separated into 25-30 small droplets in a petri dish and the number of swimming *Oxyrrhis* cells was counted under a stereo microscope. The original concentration of *Oxyrrhis* initially resulted in a total of 100 – 200 moving cells, which could be counted within 3-5 minutes. Reproducibility of

this counting procedure as checked in a preliminary experiment using filtered seawater as sample was satisfactory; repeated subsample counts of the same sample yielded a SD of 5.9 % (115.3 ± 6.8; n = 10) and triplicate subsample counts of 10 different samples one after the other pipetted in different wells yielded a SD of 8.2 % (115.2 ± 9.4; n=10). In addition to the live counting procedure, subsamples were fixed with lugol´s solution and counted with an inverted microscope. After exposure to some of the *Alexandrium* strains, *Oxyrrhis* lost its motility, thereafter became rounded, and then increased in size due to swelling and finally lysed. In the fixed samples, a cell was only scored if the normal cell shape was still visible.

Based on this counting procedure the following experiment was carried out: In multidish wells, 1.5 ml of *Oxyrrhis marina* cells were mixed with 3 ml of *Alexandrium tamarense* (strain 31/9), resulting in a final concentration of $3.9 \cdot 10^3$ *A. tamarense* $ml^{-1}$. One-quarter ml was fixed for cell counts, and 0.1 ml was counted using the droplet method outlined above at time 0, 20, 40, 60, 120, 180 min. A control experiment was carried out in a similar way using IMR 1/2 medium instead of the *Alexandrium tamarense* culture. The experiment was carried out in triplicate.

Based on the results from these experiments, the following procedure was used in all subsequent experiments: 1ml of algal sample was mixed to 0.5 ml of *Oxyrrhis marina* culture. IMR 1/2 medium was used as a control. After 1 h of exposure at ambient light and room temperature, the number of moving *Oxyrrhis marina* was counted using the droplet method. Results are always expressed as percentage of moving cells compared to the control. After 3 h of exposure, 0.25 ml were removed into small settling-chambers (diameter10 mm), fixed with lugol´s solution and counted under an inverted microscope. To compare different *Alexandrium* strains, the effects on *Oxyrrhis marina* were tested using exponentially growing algal cultures when they reached cell concentrations resulting in a final cell density of about $3 \cdot 10^3$ $ml^{-1}$. The non-toxic dinoflagellate *Scrippsiella trochoidea* was used as a control test species.

*Dependence of cell concentration*

For some strains of *Alexandrium*, algal cell concentration-dependence of the immobilisation effect on *Oxyrrhis marina* (droplet counts after 1 h incubation) was analysed. Therefore, exponentially growing cultures of *Alexandrium spp.* were tested as described above at several different points of the growth curve representing different cell concentrations (note that algae taken at different stages of the growth curve may differ in the per cell activity

potential). Percentages of immobilisation were transformed to probits (Hewlett & Placklet 1979). $EC_{50}$ values, defined as the amount of algae needed to induce 50 % immobilisation after 1 h of incubation, were calculated using linear regression analysis of probits against log-transformed *Alexandrium* concentrations.

*Test of cell-free filtrate*

In some experiments, the effect of culture filtrate of *Alexandrium* on *O. marina* was tested. A few ml of exponentially growing *Alexandrium* culture (same cell concentration as used for parallel whole cell incubations) were gently filtered using either 10 µm gauze or 0.2 µm membrane filters (Sartorius Minisart filters). The filtrate was added to *Oxyrrhis marina* and the samples analysed as described before.

*Quantitative comparison between* O. marina *and* O. rotunda

One experiment was conducted in order to quantitatively compare the immobilisation effect on *O. marina* and *O. rotunda*. A quantitative estimate using *O. rotunda* was possible using *A. tamarense* strain 31/9, because its dark pigmentation and slow swimming speed allowed for a tedious but reliable application of the life counting droplet method. One ml of a dense *O. rotunda* culture (ca. 500 cells ml-1) was mixed with 1 ml of *A. tamarense* 31/9 (final algal concentration in the mixture: 3200 cells $ml^{-1}$) or 1 ml of 0.2 µm filtrate, respectively. After 1 h of exposure at ambient light and room temperature, 500 µl of the mixture was separated into 25-30 small droplets in a petri dish and the number of swimming *Oblea* was counted under a stereomicroscope. For *O. marina*, 0.5 ml (ca. 5000 cells $ml^{-1}$) were mixed with 0.75 ml of A. *tamarense* culture and 0.25 ml IMR 1/2 medium (resulting as above in a likewise final algal concentration of 3200 $ml^{-1}$) or 0.75 ml of filtrate and 0.25 ml IMR 1/2 medium. The experiment then proceeded as described above. For both heterotrophic dinoflagellates a control experiment was carried out in a similar way using IMR 1/2 medium instead of the *Alexandrium* culture. All treatments were done in triplicate.

***Results:***

*Toxin analysis*

PSP-toxins could be detected in 5 of the 16 *Alexandrium* species/strains (Tab. 2). All these strains produced only small amounts of PSP toxins, with total PSP-toxin content ranging from 3.0 fmol $cell^{-1}$ (*A. minutum*, AL3T) to 42.3 fmol $cell^{-1}$ (*A. tamarense*, BAH181). The toxin profile was quite similar for *A. minutum* (AL3T) and *A. lusitanicum* (BAH91) with a predominance of GTX1 and GTX4. For *A. catenella* (BAH255) the sulfoxycarbamyl toxins $B_{1,2}$ and $C_{1+2}$ were predominant, but small amounts of STX also were found. The PSP toxin profiles of the two strains of *A. tamarense* (BAH181, GTPP01) were quite similar, but showed a high percentage of Neo and STX for the clone isolated near the Orkney Islands (BAH181) (Tab. 2).

*Effects of Alexandrium on heterotrophic dinoflagellates*

Preliminary qualitative observations revealed rapid negative effects on the thecate heterotrophic dinoflagellate *Oblea rotunda* when exposed to different *Alexandrium* strains. As observed under a stereomicroscope, cells tended to disappear from the water column and concentrate at the bottom, either immobilized or only slow moving. Rapidity and strength of effects appeared to vary among different *Alexandrium* strains. With some algal strains, *O. rotunda* seemed to be less affected and grazer attacks and pallium feeding event were observed. However, most *Alexandrium* strains and *O. rotunda* are very similar in size and swimming behaviour, making a clear differentiation between the species extremely difficult. Although rapid immobilisation effects were intuitively apparent for some *Alexandrium* strains, the approach using *Oblea rotunda* was not practical for detailed quantification of short-term effects. We therefore used the heterotrophic dinoflagellate *Oxyrrhis marina* as a test organism. It can be easily distinguished from *Alexandrium* by size, cell shape, and swimming pattern, even at low magnification under a dark-field stereomicroscope.

Microscopic observations showed that certain Alexandrium strains caused the heterotrophic dinoflagellate *Oxyrrhis marina* to lose motility and then become rounded. Subsequently, cells swelled and finally lysed. The time course of these effects was followed after addition of *Oxyrrhis marina* to *Alexandrium tamarense* (strain 31/9, final concentration of $3.9 \cdot 10^3$ $ml^{-1}$) (Fig. 1). The effect of immobilisation, as estimated by the life-counting droplet method, was very rapid. The first sample could not be processed before 3 min after

mixing, and by this time motility was significantly less than that of controls mixed with IMR 1/2 medium (t-test, $p<0.05$). Results of the following experiments therefore are always expressed as percentage of moving cells compared to controls. After 20 min of exposure the number of moving *Oxyrrhis marina* was drastically reduced and an exposure time of 60 min resulted in a nearly 100 % immobilisation. Cell lysis, as derived from counts of visible intact cells after fixation, was slower than immobilisation (Fig. 1 B) with a nearly complete lysis after 2-3 h of exposure. Effects of different *Alexandrium* strains thus were subsequently studied using live counts (droplet method) after 1 h exposure and fixed cell counts after 3 h of exposure.

*Comparison of different* Alexandrium *strains*

Effects of different *Alexandrium* strains were analysed at comparable final cell concentrations of about $3 \cdot 10^3$ ml$^{-1}$. The exact numbers are listed in Table 3. The percentage of *Oxyrrhis marina* which became immobilised after 1 h exposure varied considerably among the different Alexandrium strains tested (Fig. 2 A). The response varied from unaffected (e.g. *A. minutum*, AL3T) to a nearly 100 % immobilisation in some strains (e.g. *A. tamarense*, SZNB01). Cell counts of fixed samples after 3 h exposure showed comparable results (Fig. 2 B). No negative effects could be observed for the non-toxic control species *Scrippsiella trochoidea*. Differences in the strength of effects could not be related to estimates of PSP toxin content (Fig. 3). The most effective strains included both strains without PSP toxins (e.g. SZNB01) and with PSP toxins (e.g. strain BAH181). On the other hand, strains with (e.g. AL3T) and without PSP toxins (e.g. GTL129) had no effect at the cell concentration tested.

*Dependence of cell concentration*

The immobilisation effect was strongly dependent on the *Alexandrium* cell concentration for all five tested lytic strains (fig. 4). Data shown in fig. 4 are pooled from several experiments using different culture runs. Although all cultures were exponentially growing, there is some scatter in the immobilisation effect, which might be due to small differences in algal physiological conditions or in toxin sensibility of *O. marina* utilised in the different experiments. However, the data clearly show that significant negative short-term effects began to occur at cell concentrations of about $0.2 - 1 \cdot 10^3$ ml$^{-1}$, depending on the strain. $EC_{50}$ values, defined as the amount of algae needed to induce 50 % immobilisation after 1 h of incubation, of the 5 tested strains were $2.1 \cdot 10^3$ ml$^{-1}$ (*A. catenella* BAH255), $1.6 \cdot$

$10^3$ $ml^{-1}$ (*A. tamarense* 31/9), $1.5 \cdot 10^3$ $ml^{-1}$ (*A. tamarense* BAH181), $1.0 \cdot 10^3$ $ml^{-1}$ (*A. tamarense* SZNB01) and $0.6 \cdot 10^3$ $ml^{-1}$ (*A. tamarense* GTPP01).

*Effect of culture filtrate*

A negative effect on *Oxyrrhis marina* also was obvious when testing *Alexandrium* cell free culture filtrate (fig. 5). The immobilisation effect of cell free filtrate, however, was lower compared to the effect of algal suspensions (same cell concentration as used for the filtrate). There was no difference between the short-term immobilisation effect of filtrate through either gauze (10 µm) or a 0.2 µm membrane.

*Quantitative comparison between O. marina and O. rotunda*

In one experiment, the immobilisation effect of *A. tamarense* (strain 31/9) filtrate (< 0.2 µm) and cells was estimated for both heterotrophic dinoflagellates, *Oxyrrhis marina* and *Oblea rotunda* (Fig. 6). Both species are immobilised with the thecate species (O. rotunda) being even more affected compared to the athecate *O. marina*. Here again, negative effects of the cell-free filtrate were less pronounced for both target species.

***Discussion***

The results clearly show immobilising/lytic effects of several species/strains of the genus *Alexandrium*, which could not be explained by PSP toxin content. The observed toxic effects are caused by extracellular toxins, because (a) *Oxyrrhis marina* did not ingest *Alexandrium* and (b) lytic effects also are found in cell-free culture medium. Using non-axenic algal cultures it is important to consider the potential role of bacteria for the observed effects, since it is well known that bacteria may be either directly or indirectly associated with algal toxin production (Doucette et al. 1998). The immobilisation effect of whole algal culture (including bacteria) was higher compared to the effect of culture filtrate, indicating a continual release of toxic substances. However, there was no difference between the effect of filtrate gained either by gauze (10 µm, free bacteria should be unalteredly present) or membrane-filters (0.2 µm, should remove most bacteria), making involvement of extracellular toxins produced by free bacteria unlikely.

The noxious exudates released by *Alexandrium* may generally be classified as allelochemicals. Allelochemical secondary metabolites are mainly distinguished from phycotoxins (like PSP-toxins) in that phycotoxins can be vectored through the food web, accompanied by broad-based trophodynamic effects, whereas allelochemicals are usually directly targeted. The presented evidence of allelochemical activity of *Alexandrium* not related to PSP-toxins validates a couple of related indications, which can be found widespread in the literature: Ogata & Kodama (1986) described ichtyotoxic and haemolytic effects in the culture medium of *Protogonyaulax* (= *Alexandrium*) *catenella* and *P. tamarense*. As they did not found any PSP toxins in the medium, they concluded that the observed effect was caused by factor(s) other than PSP-toxins. Haemolytic effects of *Alexandrium* cell extract were confirmed later (Simonsen et al. 1995, Eschbach et al. 2001). Based on a comparison with the low haemolytic activity of purified STX and GTX1-5 standards, Simonsen et al. (1995) suggested that components other than PSP-toxins are likely to be responsible for the haemolytic effects of *A. tamarense*. Likewise, Lush & Hallegraeff (1996) attributed toxic effects of whole cells and cell-free culture medium of *A. minutum* to *Artemia salina* to a fast acting toxin distinct from PSP. Similar toxicity of exudates of *A. minutum* has been reported upon exposure to the copepod *Euterpina acutifrons* (Bagoien et al. 1996).

In addition, there are a few papers reporting negative effects of *Alexandrium* filtrate on other algal species. Blanco & Campos (1988) showed that culture filtrate of a PSP-toxin

containing *A. lusitanicum* adversely affected several flagellates (in fact most of the cells were killed), whereas growth of the algal species was not affected by filtrate of a non-PSP toxin producing *A. tamarense*. Based on that comparison, Blanco & Campos (1988) tentatively ascribed the toxic effect of culture filtrate to PSP-toxins. More recently, Arzul et al. (1999) investigated the allelopathic properties in three *Alexandrium* species. They found that the filtrate of all three species repressed growth of certain algal species. Allelopathic activity of exponentially growing *Alexandrium* species was positively related to both haemolytic activity and published values of PSP toxicity, probably reflecting correlated metabolic activity. The observed increase in allelopathy at the senescent growth phase led Arzul et al. (1999) to the suggestion, that substances other than STX are present.

In contrast to the large body of literature with copepods, there are only a few reports dealing with impacts of *Alexandrium* on heterotrophic protists. *A. tamarense* has been described as deleterious to the marine heliozoa *Heterophrys marina* (Tobiesen 1991). Hansen (1989) and Hansen et al. (1992) described effects of *Alexandrium* on the tintinnid ciliate *Favella ehrenbergii*. Hansen (1989) studied the behaviour and growth of *Favella* fed with six clones of *A. tamarense* producing different levels of PSP toxin. He clearly showed that algae are ingested by the ciliate, but the latter is only affected by exudates in the medium. The exudates induce ciliary reversal resulting in continuous backward swimming, swelling of the ciliate and subsequent cell lysis. Based on indirect evidence, Hansen (1989) suggested that the toxic effect of *Alexandrium* on ciliates is caused by PSP toxins. However, the same immobilisation effects on *F. ehrenbergii* were caused by *A. ostenfeldii*, which contained only very small amounts of PSP toxins (Hansen et al. 1992). Four out of seven *Alexandrium* tested species/strains sustained growth of the heterotrophic dinoflagellate *Polykrikos kofoidii*, whereas the protozoan grazer was rapidly killed by three strains (Matsuoka et al. 2000). Although Matsuoka et al. (2000) did not measure toxin content, they designated these three strains (*A. fundyense*, *A. lusitanicum*, *A. monilatum*) to be toxic (presumably based on literature data). *A. monilatum* is not known to produce PSP-toxins but it synthesizes an unknown ichtyotoxin (Aldrich et al. 1967). Moreover, Matsuoka et al., (2000) reported that both toxic and non-toxic strains (based again on literature data) of *A. tamarense* sustained moderate to rapid growth of *P. kofoidii*, suggesting that, in accordance with the present findings, factors other than PSP toxins are responsible for killing this heterotrophic dinoflagellate. Cho and Matsuoka (2000) described cell lysis of *P. kofoidii* feeding on a PSP-toxic *A. tamarense* strain. According to their descriptions and microphotographs, cell lysis

occurs after the ingestion of an *A. tamarense* cell, which was rapidly followed by egestion. In line with the present findings, Cho & Matsuoko (2000) observed a fast and nearly complete lysis of *P. kofoidii* within 1 h exposure to an *A. tamarense* concentration of 2000 $ml^{-1}$. However, in view of the present results, it seems unlikely that cell lysis of *P. kofoidii* is causatively linked to ingestion.

Quantitative findings in the present study were mainly based on *Oxyrrhis marina*, a species rather atypical for the marine plankton. However, detection of negative effects on *O. marina*, a marine protozoa, are likely of greater ecological significance compared to the rather unspecific impact on blood cells, as obtained with standard haemolytic tests. Both heterotrophic dinoflagellate species are immobilised, with the thecate species (*O. rotunda*) being even more affected compared to the athecate *O. marina*. Additional qualitative observations of the short term effects of *Alexandrium* on the thecate species *Oblea rotunda* as well as the similarity of cell lysis observed for *Polykrikos kofoidii* (Cho & Matsuoka 2000), suggest that other heterotrophic dinoflagellates are probably affected in the same way. Moreover, it may be attractive to ascribe the whole range of observed negative effects of *Alexandrium* culture medium on blood cells (haemolytic effects), heterotrophic protozoa (immobilisation/cell lysis, this study, Hansen 1989), algae (growth repression/cell lysis. Blanco & Campos 1988, Arzul et al. 1999) or copepods (grazing inhibition, Huntley et al. 1986) to one single chemical compound. However, Arzul et al. (1999) concluded that the allelopathic activity of *Alexandrium* is caused by a complex of chemicals, rather than by a specific substance. Almost nothing is known about the chemical composition of such compounds. The experiments with culture filtrate at least indicate that these substances are water-soluble but labile in culture media. Immobilisation activity of whole cells was higher compared to culture filtrate (Fig. 5 & 6, see also Hansen 1989) and thus probably due to a continual release of substances by the cells. Comparable lytic or allelopathic effects caused by other algal species are mainly thought to be due to glycolipids and polyunsaturated fatty acids. For example, digalactosylglycerol and octapentaenoic acid (18:5ω3) isolated by Yasumoto et al. (1990) have been shown to be both haemolytic (Yasumoto et al. 1990) and inhibitory to diatom growth (Gentien & Arzul 1990, Arzul et al. 1995).

The strength of immobilisation/lytic activity at comparable cell concentrations varied considerably among the different *Alexandrium* species/strains tested. It has been repeatedly established that cultured strains of toxic algae, such as *Alexandrium* or *Chrysochromulina*, are

typically less toxic than those collected from natural populations (White 1986, Cembella et al. 1988, Edvardsen 1993). They also may vary considerably with respect to cellular toxin content (Anderson 1990, Chang et al. 1997, Edvardsen & Paasche 1998, Parkhill & Cembella 1999) and toxin profile (Cembella 1998). Within the genus *Alexandrium*, lytic activity of the tested strains seems not to be related to certain species. Even though allelochemical effects are insignificant for all tested species of the *A. minutum/lusitanicum* species complex (strains AL3T, AL1T, BAH91, see fig. 2), there is evidence that other strains of the same species complex exude lytic compounds (*A. minutum*: Lush & Hallegraeff 1996; *A. lusitanicum*: Blanco & Campos 1988). For the tested strains which appeared to be less effective, it can not be excluded that lytic effect may be apparent at higher cell concentrations and/or longer exposure times.

Our results confirm that a large variety of combinations of PSP and lytic compounds may occur among different *Alexandrium* strains. Ecophysiological consequences of allelochemicals, however, may mimic phycotoxin effects, making it difficult to trace back observed effects to single compounds, unless many strains covering a whole range of combinations of phycotoxins and allelochemical compounds are tested. Evidence that toxic effects, grazing inhibition, and prey selection in crustacean grazers exposed to *Alexandrium* are caused by substances not associated with PSP (Huntley et al. 1986, Bagoien et al. 1996, Lush & Hallegraeff 1996, Teegarden & Cembella 1996) has already been noted above. In addition, there are a couple of reports indicating that copepods are affected by lytic exudates of other toxic algae (Gill & Harris 1987, Nielsen et al. 1990, Uye & Takamatsu 1990). Species/strain specific production of allelochemicals in addition to or instead of PSP toxins thus might partly explain some of the contradictory results on copepod grazing of *Alexandrium* species (reviewed in Turner & Tester 1997).

The power to immobilise or kill potential predators surely is of adaptive significance for a HAB species to form dense and long lasting blooms. To assess the potential impact *in situ*, however, a comparison between cell concentrations used in the present laboratory experiments and those occurring during *Alexandrium* blooms, is needed. *Alexandrium* are often considered to be „background" bloom species, in that they often are outnumbered by co-occurring phytoplankton (Anderson 1998). Indeed, a compilation presented by Wyatt & Jenkinson (1997) indicated relatively low numbers of 20 –400 cell $ml^{-1}$ as peak cell concentrations reached by *Alexandrium* during blooms in different regions. However, high-

biomass, monospecific blooms that discolour the water do occur, including those of *A. minutum* in south Australia (Hallegraeff et al. 1988), or dense blooms (>1000 $ml^{-1}$) of *A. tamarense* (*Gonyaulax excavata*) in the Argentine Sea (Carreto et al. 1986). In the Mediterranean, dense blooms of *Alexandrium* spp. with maximum concentrations up to 60000 cells per ml are repeatedly observed (Vila et al. 2001, and references therein). For the short-term immobilisation effect as observed in the present study, $EC_{50}$ cell concentrations (defined as the cell concentration which caused 50 % of immobilisation) were in the range from 1600 $ml^{-1}$ down to 600 $ml^{-1}$ for the 5 tested strains. This is in the same range of reported *Alexandrium* cell concentrations required to induce 50 % backwards swimming of the ciliate *F. ehrenbergii* within 10 minutes (Hansen 1989). Comparing these numbers with bloom concentrations cited above, we suggest that once a bloom reaches sufficiently high *Alexandrium* concentrations, allelochemicals indeed can prevent the population from any substantial protozoan grazing. A "blow out" of -at least- the protozoan grazers may explain why some *Alexandrium* blooms can persist for months (Mortensen 1985, Carreto et al. 1986). Sublethal, long-term negative effects of lower *Alexandrium* cell concentrations on protozoans are poorly known. According to Hansen (1989) *Favella ehrenbergii* is killed by a PSP toxic *A. tamarense* strain at high cell concentrations but showed ingestion and rapid growth when fed with low concentrations (< 1000 $ml^{-1}$) of the same strain. This suggests that grazing by protozooplankton might be of significance in controlling the development of a bloom if the concentration of algae is low and the concentration of predators is sufficiently high. However, extensive investigations on potential long-term effects of sublethal concentrations of allelochemicals on protozoan grazers still have to be carried out.

In addition to eliminating competitors and/or grazers, allelochemical activity may be coupled with mixotrophic nutrition, as was suggested for the haptophytes *Prymnesium patelliferum* (Tillmann 1998) and *Chrysochromulina* (Estep & MacIntyre 1989). *Alexandrium* spp. are generally considered to be mainly autotrophic, but food vacuoles containing ciliates or phytoplankton cells have been observed in *Alexandrium ostenfeldii* (Jacobson & Anderson 1996). Moreover, it recently was shown that *Alexandrium* has the capacity to take up high molecular weight organic molecules (Carlsson et al. 1998, Legrand & Carlsson 1998), to utilize organic N-substances for growth and toxin production (Ogata et al. 1996) and to remove dissolved free amino acids down to concentrations similar to those found in natural waters (John & Flynn 1999). It thus might be speculated that *Alexandrium* probably benefits from enhanced concentrations of dissolved organic matter in consequence of its lytic activity.

There can be no doubt that PSP toxins, through their neurotoxic effects on sea animals and humans, represent the most threatening property of *Alexandrium* spp. However, for the ecological success of *Alexandrium*, that is, to produce dense and long-lasting blooms, it is suggested that the allelochemical potential of *Alexandrium*, through its direct destructive effects on competing algae or unicellular grazers, is of much greater significance.

***Acknowledgment:***

The authors gratefully acknowledge Elke Jaim (Friedrich-Schiller University Jena) for the PSP-toxin measurement. We greatly appreciate the effort of all people that have isolated and made available the strains listed in table 1. Thanks are due to Allan Cembella for constructive criticism and suggestions. This work was partly supported by the German BMBF (TEPS project 03F0161) and by the European Commission (Research Directorate General-Environment Programme-Marine Ecosystems) through the BIOHAB project "Biological control of Harmful Algal Blooms in European coastal waters: role of eutrophication " (contract EVK3-CT99-00015). The BIOHAB project is part of the EC EUROHAB cluster.

Literature

Aldrich DV, Ray SM, Wilson WB (1967) *Gonyaulax monilata*: Population growth and development of toxicity in cultures. J Protozool 14:636-639

Anderson DM (1990) Toxin variability in *Alexandrium* species. In: Granéli E, Sundström B, Edler L, Anderson DM (eds) Toxic Marine Phytoplankton. Elsevier Science Publisher, New York, p 41-51

Anderson DM (1998) Physiology and bloom dynamics of toxic *Alexandrium* species, with emphasis on life cycle transitions. In: Anderson DM, Cembella AD, Hallegraeff GM (eds) Physiological Ecology of Harmful Algae Blooms. Springer, Berlin/Heidelberg/New York, p 29-48

Arzul G, Gentien P, Bodennec G, Toularastel F, Youenou A, Crassus MP (1995) Comparison of toxic effects in *Gymnodinium* cf. *nagasakiense* polyansaturated fatty acids. In: Lassus P, Arzul G, Gentien P, Marcaillou C (eds) Harmful Marine Algal Blooms. Lavoisier, Intercept Ltd., p 395-400

Arzul G, Seguel M, Guzman L, Erard-LeDenn E (1999) Comparison of allelopathic properties in three toxic *Alexandrium* species. J Exp Mar Biol Ecol 232:285-295

Bagoien E, Miranda A, Reguera B, Franco JM (1996) Effects of two paralytic shellfish toxin producing dinoflagellates on the pelagic hapacticoid copepod *Euterpina acutifrons*. Mar Biol 126:361-369

Blanco J, Campos MJ (1988) The effect of water conditioned by a PSP-producing dinoflagellate on the growth of four algal species used as food for invertebrates. Aquaculture 68:289-298

Carlsson P, Edling H, Béchemin C (1998) Interactions between a marine dinoflagellate (*Alexandrium catenella*) and a bacterial community utilizing riverine humic substances. Aquat Microb Ecol 16:65-80

Carreto JI, Benavides HR, Negri RM, Glorioso PD (1986) Toxic red-tide in the Argentine Sea. Phytoplankton distribution and survival of the toxic dinoflagellate *Gonyaulax excavata* in a frontal area. J Plankton Res 8:15-28

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. Springer Verlag, Berlin/Heidelberg/New York, p 381-403

Cembella AD, Therriault JC, Béland P (1988) Toxicity of cultured isolates and natural populations of *Protogonyaulax tamarensis* from the St. Lawrence Estuary. J Shellfish Res 7:611-621

Chang FH, Anderson DM, Kulis DM, Till DG (1997) Toxin production of *Alexandrium minutum* (Dinophyceae) from the Bay of Plenty, New Zealand. Toxicon 35:393-409

Cho HJ, Matsuoka K (2000) Cell lysis of a phagotrophic dinoflagellate, *Polykrikos kofoidii* feeding on *Alexandrium tamarense*. Plankton Biol Ecol 47:134-136

Dahl E, Lindahl O, Paasche E, Throndsen J (1989) The *Chrysochromulina polylepis* bloom in Scandinavian waters during spring 1988. In: Cosper EM, Bricelj VM, Carpenter EJ (eds) Novel Phytoplankton Blooms: causes and impacts of recurrent brown tides and other unusual blooms. Springer Verlag, Berlin, p 383-405

Doucette GJ, Kodama M, Franca S, Gallacher S (1998) Bacterial interaction with harmful algal bloom species: bloom ecology, toxigenesis, and cytology. In: Anderson DM, Cembella AD, Hallegraeff GM (eds) Physiological Ecology of Harmful Algal Blooms. Springer Verlag, Berlin/Heidelberg/New York, p 619-647

Dutz J (1998) Repression of fecundity in the neritic copepod *Acartia clausi* exposed to the toxic dinoflagellate *Alexandrium lusitanicum*: relationship between feeding and egg production. Mar Ecol Prog Ser 175:97-107

Edvardsen B (1993) Toxicity of *Chrysochromulina* species (Prymnesiophyceae) to the brine shrimp, *Artemia salina*. In: Smayda TJ, Shimizu Y (eds) Toxic Phytoplankton Bloom in the Sea. Elsevier Science Publisher B.V., p 681-686

Edvardsen B, Paasche E (1998) Bloom dynamics and physiology of *Prymnesium* and *Chrysochromulina*. In: Anderson DM, Cembella AD, Hallegraeff GM (eds) Physiological Ecology of Harmful Algae Blooms. Springer Verlag, Belin/Heidelberg/New York, p 193-208

Eppley RW, Holmes RW, Strickland JDH (1967) Sinking rates of marine phytoplankton measured with a fluorometer. J Exp Mar Biol Ecol 1:191-208

Eschbach E, Scharsack J, John U, Medlin LK (2001) Improved erythrocyte lysis assay in microtitre plates for sensitive detection and efficient measurement of haemolytic compounds from ichthyotoxic algae. J Appl Toxicol 21:in press

Estep KW, MacIntyre F (1989) Taxonomy, life cycle, distribution and dasmotrophy of *Chrysochromulina*: a theory accounting for scales, haptonema, muciferous bodies and toxicity. Mar Ecol Prog Ser 57:11-21

Fiedler PC (1982) Zooplankton avoidance and reduced grazing responses to *Gymnodinium splendens* (Dinophyceae). Limnol Oceanogr 27:961-965

Frangopulos M, Guisande C, Maneiro I, Riveiro I, Franco J (2000) Short-term and long-term effects of the toxic dinoflagellate *Alexandrium minutum* on the copepod *Acartia clausi*. Mar Ecol Prog Ser 203:161-169

Gentien P, Arzul G (1990) Exotoxin production by *Gyrodinium* cf. *aureolum* (Dinophyceae). J Mar Biol Ass UK 70:571-581

Gill CW, Harris RP (1987) Behavioural responses of the Copepods *Calanus helgolandicus* and *Temora longicornis* to dinoflagellate diets. J Mar Biol Ass UK 67:785-801

Hallegraeff GM, Steffensen DA, Wetherbee R (1988) Three estuarine Australian dinoflagellates that can produce paralytic shellfish toxins. J Plankton Res 10:533-541

Hansen PJ (1989) The red tide dinoflagellate *Alexandrium tamarense*: effects on behaviour and growth of a tintinnid ciliate. Mar Ecol Prog Ser 53:105-116

Hansen PJ, Cembella AD, Moestrup O (1992) The marine dinoflagellate *Alexandrium ostenfeldii*: paralytic shellfish toxin concentration, composition, and toxicity to a tintinnid ciliate. J Phycol 28:597-603

Hewlett PS, Placklet RL (1979) The probit and similar transformation. In: An introduction to the interpretation of quantal response in biology. Edward Arnold Ltd., London, p 12-17

Hummert C, Ritscher M, Reinhardt K, Luckas B (1997) Analysis of the characteristic PSP profiles of *Pyrodinium bahamense* and several strains of *Alexandrium* by HPLC based ion-pair chromatographic separation, post-column oxidation, and fluorescence detection. Chromatographia 45:312-316

Huntley M, Sykes P, Rohan S, Marin V (1986) Chemically-mediated rejection of dinoflagellate prey by the copepod *Calanus pacificus* and *Paracalanus parvus*: mechanism, occurence and significance. Mar Ecol Prog Ser 28:105-120

Ives JD (1985) The relationship between *Gonyaulax tamarensis* cell toxin levels and copepod ingestion rate. In: Anderson DM, White AW, Baden DG (eds) Toxic Dinoflagellates. Elsevier/North Holland, New York, p 413-418

Ives JD (1987) Possible mechanisms underlying copepod grazing responses to levels of toxicity in red tide dinoflagellates. J Exp Mar Biol Ecol 112:131-145

Jacobson DM, Anderson DM (1996) Widespread phagocytosis of ciliates and other protists by mixotrophic and heterotrophic thecate dinoflagellates. J Phycol 32:279-285

John EH, Flynn KJ (1999) Amino acid uptake by the toxic dinoflagellate *Alexandrium fundyense*. Mar Biol 133:11-19

Keller MD, Selvin RC, Claus W, Guillard RRL (1987) Media for the culture of oceanic ultraphytoplankton. J Phycol 23:633-638

Legrand C, Carlsson P (1998) Uptake of high molecular weight dextran by the dinoflagellate *Alexandrium catenella*. Aquat Microb Ecol 16:81-86

Lush GJ, Hallegraeff GM (1996) High toxicity of the red tide dinoflagellate *Alexandrium minutum* to the brine shrimp *Artemia salina*. In: Yasumoto T, Oshima Y, Fukuyo Y (eds) Harmful and toxic algal blooms. UNESCO, p 389-392

Matsuoka K, Cho HJ, Jacobson DM (2000) Observation of the feeding behaviour and growth rates of the heterotrophic dinoflagellate *Polykrikos kofoidii* (Polykrikaceae, Dinophyceae). Phycologia 39:82-86

Matsuyama Y, Miyamoto M, Kotani Y (1999) Grazing impact of the heterotrophic dinoflagellate *Polykrikos kofoidii* on a bloom of *Gymnodinium catenatum*. Aquat Microb Ecol 17:91-98

Mortensen AM (1985) Massive fish mortalities in the Faroe Islands caused by a *Gonyaulax excavata* red tide. In: Anderson DM, White AW, Baden DG (eds) Toxic Dinoflagellates. Elsevier/North Holland, New York, p 165-170

Nielsen TG, Kiorboe T, Bjornsen PK (1990) Effects of a *Chrysochromulina polylepis* subsurface bloom on the planktonic community. Mar Ecol Prog Ser 62:21-35

Ogata T, Kodama M (1986) Ichthyotoxicity found in culture media of *Protogonyaulax* spp. Mar Biol 92:31-34

Ogata T, Koike K, Nomura S, Kodama M (1996) Utilization of organic substances for growth and toxin production by *Alexandrium tamrense*. In: Yasumoto T, Oshima Y, Fukuyo Y (eds) Harmful and Toxic Algal Blooms. Intergovernal Oceanographic Commision of UNESCO, Paris, p 343-346

Parkhill JP, Cembella AD (1999) Effects of salinity, liht and inorganic nitrogen on growth and toxigenity of the marine dinoflagellate *Alexandrium tamarense* from northeastern Canada. J Plankton Res 21:939-955

Prakash A (1963) Source of paralytic shellfish toxin in the Bay of Fundy. J Fish Res Bd Canada 20:983-996

Prakash A, Medcof JC, Tennant AD (1971) Paralytic shellfish poisoning in eastern Canada. Bull Fish Res Board Can 168:1-87

Sampayo MA (1998) *Polykrikos kofoidii* Chatton predatopn on *Gymnodinium catenatum* Graham and its effects. In: Reguera B, Blanco J, Fernandez ML, Wyatt T (eds) Harmfull Algae. Xunta de Galicia and Intergovernmental Oceanographic Commision of UNESCO, p 182-183

Scholin CA (1998) Morphological, genetic, and biogeographic relatioships of the toxic dinoflagellates *Alexandrium tamarense*, *A. catenella*, and *A. fundyense*. In: Anderson DM, Cembella AD, Hallegraeff GM (eds) Physiological Ecology of Harmful Algal Blooms. Springer-Verlag, Berlin, p 13-27

Shaw BA, Andersen RJ, Harrison PJ (1997) Feeding deterrent and toxicity effects of apo-fucoxanthinoids and phycotoxins on a marine copepod (*Tigriopus californicus*). Mar Biol 128:273-280

Shimizu Y (1996) Microalgal metabolites: a new perspective. Ann Rev Microbiol 50:431-465

Simonsen S, Moller BL, Larsen J, Ravn H (1995) Haemolytic activity of *Alexandrium tamarense* cells. In: Lassus P, Arzul G, Gentien P, Marcaillou C (eds) Harmful Marine Algal Blooms. Lavoisier, Intercept Ltd., p 513-517

Smayda TJ (1997) Harmful algal blooms: Their ecophysiology and general relevance to phytoplankton blooms in the sea. Limnol Oceanogr 42:1137-1153

Stoecker D, Guillard RRL, Kavee RM (1981) Selective predation by *Favella ehrenbergii* (Tintinnida) on and among Dinoflagellates. Biol Bull 160:136-145

Teegarden GJ (1999) Copepod grazing selection and particle discrimination on the basis of PSP toxin content. Mar Ecol Prog Ser 181:163-176

Teegarden GJ, Cembella AD (1996) Grazing of toxic dinoflagellates, *Alexandrium* spp., by adult copepods of coastal Maine: Implications for the fate of paralytic shellfish toxins in marine food webs. J Exp Mar Biol Ecol 196:145-176

Thielert G, Kaiser I, Luckas B (1991) HPLC determination of PSP toxins. In: Fremy JM (ed) Proceedings of symposium on marine biotoxins. Editions CNEVA, Maisons-Alfort, p 121-125

Tillmann U (1998) Phagotrophy of a plastidic haptophyte, *Prymnesium patelliferum*. Aquat Microb Ecol 14:155-160

Tillmann U, Reckermann M (in press) Dinoflagellate grazing on the raphidophyte *Fibrocapsa japonica*. Aquat Microb Ecol

Tobiesen A (1991) Growth rates of *Heterophrys marina* (Heliozoa) on *Chrysochromulina polylepis* (Prymnesiophyceae). Ophelia 33:205-212

Turner JT, Tester PA (1997) Toxic marine phytoplankton, zooplankton grazers, and pelagic food webs. Limnol Oceanogr 42:1203-1214

Turriff N, Runge JA, Cembella AD (1995) Toxin accumulation and feeding behaviour of the planktonic copepod *Calanus finmarchicus* exposed to the red-tide dinoflagellate *Alexandrium excavatum*. Mar Biol 123:55-64

Uye S, Takamatsu K (1990) Feeding interactions between planktonic copepods and red tide flagellates from Japanese coastal waters. Mar Ecol Prog Ser 59:97-107

Vila M, Camp J, Garcés E, Masó M, Delgado M (2001) High resolution spatio-temporal detection of potentially harmful dinoflagellates in confined waters of the NW Mediterranean. J Plankton Res 23:497-514

Watras CJ, Carcon VC, Olson RJ, Chisholm SW, Anderson DM (1985) The effect of zooplankton grazing on estuarine blooms of the toxic dinoflagellate *Gonyaulax tamarensis*. J Plankton Res 7:891-908

White AW (1986) High toxin content in the dinoflagellate *Gonyaulax excavata* in nature. Toxicon 24:605-610

Wyatt T, Jenkinson IR (1997) Notes on *Alexandrium* population dynamics. J Plankton Res 19:551-575

Yasumoto T, Underdahl B, Aune T, Hormazabal V, Skulberg OM (1990) Screening for hemolytic activity and ichtyotoxic components of *Chrysochromulina polylepis* and *Gyrodinium aureolum* from Norwegian coastal waters. In: Granéli E, Sundström B, Edler L, Anderson DM (eds) Toxic Marine Phytoplankton. Elsevier, New York, p 436-440

Yu RC, Hummert C, Luckas B, Qian PY, Yhou MJ (1998) Modified HPLC method for analysis of PSP toxins in algae and shellfish from China. Chromatographia 48:671-675

**Tables:**

**Tab. 1**: Overview of *Alexandrium* species/strains tested

| species | strain nr. | origin; collector | culture medium | doubling time (h) |
|---|---|---|---|---|
| ***A. affine*** | CCMP112 | Ria de Vigo, Spain (1985); I. Bravo | K | 63 |
| ***A. catenella*** | BAH255 | Spain; M. Delgado | IMR 1/2 | 40 |
| ***A. lusitanicum*** | BAH91 | Laguna de Obidos, Portugal (1996) | K | 77 |
| ***A. minutum*** | AL1T | Mediterranean, Gulf of Trieste; A. Beran | K | 41 |
| ***A. minutum*** | AL3T | Mediterranean, Gulf of Trieste; A. Beran | K | 32 |
| ***A. ostenfeldii*** | BAH136 | New Zealand, Timaru (1992); N. Berkett | K | 150 |
| ***A. ostenfeldii*** | k-0324 | Limfjord, Denmark | K | 82 |
| ***A. ostenfeldii*** | k-0287 | Limfjord, Denmark | IMR 1/2 | 95 |
| ***A. pseudogonyaulax*** | AP2T | Mediterranean, Gulf of Trieste; A. Beran | K | 75 |
| ***A. tamarense*** | GTPP01 | Perch Pond, Falmouth, MA (1984); D. Kulis | IMR 1/2 | 50 |
| ***A. tamarense*** | SZNB01 | Mediterranean, Gulf of Naples (1999); M. Montresor | IMR 1/2 | 58 |
| ***A. tamarense*** | BAH181 | Orkney Island (1997); M. Elbrächter | IMR 1/2 | 40 |
| ***A. tamarense*** | CCMP115 | Tamar estuary, U.K. (1957); I. Adams | IMR 1/2 | 59 |
| ***A. tamarense*** | 31/9 | South England; W. Higman | IMR 1/2 | 44 |
| ***A. tamarense*** | GTLI21 | Mud Creek, Long Island (1981); D. Anderson | IMR 1/2 | 55 |
| ***A. taylori*** | AY1T | Mediterranean, Lagoon of Marano; A. Beran | K | 130 |

**Tab. 2:** PSP toxin content and toxin profile of *Alexandrium* species/strains. For all other species/strains tested (see Tab.1), no PSP toxins could be detected.

| strain | PSP-toxin content fmol cell$^{-1}$ | PSP-toxin profile (mol %) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | GTX1 | GTX2 | GTX3 | GTX4 | Neo | STX | $B_1$ | $B_2$ | $C_{1+2}$ |
| *A. minutum* (AL3T) | 3.0 | 56.6 | 5.3 | 3.3 | 34.7 | 0 | 0 | 0 | 0 | 0 |
| *A. lusitanicum* (BAH91) | 16.6 | 51.5 | 4.1 | 3.7 | 41.4 | 0 | 0 | 0 | 0 | 0 |
| *A. catenella* (BAH255) | 9.9 | 0 | 0 | 0 | 0 | 0 | 0.6 | 20.2 | 26.1 | 53.1 |
| *A. tamarense* (BAH181) | 42.3 | 11.9 | 6.9 | 7.6 | 7.2 | 12.8 | 11.6 | 3.1 | 3.5 | 35.5 |
| *A. tamarense* (GTPP01) | 33.4 | 14.2 | 0.9 | 1.2 | 16.0 | 3.6 | 0.3 | 7.0 | 1.5 | 55.4 |

**Tab. 3**: Final cell concentrations of *Alexandrium* species/strains tested for their effects on *Oxyrrhis marina* (see. fig. 2)

| species | strain nr. | final conc. $10^3$ $ml^{-1}$ |
|---|---|---|
| *A. affine* | CCMP112 | 2.6 |
| *A. catenella* | BAH255 | 2.6 |
| *A. lusitanicum* | BAH91 | 4.0 |
| *A. minutum* | AL1T | 5.0 |
| *A. minutum* | AL3T | 3.2 |
| *A. ostenfeldii* | BAH136 | 2.9 |
| *A. ostenfeldii* | k-0324 | 2.7 |
| *A. ostenfeldii* | k-0287 | 3.0 |
| *A. pseudogonyaulax* | AP2T | 2.4 |
| *A. tamarense* | GTPP01 | 3.0 |
| *A. tamarense* | SZNB01 | 3.8 |
| *A. tamarense* | BAH181 | 3.4 |
| *A. tamarense* | CCMP115 | 3.0 |
| *A. tamarense* | 31/9 | 2.8 |
| *A. tamarense* | GTLI21 | 4.0 |
| *A. taylori* | AY1T | 3.2 |

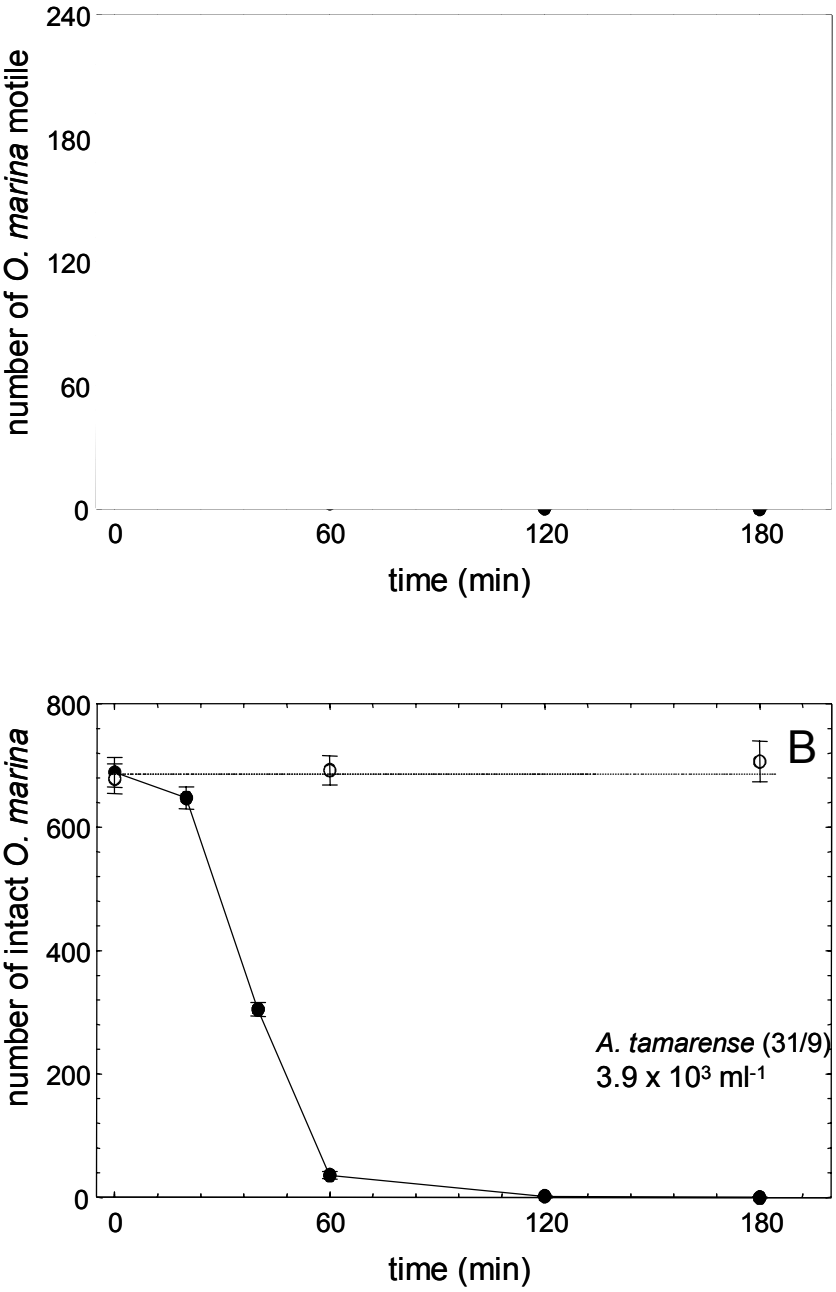



**Fig. 1**: Number of (A) motile *Oxyrrhis* (live counts) or (B) intact *Oxyrrhis* (fixed cell counts) as a function of exposure time to (•) *A. tamarense* (31/9, added to a final concentration of 3.9 $10^3$ cells $ml^{-1}$), or to (O) control (IMR 1/2 medium added). Data points refer to treatment mean ± 1SD (n=3).

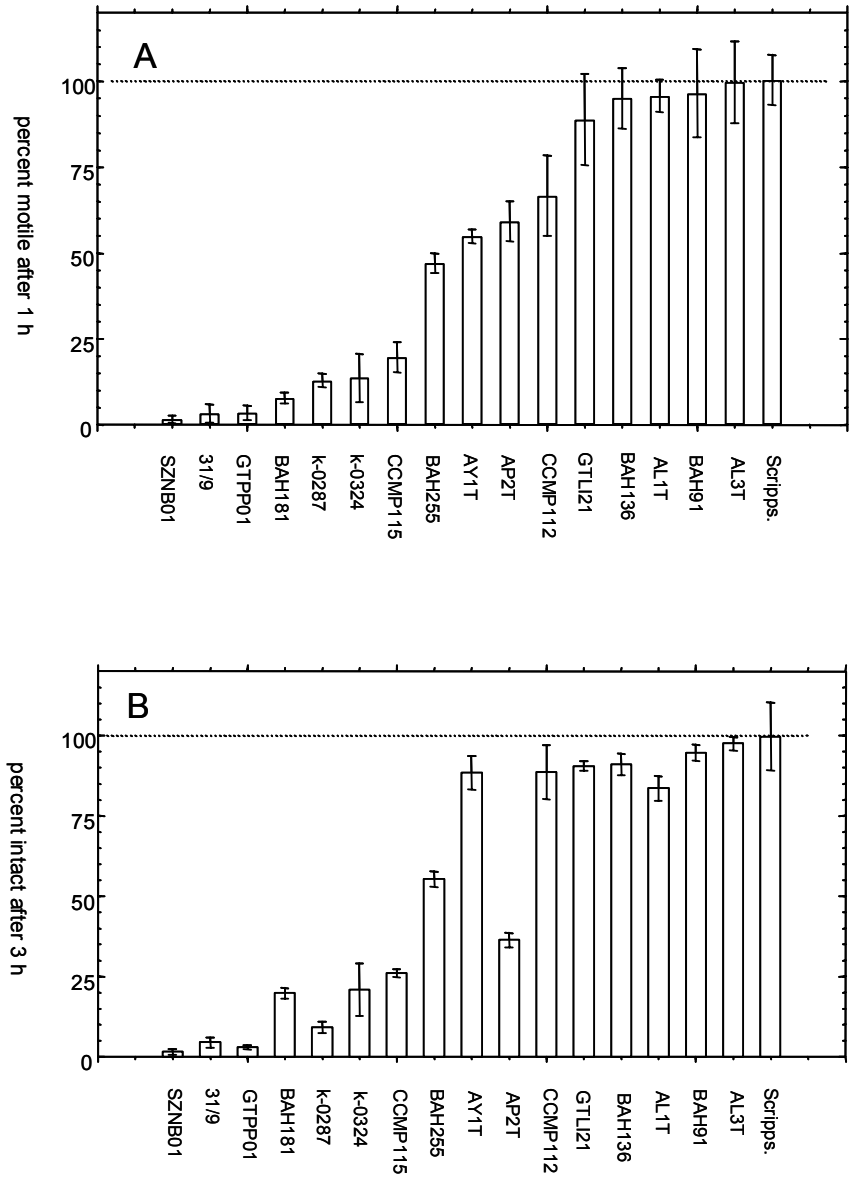



**Fig. 2**: Percentage of (A) motile *Oxyrrhis marina* after 1 hour exposure (live counts) or (B) intact *Oxyrrhis marina* after 3 hours exposure (fixed cell counts) to different species/strains of *Alexandrium*. Final algal cell concentrations are listed in Tab. 3. Results are expressed as triplicate mean ± 1SD.

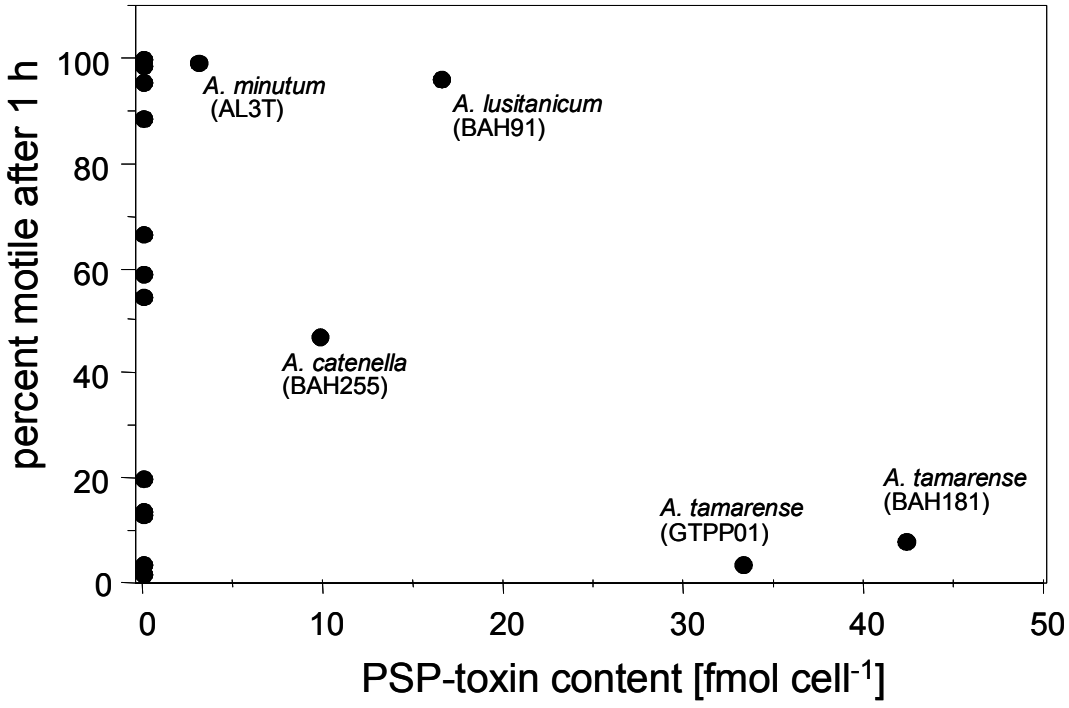



**Fig. 3**: Relationship between the percentage of motile *Oxyrrhis* after 1 hour of exposure (data from Fig. 2) and PSP-toxin content (fmol cell$^{-1}$) of the respective *Alexandrium* species/strain (data from Tab. 2).

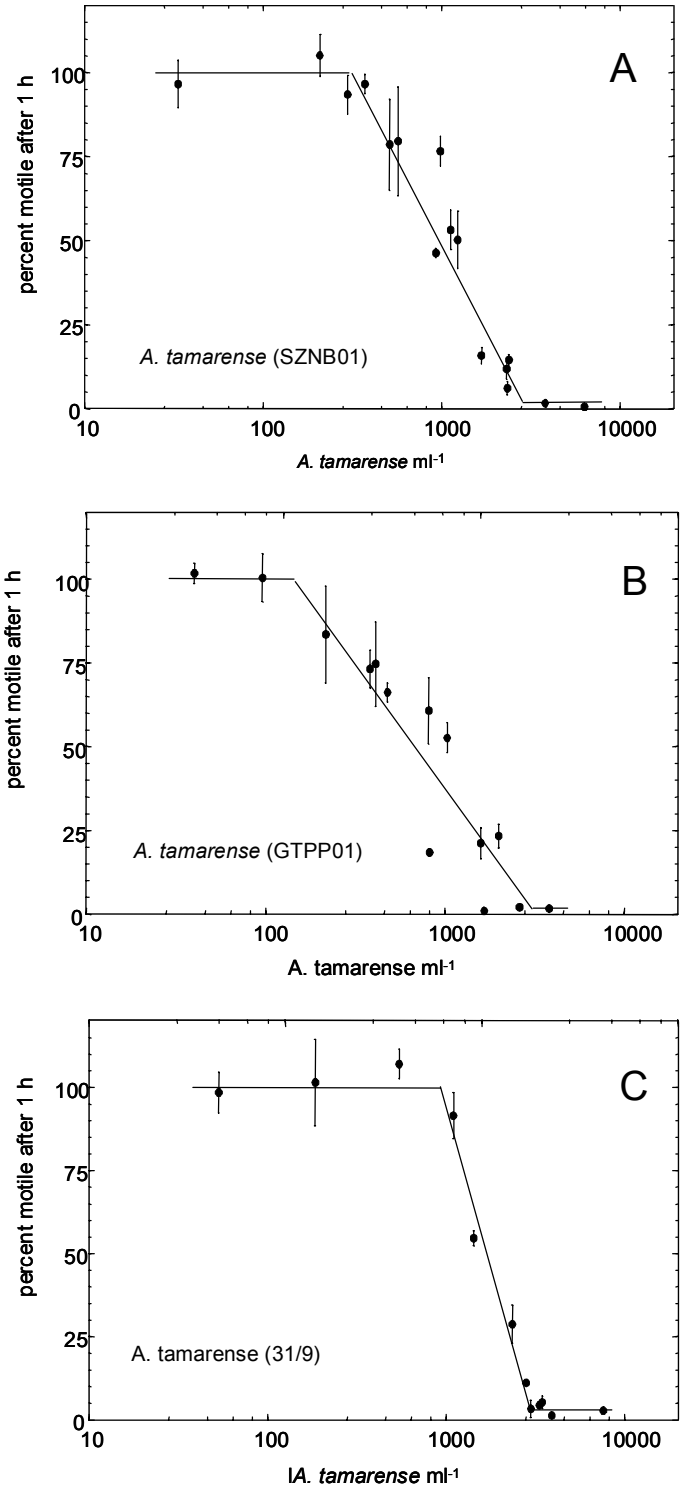



Fig. 4: continuous on the next pape.

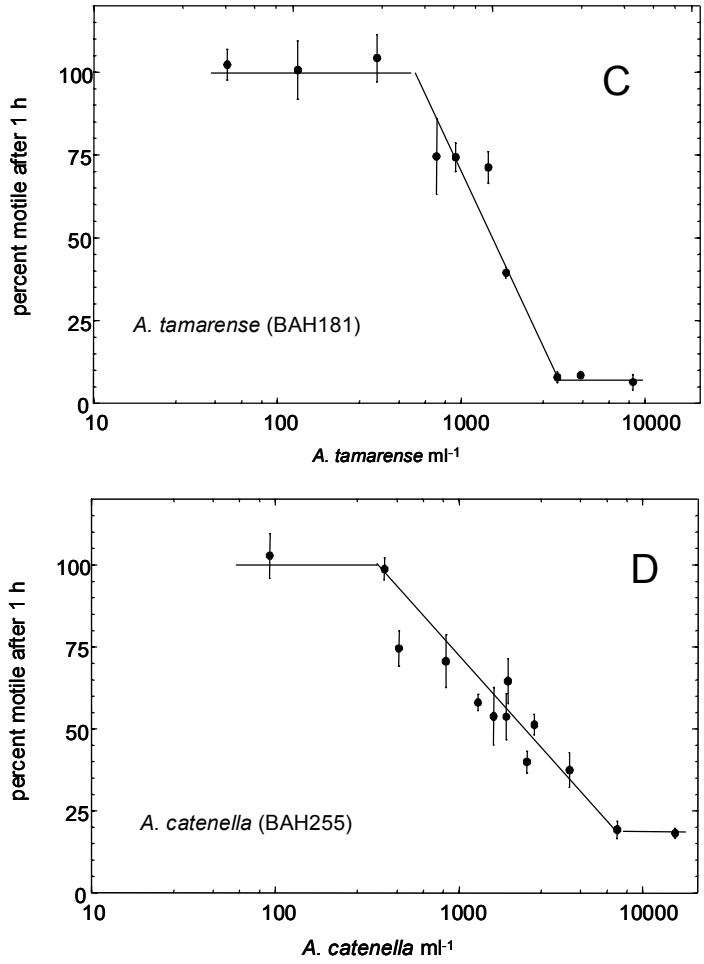



**Fig. 4**: Percentage of motile *Oxyrrhis* after 1 h exposure (live counts) as a function of algal cell concentration for (A) *A. tamarense* (SZNB01), (B) *A. tamarense* (GTPP01), (C) *A. tamarense* (31/9), (D) *A. tamarense* (BAH181) and (E) *A. catenella* (BAH255). Results are expressed as triplicate mean ± 1SD.

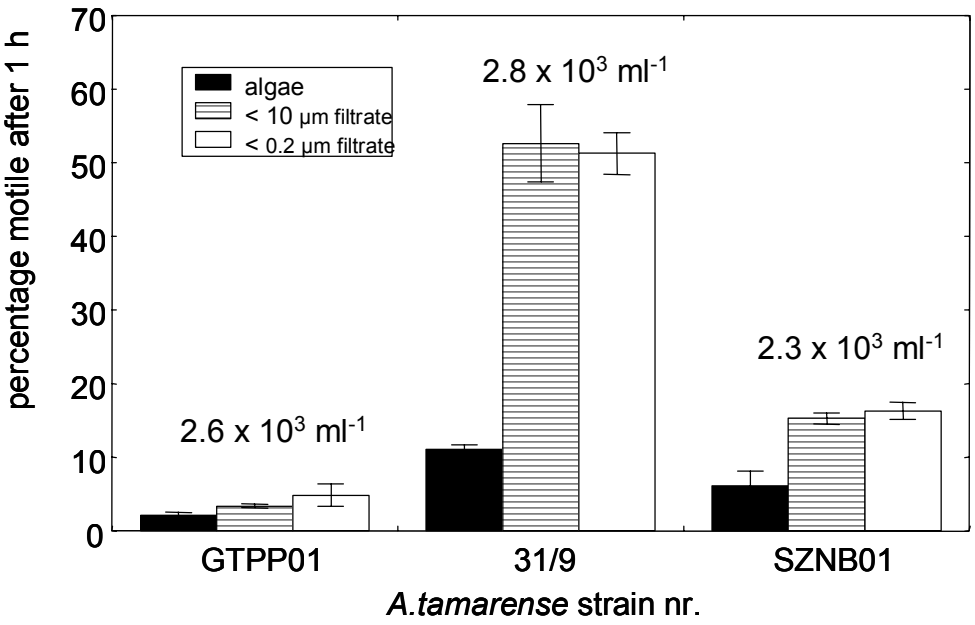



**Fig. 5**: Percentage of *Oxyrrhis* motile after 1 hour exposure to whole cells, < 10 µm filtrate or < 0.2 µm filtrate of three strains of *A. tamarense* (GTPP01, 31/9, SZNB01). Numbers above bars indicate final algal cell concentration. Results are expressed as triplicate mean ± 1SD.

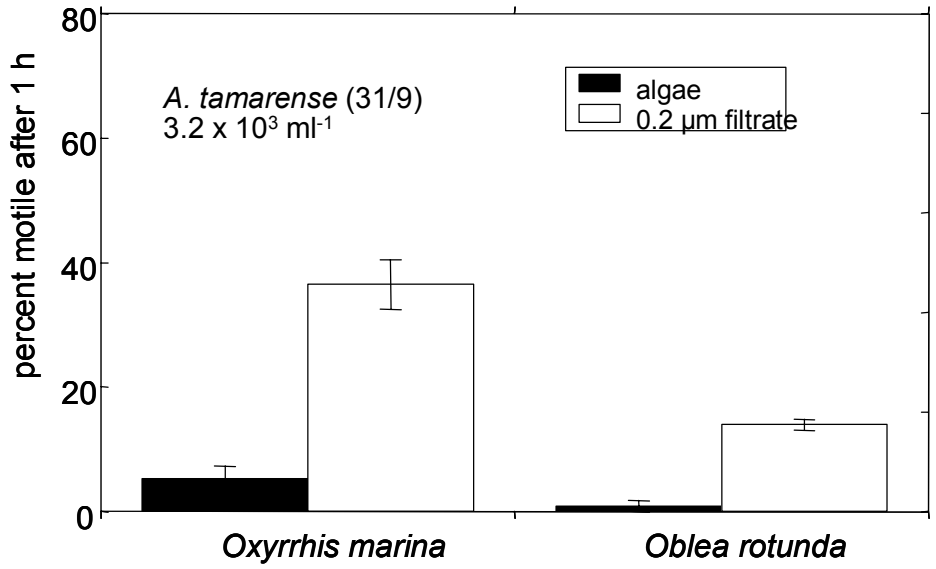



**Fig. 6**: Effects of *A. tamarense* (31/9) whole cells and 0.2 µm filtrate on *Oxyrrhis marina* and *Oblea rotunda* motility after 1 hour of exposure. Results are expressed as triplicate mean ± 1SD.

## *2.4. Publication II:* A comparative approach to study inhibition of grazing and lipid composition of a toxic and non-toxic clone of *Chrysochromulina polylepis* (Prymnesiophyceae*)*

Uwe John[1], Urban Tillmann and Linda K. Medlin

Alfred Wegener-Institute for polar and marine research
Am Handelshafen 12, D-27570 Bremerhaven, Germany

[1] corresponding author
phone : +49 471 4831 1841
fax: +49 471 4831 1425
e-mail: ujohn@awi-bremerhaven.de

***Abstract***

Since the massive bloom in 1988 in the North Sea, the prymnesiophyte flagellate *Chrysochromulina polylepis* Manton et Parke has been known for its ichtyotoxicity. Laboratory experiments using two clones of *C. polylepis* were conducted in a comparative approach. The clones were similar in size and shape, but differed in their toxicity, as demonstrated by the *Artemia* bioassay. To study the effects of toxic *C. polylepis* on protozooplankton grazers, grazing experiments were performed with the heterotrophic dinoflagellate *Oxyrrhis marina* Dujardin as grazer. A first experiment was carried out in order to follow batch culture growth and initial grazing of *O. marina* when fed toxic or non-toxic clones of *C. polylepis*. Ingestion of the toxic clone was 27 % of ingestion when fed with the non-toxic clone. When *O. marina* was fed with the toxic clone, vacuoles within *O. marina* cells contained fewer food particles and the cells grew slower (58 % of the division rate estimated for the non-toxic clone). A second experiment was conducted to determine the grazing and growth response of *O. marina* as a function of algal food concentration. Profound differences in ingestion, clearance, division, and gross growth efficiency of *O. marina* when fed the two clones of *C. polylepis* were again apparent. the toxin acts as a grazers deterrent, even at algal concentrations of 400 x $10^3$ $ml^{-1}$, *O. marina* was not killed by the presence or ingestion of toxic *C. polylepis*. In addition to grazing experiments, lipid classes and fatty acids of both algal clones were analysed and compared in order to follow the hypothesis that toxicity of *C. polylepis* is caused by liposaccharides, lipids, or fatty acids. However, the chemical composition with respect to lipid classes and fatty acids of both clones were quite similar, making it unlikely that these substances are involved in the toxicity towards *Artemia* and *O. marina*.

Key words:*Chrysochromulina polylepis*; grazing inhibition; lipid composition; *Oxyrrhis marina*; toxic algae

***Introduction***

The prymnesiophyte *Chrysochromulina polylepis*was described from the North Sea in the mid 1950s (Manton and Parke 1962). Although this species previously was believed to be non-toxic to fish (Manton and Parke 1962), an extraordinary bloom of *C. polylepis* in the Kattegat/Skagerrak area and off the Norwegian Coast in 1988 caused extensive fish kills. Since then, a number of blooms of different *Chrysochromulina* species have been reported, some of which have caused fish mortality (reviewed by Edvardsen and Paasche 1998). The 1988 bloom extended over an area of approximately 75,000 $km^2$ (Granéli et al. 1993), and was characterised by pronounced toxic effects to various organisms. These included farm fish and wild fish populations, plus mussels, echinoderms, polychaetes, ascidians, cnidarians, sponges, red and brown algae (Rosenberg et al. 1988), as well as bacteria, protozoans, copepods (Nielsen et al. 1990) and other microalgae (Dahl et al. 1989, Johnsen and Lomsland 1990). The factors and mechanism that led to the bloom have been intensively reviewed and discussed (Maestrini and Granéli 1991, Gjøsæter et al. 2000). In addition to unusual physical and chemical conditions in 1988, adverse effects of *C. polylepis* to planktonic grazers may have played an important role in the development of the almost monospecific bloom, as reduced or inhibited grazing is generally believed to be an important factor in harmful bloom dynamics (Smayda 1997).

Generally, toxins produced by *C. polylepis* are non-selective, interfering mainly with membrane function, and thus may affect organisms ranging from protozoa to fish. Their chemical structures are not fully elucidated, although Yasumoto et al. (1990) described them as lipids and/or fatty acids. Toxicity of *Chrysochromulina* was demonstrated to be highly variable even within the same species (Edvardsen and Paasche 1998 and references therein), however, little is known of the factors triggering toxicity. Phosphate deficiency (Edvardsen et al. 1990, Edvardsen 1993), cellular N:P ratio (Johansson and Granéli 1999), as well as growth phase and pH (Schmidt and Hansen 2001) are known to influence toxicity of *C. polylepis*.

Field and laboratory experiments demonstrated that *C. polylepis* inhibited the activity of a broad range of planktonic organisms, including bacteria, heterotrophic protists, copepods and other algae (Carlsson et al. 1990, Nielsen et al. 1990, Tobiesen 1991, Myklestad et al. 1995, Schmidt and Hansen 2001). In laboratory experiments using protistan grazers, Carlsson et al. (1990) showed that the grazing activity of the tintinnid *Favella ehrenbergii* on

*Heterocapsa_triquetra* was negatively influenced by the addition of *C. polylepis*. In the case of the heliozoan *Heterophrys marina*, the rapid growth, which had been observed at *C. polylepis* concentrations of 2 x $10^3$ cells $ml^{-1}$, decreased with increasing *C. polylepis* concentrations, coming to a complete halt at around 75 x $10^3$ cells $ml^{-1}$ (Tobiesen 1991). An adverse effect on *H. marina* even at low *C. polylepis* concentrations was indicated by the fact that other, non-toxic food organisms permitted more rapid cell growth (Tobiesen 1991).

Nevertheless, it can not easily be ruled out that harmful effects of senescent dense algal cultures on test organisms might be caused by factors other than toxins, e.g., by high pH in the culture medium (Schmidt and Hansen 2001). In addition, in comparative studies using different algal species as food, it is difficult to link causatively differences in growth and grazing activity to algal toxicity, as cell properties other than toxin content, such as size, cell shape or swimming speed, are important factors determining grazing efficiency (e.g. Hansen 1992, Buskey 1997, Tillmann and Reckermann 2002). An elegant way to overcome these problems is to compare grazing on toxic versus non-toxic clones of the same algal species, which can be assumed to be virtually identical in all aspects except toxicity (Teegarden 1999).

In the present study, we conducted grazing experiments with two different clones of *C. polylepis* that differ in toxicity (as defined by their toxic effects on the brine shrimp *Artemia franciscana*) using the heterotrophic dinoflagellate *Oxyrrhis marina* as grazer. To understand the mode of toxin action, detailed knowledge on the chemical identity of the reactive compounds is a prerequisite. Therefore, lipid classes and fatty acids of both algal clones were analysed and compared in order to follow the hypothesis that toxicity of *C. polylepis* is caused by liposaccharide, lipids, or fatty acids (Yasumoto et al. 1990, Simonsen and Moestrup 1997).

## *Material & Methods*

### *Cultures*

The experiments were conducted with two haploid clones of *Chrysochromulina polylepis* Manton et Parke, named B1511 and B11. Both clones were isolated by Bente Edvardsen from a toxic strain B1, which was isolated from the Oslo Fjord (Norway). Each clone represents one of the two different cell types of *C. polylepis* described in detail by Paasche et al. (1990), Edvardsen and Paasche (1992), Edvardsen and Vaulot (1996), and Edvardsen and Medlin (1998). Briefly, both cell types are virtually indistinguishable with light microscopy, but electron microscopy observation has shown differences in the fine

structure of the organic scales covering the cells. Based on the original description by Manton and Parke (1962), one cell type was termed authetic (α), whereas the second type was termed alternate (β) (Edvardsen and Paasche 1992). The clone B1511 consists of authentic cells, whereas the clone B11 contained only cells of the alternate type (Edvardsen and Vaulot 1996). Both clones were grown in axenic batch cultures in IMR ½ medium (Eppley et al. 1967), supplemented with selenite (Dahl et al. 1989) under controlled conditions of 15° C with artificial light of 45 µmol $m^{-2}$ $s^{-1}$ and a light/dark cycle of 14:10. The heterotrophic dinoflagellate *Oxyrrhis marina* (Göttingen culture collection, strain B21.89) was grown under the same conditions with chlorophyte *Dunaliella sp.* as food.

*Artemia Test*

*Artemia* tests were performed following the protocol of the Artemia Reference Centre (ARC Gent, Belgium) with slight modifications. Approximately 100 mg of *Artemia franciscana* cysts (Batch number: AF/N2000) were incubated for 48 h in 500 ml IMR ½ under constant light at room temperature and moderate aeration to achieve continuous suspension and at least 90 % oxygen saturation. After 48 h, an aliquot of *Artemia* nauplii was collected in a petri dish and 20-30 instar II nauplii were transferred with approximately 50 µl into each well of a 24 well plate (Nunc). The well was filled with 2 ml of IMR ½ (control) or varying concentrations of algal culture. All tests were done in triplicate. After 24h in darkness at room temperature, living and dead nauplii in each well were counted. Death of nauplii was defined as non-motility for more than 10 s. The corresponding mortality was transformed into probit units (Hewlett and Placklet 1979) and plotted against log-transformed cell concentration. The algal concentration causing 50% mortality of *A. franciscana* ($LC_{50}$) were determined from the regression line, where a probit of 5 corresponds to 50% mortality.

*Lipid determination*

Five litres of algal culture were collected by centrifugation and stored at – 80 °C until analyzed. The algal pellet was resuspended in dichlormethane : methanol (2 : 1 by vol.) and sonicated for 3 min with a stainless-steel probe. After the sonication the suspension was washed in 0.88% KCl. Lipid class composition was analyzed using thin layer chromatography (TLC) according to Olsen and Henderson (1989) using the standards for each lipid class obtained from Sigma Chemical Pool, U.K. For the determination of fatty acids, the algal extracts extracted as described above were transmethylated in methanol containing 2%

sulphuric acid for 4 h at 80 °C to produce fatty methyl esters (FAME) (Kattner and Fricke 1986). FAMEs were extracted with hexane and analyzed by gas chromatography.

*Grazing and growth of Oxyrrhis marina*

A first experiment was carried out in order to follow batch culture growth and initial grazing of *O. marina* when fed each clone of *C. polylepis*. Ten ml of a dense culture of *O. marina* were mixed with *C. polylepis* in triplicate in 200 ml Erlenmeyer flasks resulting in final concentrations of 0.55 x $10^3$ $ml^{-1}$ and 20 x $10^3$ $ml^{-1}$ for predator and prey, respectively. The *O. marina* culture had been pre cultured with *Duniella*, but was starved for 2 days prior to the onset of the experiment. Flasks containing only phytoplankton served as control. All flasks were incubated at 15 °C at photon flux density of 45 µmol $m^{-2}$ $s^{-1}$ and a photoperiod of 14L:10D. For cell counts, subsamples were taken twice a day. *Oxyrrhis marina* was counted in 1 ml Lugol´s iodine-fixed subsamples with an inverted microscope, whereas *C. polylepis* cell numbers were estimated using a Coulter Counter Model II. Division rate, µ ($d^{-1}$) was calculated from the regression coefficient of the natural logarhism (ln) of cell number versus time. For elemental analysis, triplicate subsamples of control flasks (*C. polylepis* only) were gently filtered onto precombusted Whatman GF/C filters, gently treated with 3-5 drops of 0.1 N HCl, dried at 60° C (24 h), and combusted in a Carlo-Erba NA 1500 elemental analyser. To quantify ingestion, 1 ml subsamples were taken at times 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 22, and 46 h. Samples were fixed with 1 % glutaraldehyde and inspected under an inverted microscope. For at least 100 *O. marina* cells per sample, whether or not the grazer had ingested algal cells (regardless the exact number of ingested cells), was scored. From these data, the probability that a grazer had not ingested any prey cell could be calculated. Data were fitted by non-linear regression to the equation $P_{0(t)} = (1\text{-}z)\ e^{-\lambda t} + z$, assuming that the sample distribution fit a Poisson with extra zeros (Bratvold et al. 2000). Z is the fraction of the population that do not feed at all and λ is the Poisson parameter in units of ingested food cells per grazer per hour (Bratvold et al. 2000). In addition, in samples taken after 22 and 46 h the number of algal cells inside food vacuoles of at least 100 grazers was estimated and divided into categories of <5, 5-10, 10-20 and > 20 algal cells per grazer.

A second experiment was conducted to determine the grazing and growth response of *O. marina* as a function of algal food concentration. A series of equivalent grazer inoculate (final 400 x $10^3$ cells $ml^{-1}$. Different food concentrations were established by appropriate dilution of a exponential batch culture of *C. polylepis*. Three replicates were set up for each

food concentration. Triplicate flasks containing the same concentrations of algae only served as control. At time t=0, initial samples were taken for determination of cell concentrations. After 2 h incubation, 1 ml subsamples were taken for the determination of initial grazing rates and fixed with 1% glutaraldehyde. After settlement in 1 ml Utermöhl chambers, samples were inspected under an inverted microscope using fluorescence light (Zeiss filter set 14). Counts of the number of ingested algal cells of at least 200 individuals of *O. marina* allowed for the determination of initial grazing rate (algae per grazer per hour). Final samples for determination of cell numbers were taken 48 h after initial sampling. Final samples were fixed with Lugol´s iodine solution and grazer cells were counted microscopically. In each final sample, as well as in initial samples, the cell size of at least 20 fixed grazers was measured by means of a calibrated ocular micrometer. Size of both *C. polylepis* clones was estimated using a Coulter Counter Multisizer II. From size measurements, cell volumes were calculated using a prolate spheroid and a sphere as a geometric shape for *O. marina* and *C. polylepis*, respectively. Algal cell concentrations of experimental and control flasks were estimated using a Coulter Counter Multisizer II, except for final counts for the two lowest food concentrations. In these samples, algal cells were counted microscopically as algal density were two low for a reliable estimation by Coulter Counter. Division rate, ingestion and clearance for the 48 h period were calculated using the equations of Frost (1972) and Heinbokel (1978). Apparent gross growth efficiency (aGGE) was calculated as the grazer biomass produced per unit algal biomass consumed using measured *C. polylepis* carbon values, calculated volume of *O. marina*, and a C:cell-volume conversion factor of 0.14 (Lessard 1991). All rates were plotted against initial food concentrations. To perform grazing experiments with a mixture of both clones, an attempt was made to stain *C. polylepis* with the green fluorescent vital stain CMFDA (5-chloromethylfluorescein diacetate) according to Li et al. (1996) with slight modifications. Dense *C. polylepis* cultures were stained with a final dye concentrations of 1 µM CMFDA for 3 h.

***Results:***

Both C. polylepis strains were tested for their toxicity to Artemia franciscana nauplii. No mortality of Artemia was observed for the seawater control, nor for the strain B11 up to the highest test concentration of $4 \times 10^5$ cells $ml^{-1}$. When exposed to strain B1511, however, mortality of Artemia was high, resulting in a LC $50_{24h}$ of $4.1 \times 10^3$ cells $ml^{-1}$ (Fig. 1). In contrast to this marked difference in toxicity, no obvious differences were detected in fatty

acid composition between the two clones. For both strains, 18:5 (ω3), 20:5 (ω3) and 22:6 (ω3) fatty acids were predominant (Table 1). With respect to lipid classes, again no obvious differences between the two clones could be detected (Table 2). Elemental analysis revealed slightly higher carbon and nitrogen content for the non-toxic clone B11 compared to the toxic clone B1511 (Tab. 3). Based on Coulter counter size measurement, the median size of the two C. polylepis clones was 6.65 µm and 6.31 µm, respectively. Due to these differences in size, the calculated ratio of carbon to volume was nearly the same for both clones (Tab. 3).

After inoculating starved *O. marina* with *C. polylepis* cultures, the dinoflagellate immediately started to ingest algal cells. The time-series of estimates of the percentage of *O. marina* that did not ingest algal cells, however, shows clear differences between the two *C. polylepis* clones (Fig. 2). The numbers of *Oxyrrhis* feeding on the non-toxic clone B11 increased sharply with time (i.e., the probability of having no ingested prey decreased), whereas ingestion of the toxic clone B1511 is much lower. Calculation of the grazing rates from a probability of zero curve ($P_{0(t)} = (1\text{-}z)\ e^{-\lambda t} + z$ ) revealed λ as the grazing rate to be 0.55 and 0.15 cells grazer$^{-1}$ h$^{-1}$ for the non-toxic and toxic clone, respectively. The percentage of *O. marina* not feeding at all (Z), was estimated to be about 5 % and 10 % for the non-toxic and toxic clones, respectively. Reduced grazing on the toxic clone is also evident when comparing the numbers of ingested cells after 22 and 46 h of incubation (Fig. 3). Most *O. marina* cells feeding upon the toxic clone had ingested 1-5 algal cells, whereas few grazers were observed with 10 or more algal cells inside food vacuoles. In contrast, most of *O. marina* feeding upon the non-toxic clone had ingested 10-20 algal cells after 22 or 46 h of incubation. Whereas cell numbers in non-grazed control bottles of the non-toxic clone B11 increased exponentially with time ($\mu = 0.51\ d^{-1}$), grazing in the experimental bottles led to a strong decline of algal numbers, to about $10^3$ cells ml$^{-1}$ (Fig. 4). Growth rate of the toxic clone B1511 in non-grazed control bottles ($\mu = 0.40\ d^{-1}$) was comparable to the growth rate of the non-toxic clone. For B1511, however, growth rate in the presence of *O. marina* as grazer was only slightly depressed ($\mu = 0.33\ d^{-1}$, Fig. 4A). Growth curves of *O. marina* are presented in Fig. 5. Cell numbers of *O. marina* increased exponentially during the entire period in both treatments. However, growth rate of *O. marina* with the toxic strain as food ($\mu = 0.22\ d^{-1}$) was obviously lower compared to the growth rate with the non-toxic clone ($\mu = 0.38\ d^{-1}$). Under identical incubation conditions *O. marina* reached a growth rate of $\mu = 0.45\ d^{-1}$ when *Dunaliella* sp. was offered as food algae (data not shown).

In the 48h incubation experiment using different initial *C. polylepis* concentrations, phytoplankton in control bottles increased on average to 143 % in respect of the start concentration. In experimental bottles of the toxic clone B1511, final algal cell concentration accounted for 135 % of the start concentration. In contrast, the non-toxic clone B11 was reduced to 1 %, 14% and 75% of the start concentration for the three lowest algal concentrations (20 – 100 x $10^3$ cells $ml^{-1}$), respectively, and remained nearly constant for the two highest concentrations (data not shown).

Ingestion was estimated using two different approaches. The initial grazing rates of starved *O. marina* , as estimated by fluorescence microscopy, are presented in Fig. 6. For both *C. polylepis* clones, ingestion rate of *O. marina* increased with increasing food concentration until it became saturated at about 100 x $10^3$ cells $ml^{-1}$. However, both the initial increase, as well as the highest ingestion rate at food saturation, were obviously lower when fed with the toxic clone B1511. Ingestion rates calculated using the decline of food cells during the 48 h incubation period showed the same pattern (Fig. 7A), although values are generally lower by a factor of about two. Clearance calculated for *O. marina* feeding on the non-toxic clone of *C. polylepis* showed a typical pattern, increasing at low food concentration up to 102 nl $grazer^{-1}$ $h^{-1}$. When fed with the toxic clone, calculated clearance rates of 0.4 – 7 nl $grazer^{-1}$ $h^{-1}$ remained remarkably low for all food concentrations tested (Fig. 7B). Maximum specific division rates of *O. marina* were 0.46 and 0.27 $d^{-1}$ for the non-toxic and toxic clone, respectively. However, with both algal clones as food, division rate of *O.marina* was almost constant over the whole range of different food concentrations (Fig. 8A). This is mainly because of size differences of *O. marina* in the different treatments (data not shown). When growth is calculated on the basis of an increase in total volume of *O. marina* (Fig. 8 B), both curves show a typical increase of specific growth rate with increasing food concentration. However, volume-specific growth rate is explicitly higher compared to division rate based on the increase of cell numbers, probably reflecting an overall increase of cell volume of starved *O. marina* as a consequence of food supply. Based on the calculated biomass increase of *O. marina* and total algal carbon ingested, the apparent gross growth efficiency (aGGE) was calculated (Fig. 9). When plotted for food concentration up to 200 x $10^3$ $ml^{-1}$, as numbers calculated for the highest food concentration were erratically high, aGGE for the non-toxic clone B11, ranged from 0.16 to 0.45 had low standard deviations and was relatively constant for higher algal concentrations. For the toxic clone B1511, however, the calculation yielded highly scattered aGGE values predominantly above the theoretical maximum value of 1 (see discussion).

Grazing experiments using a mixture of both clones as prey, one of them stained with the live stain CMFDA, were unsuccessful. As frequently observed under the microscope, intense staining of *C. polylepis*, concentrated in the posterior part of the cell, was lost spontaneously, probably due to leakage or excretion. Hence, a reliable application of this technique was not possible.

***Discussion***

In the present study, the feeding efficiency of *Oxyrrhis marina* on two different clones of *C. polylepis* was analyzed. The basic assumption for the interpretation of our grazing experiments is that, from the grazers perspective, both clones are virtually identical except for toxicity. In fact, the two clones representing different cell types (see Material & Methods) at least differ slightly with respect to the morphology of body scales (Paasche et al. 1990, Edvardsen and Paasche 1992). However, it is not known if these small morphological differences might influence feeding interaction, e.g., by prey recognition and/or handling. Among the main known prey features affecting ingestion are size and motility (e.g. Hansen et al. 1994, Buskey 1997, Tillmann and Reckermann 2002). Although motility was not explicitly quantified, qualitative microscopical observation revealed no basic differences between the two clones of *C. polylepis* with respect to swimming behavior and/or swimming speed. Size may play an important role for food selection of *O. marina* (Hansen et al. 1996). Initial flow cytometric analysis of Edvardsen & Paasche (1992) indicated size differences between both cell types with the alternate cell type about twice the size of the authentic cell type. For the two clones used in the present study, size measurements using a Coulter Counter revealed only minor differences in median cell size (Table 3) and, hence, in calculated cell volume. Hansen et al. (1996) observed a shift in the size spectrum of a single food algae as a result of *O. marina* grazing activity and interpreted this as size-selective grazing even within a small size range. However, it is well known that protistan grazers release fecal particles (Nöthig and Bodungen 1989, Elbrächter 1991, Tillmann and Reckermann 2002) that may overlap in size with their algal food. Particle production may thus confound results of experiments using a Coulter Counter, with respect to particle size selectivity (Stoecker 1984). We therefore argue that the rather small differences in size between the two *C. polylepis* clones are unlikely to be the cause of the large differences in grazing rate of *O. marina*.

The crucial difference between the two clones tested is their toxicity to nauplii of *Artemia franciscana*. This is in accordance with results of Edvardsen & Paasche (1992), who found a culture containing 90% alternate cells to be only slightly toxic to *Artemia* nauplii (24-h $LC_{50} > 350 \times 10^3$ cells $ml^{-1}$), whereas 24-h $LC_{50}$ of cultures containing 100% authentic cells was about $2.6 \times 10^3$ cells $ml^{-1}$. A culture apparently containing 100% alternate cells appeared non-toxic to *Artemia* at concentrations up to $100 \times 10^3$ $ml^{-1}$, whereas authentic cells had a 24h-LC50 of $6.9 \times 10^3$ $ml^{-1}$ (Edvardsen 1993). This is close to our estimate of toxicity of clone B1511 with a 24-h $LC_{50}$ of $4.1 \times 10^3$ cells $ml^{-1}$, which is also within the range estimated by Simonsen & Moestrup (1997). The differences in toxin content/production between the two *C. polylepis* clones used in our experiments are probably the cause of the small differences in cellular nitrogen content and growth rate (Table 3), which are unlikely to be the cause of the large differences in grazing rate of *O. marina*. In summary it is reasonable to conclude that both strains are nearly identical except for their toxicity, implying that clone-specific differences in grazing and growth of the predator are causatively linked to toxicity effects.

Profound quantitative differences in grazing and growth of *O. marina* when fed the two clones of *C. polylepis* were apparent in all experiments (Exp. 1: Fig. 2, 3 and 5; Exp. 2: Fig. 6-8). However, the shape of the numerical- and functional response curves for the two clones was quite similar. The growth rate of *O. marina* was never inhibited with increasing concentrations of the toxic clone B1511, even up to concentrations of $4 \times 10^5$ cells $ml^{-1}$ (Fig. 8). Therefore the negative effects to the grazers are not related to the algal concentration and hence not to the concentration of a putative toxin released into the water. Rather, grazing inhibition seems to be related to each single feeding process; *O. marina* apparently avoided ingestion of the toxic clone. This might be because of reception of some toxin-related properties of the algal cell, e.g. olfactory or glutatory factors, as chemosensory capabilities are well known among heterotrophic dinoflagellates (Hauser et al. 1975, Spero 1985, Buskey 1997). Nevertheless, ingestion of toxic cells was not totally suppressed. No other particulate food was available and *O. marina* was starved prior to the experiments. Starved *O.marina* have been shown to be less selective (Tarran 1991), therefore this conditions might have been acute enough to permit limited grazing. There is no evidence that toxic cells, after being ingested, caused grazing inhibition or substantial harm to the grazer. The number of grazers having ingested prey cells after only 30 min of incubation was obviously lower when incubated with the toxic clone B1511 (Fig. 2), therefore it is unlikely that internal toxicity was

invloved. Moreover, although the number of ingested toxic cells increased three-fold with increasing food concentrations (Fig. 7), the corresponding volume-specific growth rate did not decrease, as would be expected as a consequence of lethal effects of incorporated toxins.

From an ecological point of view, it is a important to distinguish whether or not toxic algae are avoided by a predator, or if the predator is eliminated by toxins. In the former case, the predator is able to continue feeding on other co-existing algal species, thereby releasing the toxic species from competition. In addition, grazing on other algae and subsequent DOC release (Strom et al. 1997) might stimulate the growth of bacteria, which in turn can be taken up by mixotrophic algae (Jones et al. 1993, Nygaard and Tobiesen 1993). In the latter case, elimination of grazers is also removes a constraint on competing algal species, making it more difficult to explain the formation of monospecific blooms. The results in the present study clearly showed that *O. marina* was not killed by the presence or by the ingestion of toxic *C. polylepis*. This is in accord with results of Carlsson et al. (1990) and Nielsen et al. (1990), who showed that mortality of the tintinnid *Favella ehrenbergii* upon addition of *C. polylepis* came close to but never surpassed the population decline resulting from simple starvation. Growth rate of the heliozoan *Heterophrys marina* decreased at increasing *C. polylepis* concentrations approaching zero at concentrations above 75 x $10^3$ cells $ml^{-1}$ (Tobiesen 1991). However, *H. marina* cells survived and regained normal growth after transfer into fresh medium. From our experiments using monoculture prey, it is impossible to state whether or not ingestion of other algae might be affected by the presence of toxic *C. polylepis*. Unfortunately, the attempt to perform grazing experiments with a mixture of both *C. polylepis* clones, one selectively stained with CMFDA, failed. It is important to note that growth of *O. marina* is less effected by the presence of toxic *C. polylepis* than ingestion. In experiment 1, ingestion rate of *O. marina* fed with the toxic clone reached 27 % of the rate estimated for *O. marina* feeding on the non-toxic clone, whereas the division rate of *O. marina* fed the toxic clone was 58 % of the division rate of *O. marina* fed the non-toxic clone. The corresponding numbers for experiment 2 (mean for all food concentrations) were 30 % and 54 % for ingestion and division, respectively. The calculated numbers of the apparent gross growth efficiency for *O. marina* feeding on the toxic clone above the theoretical maximum of 1 (Fig. 9) provite evidence that *O. marina* mixed with the toxic clone may have used clumps of bacteria and detritus or dissolved organic substances present in the algal medium for growth. *Oxyrrhis* had been previously shown to be capable of growth in axenic medium, relying on osmotrophy to support growth (Droop 1959). Thus, in the presence of toxic *C. polylepis*, *O.*

*marina* seems to be able to switch to an alternate food source. Experiments using *Favella ehrenbergii* fed with mixed algal cultures indicated that ingestion by the ciliate is generally suppressed by the presence of high concentrations of *C. polylepis* (Carlsson et al. 1990, Nielsen et al. 1990). For copepods, Nielsen et al. (1990) observed no negative effect with *Acartia tonsa* feeding on palatable cells in experiments with mixtures of the rhodophyte *Rhodomonas baltica* and different concentrations of cultivated *C. polylepis*. When incubated with natural populations of *C. polylepis* from the 1988 bloom, however, the mortality rate of copepods significantly increased.

Clearly, more experiments using mixed algal cultures are needed to address the question of whether or not grazers are eliminated or merely deterred the *C. polylepis* toxins. In addition to reduction of grazing pressure, *C. polylepis* toxin may directly effect other algae as well. Whereas Riegman et al. (1996) ascribed the success of *C. polylepis* in multispecies culture experiments to the ability of *C. polylepis* to outcompete other algae, Schmidt & Hansen (2001) recently showed that toxins released by *C. polylepis* had a direct harmful effect on a whole range of tested algae in mixed cultures. This allelochemical activity, in addition to a reduced grazing pressure, may explain why a bloom like the one in 1988 in the North Sea became essentially monospecific at high cell concentrations. According to Schmidt & Hansen (2001), all but one autotrophic dinoflagellate tested were immobilized at *C. polylepis* concentrations of $1.9 \times 10^5$ cells $ml^{-1}$. Although heterotrophic dinoflagellates might be expect to respond in a similar way, no signs of immobilisation of *O. marina* could be detected in our experiments. The dinoflagellate *O. marina* might be particularly resistant against *C. polylepis* toxin, although it has been shown to be rapidly immobilized by *Alexandrium* toxins (Tillmann and John 2002). However, it is well known that different strains of *C. polylepis* may vary considerably with respect to their toxicity (Edvardsen and Paasche 1998), which may be regulated by phosphorous deficiency (Edvardsen et al. 1990, Edvardsen 1993), cellular N:P ratio (Johansson and Granéli 1999), as well as growth phase and pH (Schmidt and Hansen 2001). As Schmidt & Hansen did not perform *Artemia* tests, therefore results can not be directely compared and differences in *C. polylepis* toxicity therefore might explain the different reaction of autotrophic and the heterotrophic dinoflagellates.

To date, toxicity of *C. polylepis* has been defined towards a wide range of different bioassays, including haemolytic tests (e.g. Edvardsen et al. 1990, Eschbach et al. 2001), the standard *Artemia* test (Edvardsen 1993), toxicity towards rat hepatocytes (Underdahl et al.

1989), the inhibition of the uptake of neutrotransmitters into synaptosomes and synaptic vesicles of rat brain (Meldahl et al. 1994), the immobilisation effect on *Heterocapsa triquetra* (Schmidt and Hansen 2001), or various negative effects towards a number of zooplankton grazers (Carlsson et al. 1990, Tobiesen 1991). However, there is a clear need to chemically identify the toxic principles of *C. polylepis*. Thus far, screening for bioactive compounds in *C. polylepis* suggests that toxicity could be related to certain lipid classes or fatty acids. Based on chromatographic and mass spectral data of isolated haemolytic compounds, Yasumoto et al. (1990) suggested monoacyldigalactosylglycerol and octadecapentaenoic acid, are implicated in the toxicity of *C. polylepis*. However, haemolytic compounds are quite common in several algal species without any substantial correlation between haemolytic activity and toxicity in other bioassays (Yasumoto et al. 1987, Simonsen and Moestrup 1997). Simonsen & Moestrup (1997) detected several haemolytic compounds in *C. polylepis*, but only one spot was toxic to *Artemia*. These authors suggested that this substance from *C. polylepis* was characteristic of a liposaccharide. In our study, however, we found no obvious differences in the lipid classes and/ or fatty acid composition between the toxic and non-toxic clones (Table 1 and 2), suggesting that other compounds are likely responsible for the *Artemia* toxicity and grazer deterrent properties of *C. polylepis*. There are suggestions in the literature that *C. polylepis* produces toxins without hemolytic properties in addition to the haemolytic ones (Stabell et al. 1993). Thus, *C. polylepis* toxins might be a multiple group of unrelated compounds (Underdahl et al. 1989), probably causing different toxic effects. Clearly, detailed comparative studies have to be carried out between the various bioassays for each purified toxic component in question.

Using a toxic and a non-toxic clone of a single species is a powerful tool for comparative studies of the ecological effect and of chemical properties of toxic algae. In this study, we demonstrated a clear effect of the toxic *C. polylepis* clone upon a microzooplankton grazer. The chemical composition with respect to lipid classes and fatty acids provided no hint for an involvement of these substances in the toxicity towards *Artemia* and *O. marina*. In future, we will use this model of comparative studies for further chemical analysis to clarify the chemical identity of *C. polylepis* toxins.

***Acknowledgment:***

Special thanks are due to Bente Edvarsen for providing the two clones of *C. polylepis*. Thanks to Martin Graeve for his technical support in fatty acid and lipid analysis. This work was supported by the German BMBF (TEPS 03F0161) and the European Commission (Research Directorate General-Environment Programme-Marine Ecosystems) through the BIOHAB project "Biological control of Harmful Algal Blooms in European coastal waters: role of eutrophication " (contract EVK3-CT99-00015). The BIOHAB project is part of the EC EUROHAB cluster.

***Literature:***

Bratvold, D., Scienc, F., Taub, S.R., 2000. Analysis of the distribution of ingested bacteria in nanoflagellates and estimates of grazing rates with flow cytometry. Aquat. Microb. Ecol. 21, 1-12.

Buskey, E.J., 1997. Behavioral components of feeding selectivity of the heterotrophic dinoflagellate *Protoperidinium pellucidum*. Mar. Ecol. Prog. Ser. 153, 77-89.

Carlsson, P., Granéli, E., Olsson, P., 1990. Grazer elimination through poisoning: one of the mechanisms behind *Chrysochromulina polylepis* blooms? In: Granéli, E., Sundström, B., Edler, L.,Anderson, D.M. (Eds.), Toxic Marine Phytoplankton. Elsevier, New York, pp. 116-122.

Dahl, E., Lindahl, O., Paasche, E., Throndsen, J., 1989. The *Chrysochromulina polylepis* bloom in Scandinavian waters during spring 1988. In: Cosper, E.M., Bricelj, V.M.,Carpenter, E.J. (Eds.), Novel Phytoplankton Blooms: causes and impacts of recurrent brown tides and other unusual blooms. Springer Verlag, Berlin, pp. 383-405.

Droop, M.R., 1959. Water soluble compounds in the nutrition of *Oxyrrhis marina*. J. Mar. Biol. Ass. UK 38, 605-620.

Edvardsen, B., 1993. Toxicity of *Chrysochromulina* species (Prymnesiophyceae) to the brine shrimp, *Artemia salina*. In: Smayda, T.J.,Shimizu, Y. (Eds.), Toxic Phytoplankton Bloom in the Sea. Elsevier Science Publisher B.V., pp. 681-686.

Edvardsen, B., Medlin, L., 1998. Genetic analyses of authentic and alternate forms of *Chrysochromulina polylepis* (Haptophyta). Phycologia 37, 275-283.

Edvardsen, B., Moy, F., Paasche, E., 1990. Hemolytic activity in extracts of *Chrysochromulina polylepis* grown at different levels of selenite and phosphate. In: Granéli, E., Sundström, B., Edler, L.,Anderson, D.M. (Eds.), Toxic Marine Phytoplankton. Elsevier, New York, pp. 284-289.

Edvardsen, B., Paasche, E., 1992. Two motile stages of *Chrysochromulina polylepis* (Prymnesiophyceae): morphology, growth, and toxicity. J. Phycol. 28, 104-114.

Edvardsen, B., Paasche, E., 1998. Bloom dynamics and physiology of *Prymnesium* and *Chrysochromulina*. In: Anderson, D.M., Cembella, A.D.,Hallegraeff, G.M. (Eds.), Physiological Ecology of Harmful Algae Blooms. Springer Verlag, Belin/Heidelberg/New York, pp. 193-208.

Edvardsen, B., Vaulot, D., 1996. Ploidy analysis of the two motile forms of *Chrysochromulina polylepis* (Prymnesiophyceae). J. Phycol. 32, 94-102.

Elbrächter, M., 1991. Faeces production by dinoflagellates and other small flagellates. Mar. Microb. Food Webs 5, 189-204.

Eppley, R.W., Holmes, R.W., Strickland, J.D.H., 1967. Sinking rates of marine phytoplankton measured with a fluorometer. J. Exp. Mar. Biol. Ecol. 1, 191-208.

Eschbach, E., Scharsack, J., John, U., Medlin, L.K., 2001. Improved erythrocyte lysis assay in microtitre plates for sensitive detection and efficient measurement of haemolytic compounds from ichthyotoxic algae. J. Appl. Toxicol. 21, 513-519.

Frost, B.W., 1972. Effects of size and concentration of food particles on the feeding behavior of the marine planktonic copepod *Calanus pacificus*. Limnol. Oceanogr. 17, 805-815.

Gjøsæter, J., Lekve, K., Stenseth, N.C., Leinaas, H.P., Christie, H., Dahl, E., Danielssen, D.S., Edvardsen, B., Olsgard, F., Oug, E., Paasche, E., 2000. A long-term perspective on the *Chrysochromulina* bloom on the Norwegian Skagerrak coast 1988: a catastrophe or an innocent incident? Mar. Ecol. Prog. Ser. 207, 201-218.

Granéli, E., Paasche, E., Maestrini, S.Y., 1993. Three years after the *Chrysochromulina polylepis* bloom in Scandinavian waters in 1988: some conclusions of recent research and monitoring. In: Smayda, T.J.,Shimizu, Y. (Eds.), Toxic Phytoplankton Bloom in the Sea. Elsevier Science Publisher B.V., pp. 23-32.

Hansen, B., Bjornsen, P.K., Hansen, P.J., 1994. The size ration between planktonic predators and their prey. Limnol. Oceanogr. 39, 395-403.

Hansen, F.C., Witte, H.J., Passarge, J., 1996. Grazing in the heterotrophic dinoflagellate *Oxyrrhis marina*: size selectivity and the preference for calcified *Emiliania huxlyi* cells. Aquat. Microb. Ecol. 10, 307-313.

Hansen, P.J., 1992. Prey size selection, feeding rates and growth dynamics of heterotrophic dinoflagellates with special emphasis on *Gyrodinium spirale*. Mar. Biol. 114, 327-334.

Hauser, D.C.R., Levandowski, M., Hunter, S.H., Chunosoff, L., Hollwitz, J.S., 1975. Chemosensory responses by the heterotrophic dinoflagellate, *Crypthecodinium cohnii*. Microb. Ecol. 1, 246-254.

Heinbokel, J.F., 1978. Studies on the functional role of tintinnids in the southern California Bight. I. Grazing and growth rates in laboratory cultures. Mar. Biol. 47, 177-189.

Hewlett, P.S., Placklet, R.L., 1979. The probit and similar transformation. In: An introduction to the interpretation of quantal response in biology. Edward Arnold Ltd., London, pp. 12-17.

Johansson, N., Granéli, E., 1999. Cell density, chemical composition and toxicity of *Chrysochromulina polylepis* (Haptophyta) in relation to different N:P supply ratios. Mar. Biol. 135, 209-217.

Johnsen, T.M., Lomsland, E.R., 1990. The culmination of the *Chrysochromulina polylepis* (Manton & Parke) bloom along the western coast of Norway. In: Granéli, E., Sundström, B., Edler, L.,Anderson, D.M. (Eds.), Toxic Marine Phytoplankton. Elsevier, New York, pp. 177-182.

Jones, H.L.J., Leadbeater, B.S.C., Green, J.C., 1993. Mixotrophy in marine species of *Chrysochromulina* (Prymnesiophyceae): Ingestion and digestion of a small green flagellate. J. Mar. Biol. Ass. U.K. 73, 283-296.

Kattner, G., Fricke, H.S.G., 1986. Simple gas-liquid chromatographic method for the simultaneous determination of fatty acids and alcohols in wax esters of marine organisms. Journal of Chromatography 361, 263-268.

Lessard, E.J., 1991. The trophic role of heterotrophic dinoflagellates in diverse marine environments. Mar. Microb. Food Webs 5, 49-58.

Li, A., Stoecker, D.K., Coats, D.W., Adam, E.J., 1996. Ingestion of fluorescently labeled and phycoerythrin-containing prey by mixotrophic dinoflagellates. Aquat. Microb. Ecol. 10, 139-147.

Maestrini, S.Y., Granéli, E., 1991. Environmental conditions and ecophysiological mechanisms which led to the 1988 *Chrysochromulina polylepis* bloom: an hypothesis. Oceanol. Acta 14, 397-413.

Manton, I., Parke, M., 1962. Preliminary observations on scales and their mode of origin in *Chrysochromulina polylepis* sp. nov. J. Mar. Biol. Ass. U.K. 42,

Meldahl, A.S., Edvardsen, B., Fonnum, F., 1994. Toxicity of four potentially ichthyotoxic marine phytoflagellates determined by four different test methods. J. Toxicol. Environ. Health 42, 289-301.

Myklestad, M.M., Ramlo, B., Hestmann, S., 1995. Demonstration of strong interaction between the flagellate *Chrysochromulina polylepis* (Prymnesiophyceae) and a marine diatom. In: Lassus, P., Arzul, G., Gentien, P.,Marcaillou, C. (Eds.), Harmful Marine Algal Blooms. Lavoisier, Intercept Ltd., pp. 633-638.

Nielsen, T.G., Kiorboe, T., Bjornsen, P.K., 1990. Effects of a *Chrysochromulina polylepis* subsurface bloom on the planktonic community. Mar. Ecol. Prog. Ser. 62, 21-35.

Nöthig, E.-M., Bodungen, B.v., 1989. Occurence and vertical flux of faecal pellets of probably protozoan origin in the southeastern Weddel Sea (Antartica). Mar. Ecol. Pog. Ser. 56, 281-289.

Nygaard, K., Tobiesen, A., 1993. Bacterivory in algae: a survival strategy during nutrient limitation. Limnol. Oceanogr. 38, 273-279.

Olsen, R.E., Henderson, R.J., 1989. The rapid analysis of neutral and polar marine lipids using double-development HPTLC and scanning densitometry. J. Exp. Mar. Biol. Ecol. 129, 189-197.

Paasche, E., Edvardsen, B., Eikrem, W., 1990. A possible alternate stage in the life cycle of *Chrysochromulina polylepis* Manton et Parke (Prymnesiophyceae). Nova Hedwigia Beih. 100, 91-99.

Riegman, R., de Boer, M., de Senerpont Domis, L., 1996. Growth of harmful marine algae in multispecies cultures. J. Plankton Res. 18, 1851-1866.

Rosenberg, R., Lindahl, O., Blanck, H., 1988. Silent Spring in the Sea. Ambio 17, 289-290.

Schmidt, L.E., Hansen, P.J., 2001. Allelopathy in the prymnesiophyte *Chrysochromulina polylepis*: effect of cell concentration, growth phase and pH. Mar. Ecol. Prog. Ser. 216, 67-81.

Simonsen, S., Moestrup, O., 1997. Toxicity tests in eight species of *Chrysochromulina* (Haptophyta). Can. J. Bot. 75, 129-136.

Smayda, T.J., 1997. Harmful algal blooms: Their ecophysiology and general relevance to phytoplankton blooms in the sea. Limnol. Oceanogr. 42, 1137-1153.

Spero, H.J., 1985. Chemosensory capabilities in the phagotrophic dinoflagellate *Gymnodinium fungiforme*. J. Phycol. 21, 181-184.

Stabell, O.B., Pedersen, K., Aune, T., 1993. Detection and separation of toxins accumulated by mussels during the 1988 bloom of *Chrysochromulina polylepis* in Norwegian coastal waters. Marine Environmental Research 36, 185-196.

Stoecker, D.K., 1984. Particle production by planktonic ciliates. Limnol. Oceanogr. 29, 930-940.

Strom, S.L., Benner, R., Ziegler, S., Dagg, M.J., 1997. Planktonic grazers are a potentially important source of marine dissolved organic carbon. Limnol. Oceanogr. 42, 1364-1374.

Tarran, G., 1991, Aspects of the grazing behaviour of the marine dinoflagellate *Oxyrrhis marina*, Dujardin. Pages 188. *University of Southampton*.

Teegarden, G.J., 1999. Copepod grazing selection and particle discrimination on the basis of PSP toxin content. Mar. Ecol. Prog. Ser. 181, 163-176.

Tillmann, U., John, U., 2002. Toxic effects of *Alexandrium* spp. on heterotrophic dinoflagellates: an allelochemical defence mechanism independent of PSP toxins. Mar. Ecol. Prog. Ser. in press.

Tillmann, U., Reckermann, M., 2002. Dinoflagellate grazing on the raphidophyte *Fibrocapsa japonica*. Aquat. Microb. Ecol. 26, 247-257.

Tobiesen, A., 1991. Growth rates of *Heterophrys marina* (Heliozoa) on *Chrysochromulina polylepis* (Prymnesiophyceae). Ophelia 33, 205-212.

Underdahl, B., Skulberg, O.M., Dahl, E., Aune, T., 1989. Disastrous bloom of *Chrysochromulina polylepis* (Prymnesiophyceae) in Norwegian coastal waters 1988. - Mortality in marine biota. Ambio 18, 265-270.

Yasumoto, T., Seino, N., Murakami, Y., Murata, M., 1987. Toxins produced by benthic dinoflagellates. Biol. Bull. 172, 128-131.

Yasumoto, T., Underdahl, B., Aune, T., Hormazabal, V., Skulberg, O.M., 1990. Screening for hemolytic activity and ichtyotoxic components of *Chrysochromulina polylepis* and *Gyrodinium aureolum* from Norwegian coastal waters. In: Granéli, E., Sundström, B., Edler, L.,Anderson, D.M. (Eds.), Toxic Marine Phytoplankton. Elsevier, New York, pp. 436-440.

Table 1. Fatty acid composition of the toxic clone B1511 and the non-toxic clone B11 of *C. polylepis*. Data represent triplicate mean ± 1SD.

| Fatty Acid | B11 (%) | B1511 (%) |
|---|---|---|
| 14:0 | 10.1 ± 0.7 | 12.2 ± 1.1 |
| 15:0 | 1.7 ± 0.3 | 1.6 ± 0.2 |
| 16:0 | 13.1 ± 1.1 | 11.2 ± 0.9 |
| 16:1 | 1.8 ± 0.1 | 3.1 ± 0.3 |
| 16:2 (n-6) | 1.2 ± 1 | 1.3 ± 1.0 |
| 16:3 (n-3) | 1.2 ± 0.1 | 1.2 ± 0.4 |
| 16:4 | 1.5 ± 0.2 | 1.3 ± 0.8 |
| 18:1 (n-9) | 3.4 ± 0.6 | 3.2 ± 0.5 |
| 18:1 (n-7) | 4.0 ± 0.3 | 5.3 ± 0.3 |
| 18:2 (n-6) | 1.3 ± 0.1 | 0.9 ± 0.5 |
| 18:4 (n-3) | 6.1 ± 1.2 | 8.5 ± 1.6 |
| 20:1 | 1.1 ± 0.1 | 1.0 ± 0.1 |
| 18:5 (n-3) | 18.6 ± 2 | 15.7 ± 3.6 |
| 20:4 (n-3) | 0.7 ± 0.01 | 1.9 ± 0.2 |
| 20:5 (n-3) | 18.8 ± 2.8 | 17.5 ± 3.2 |
| 22:1 (n-11) | 1.5 ± 0.4 | 1.3 ± 0.3 |
| 22:6 (n-3) | 14.1 ± 1.7 | 12.8 ± 2.1 |

Table 2: Lipid class composition (% total lipid) of the toxic clone B1511 and the non-toxic clone B11 of *C. polylepis* (n.d. = not detected).

| **Lipid classes** | **Abbr.** | **B11** | **B1511** |
|---|---|---|---|
| Phosphatidylcholine | PC | 43.4 % | 47.9 % |
| Phophatidylinositol | PI | 0.6 % | 1.3 % |
| Phosphatidylethanolamine/ Phosphatidylglycerol | PE/ PG | 3.9 % | 7.4 % |
| Diagalactosyldiacylglycerol | DGDG | 31.5 % | 27.4 % |
| Sulfoquinovosyldiacylglycerol | Sulf | n.d. | n.d. |
| Monogalactosyldiacylglycerol | MGDG | 6.5 % | 5.5 % |
| Triacylglycerol | TAQ | 14.1 % | 10.4 % |

Tab. 3 : Comparison of cellular properties of the toxic clone B1511 and the non-toxic clone B11 of *C. polylepis* (error terms represents standard deviation, n = 3)

| ***Chryschromulina polylepis*** | **B11** | **B1511** |
|---|---|---|
| toxicity to Artemia nauplii | no | yes |
| C-content (pg $cell^{-1}$) | 55.07 ± 0.85 | 48.87 ± 0.48 |
| N-content (pg $cell^{-1}$) | 7.81 ± 0.10 | 5.93 ± 0.03 |
| C:N (g:g) | 7.05 ± 0.03 | 8.24 ± 0.09 |
| cell volume ($\mu m^3$) | 153.9 | 131.6 |
| C/volume (pg $\mu m^{-3}$) | 0.36 | 0.37 |
| median cell size (µm) | 6.65 | 6.31 |
| division rate µ ($d^{-1}$) | 0.51 | 0.40 |

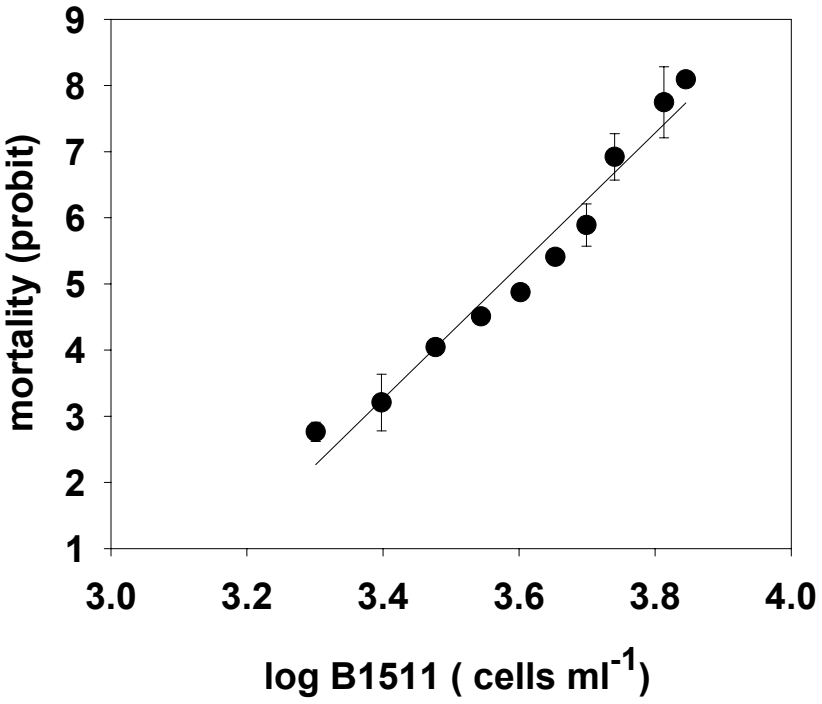



**Fig. 1:** Probit transformed percentage mortality of *Artemia franciscana* nauplii as a function of log cell concentration of *Chrysochromulina polylepis*, clone B1511. Data points represent triplicate mean ± 1SD. Probit value 5 represents 50 % mortality and corresponds to the 24h-$LC_{50}$ value of 4132 cells $ml^{-1}$ ($r^2 = 0.988$).

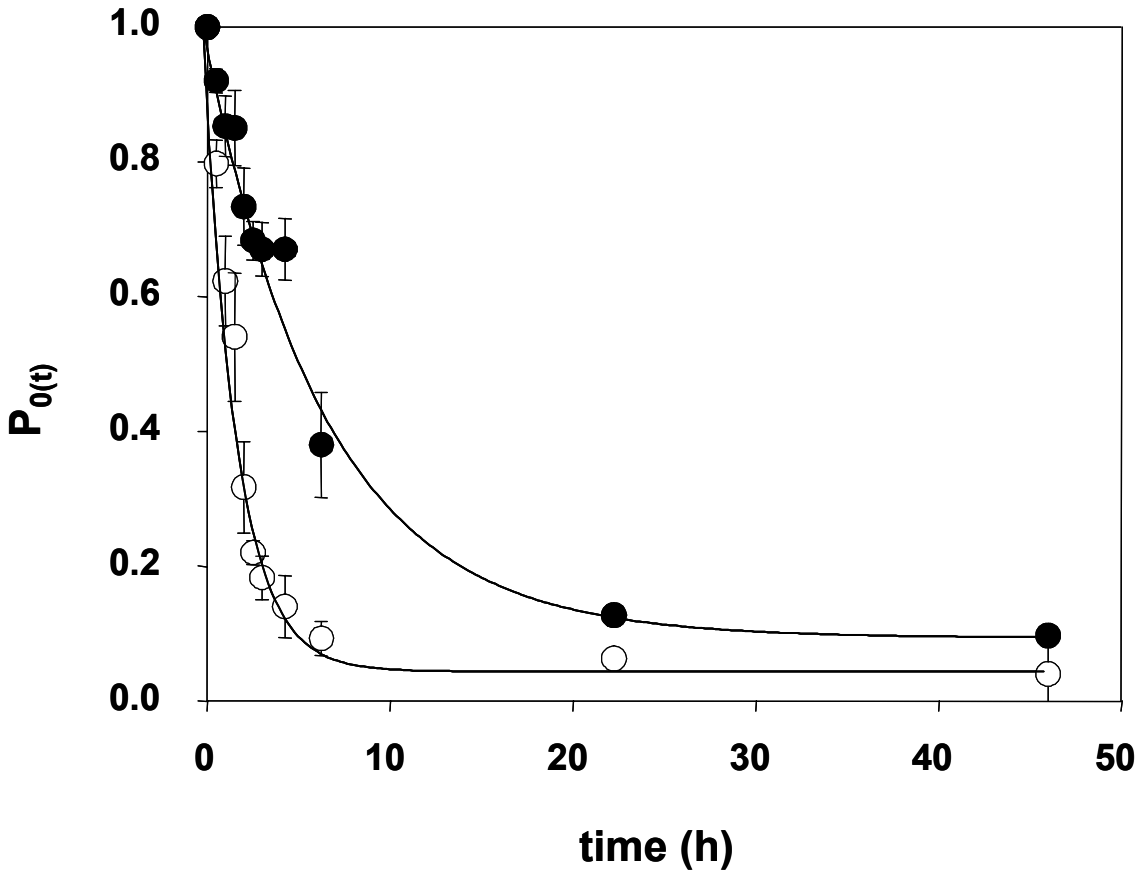



**Fig. 2:** Probability of *O. marina* having no ingested prey at time t when fed with the toxic clone B1511 (closed circles) or the non-toxic clone B11 (open circles) of *C. polylepis*. Data points represent triplicate mean ± 1SD. Data were fitted to the equation $P_{0(t)} = (1\text{-}z)\, e^{-\lambda t} + z$ (see Materials and Methods).

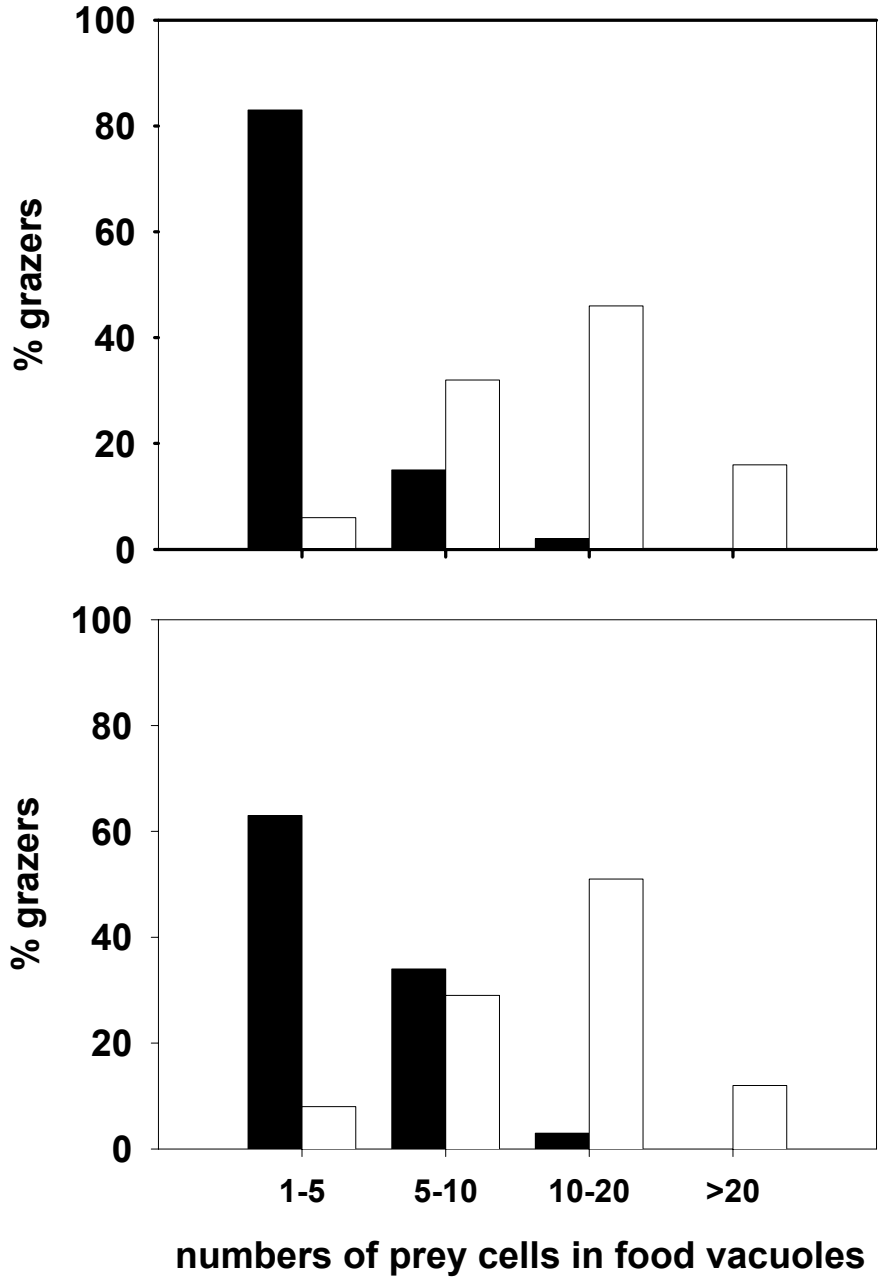



**Fig. 3:** Percentage distribution of *O. marina* with different numbers of ingested prey cells in food vacuoles when fed with the toxic clone B1511 (closed bars) or non-toxic clone (open bars) of *C. polylepis*. A: after 22 h of incubation. B: after 46 h of incubation.

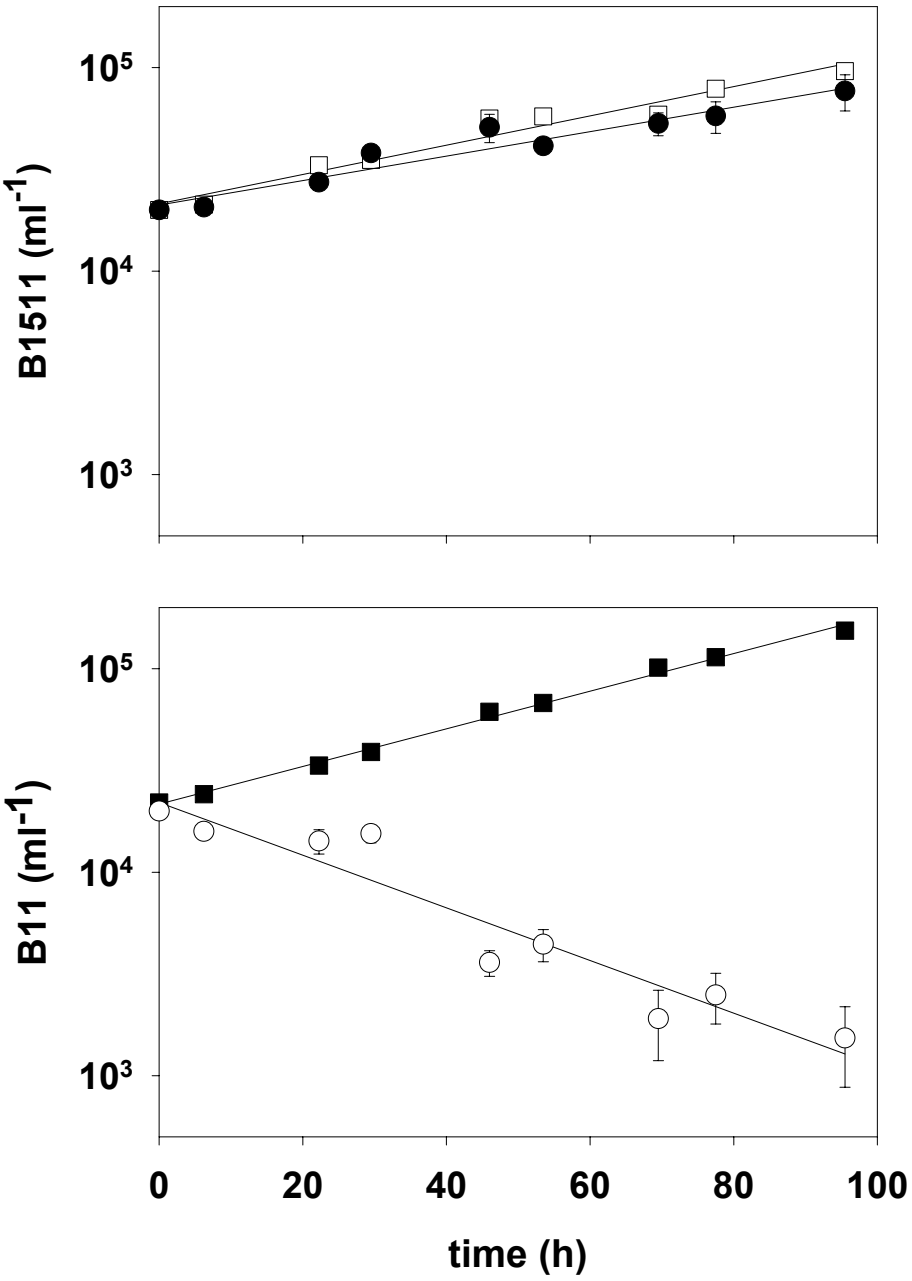



**Fig. 4:** Growth curves of *C. polylepis*. A: the toxic clone B1511 grown with *O. marina* (closed circles; $\mu = 0.33\ d^{-1}$, $r^2 = 0.931$) and grown in monoculture (open squares; $\mu = 0.40\ d^{-1}$, $r^2 = 0.959$). B: the non-toxic clone B11 grown with *O. marina* (open circles; $\mu = -0.72\ d^{-1}$, $r^2 = 0.911$) and grown in monoculture (closed squares; $\mu = 0.51\ d^{-1}$, $r^2 = 0.996$). Data points represent triplicate mean ± 1SD.

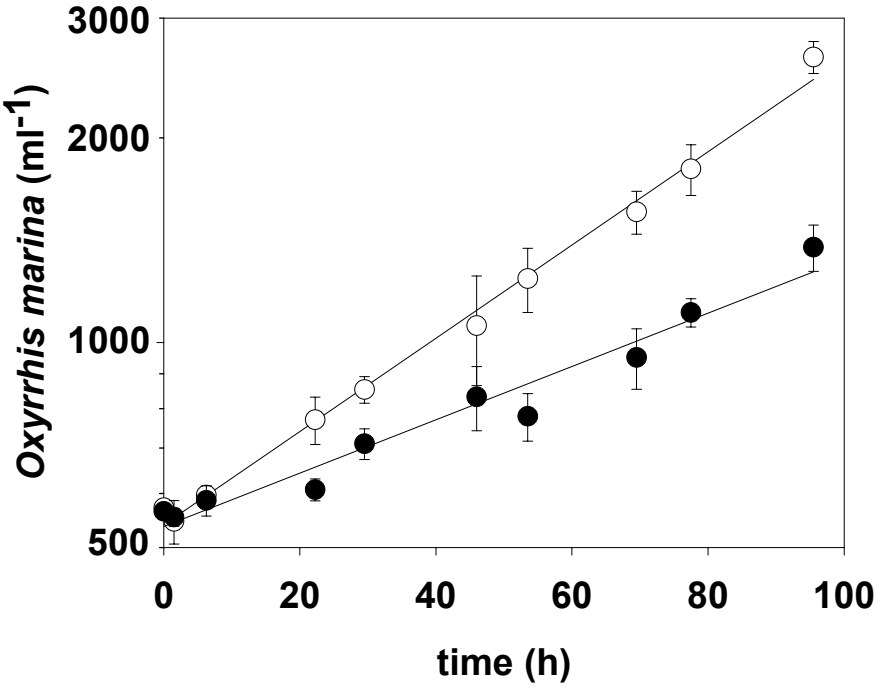



**Fig. 5:** Growth curves of *O. marina* when fed with the toxic clone B1511 (closed circles; $\mu$ = 0.22 $d^{-1}$, $r^2$ = 0.962) or the non-toxic clone B11 (open circles; $\mu$ = 0.38 $d^{-1}$, $r^2$ = 0.995) of *C. polylepis*. Data points represent triplicate mean ± 1 SD.

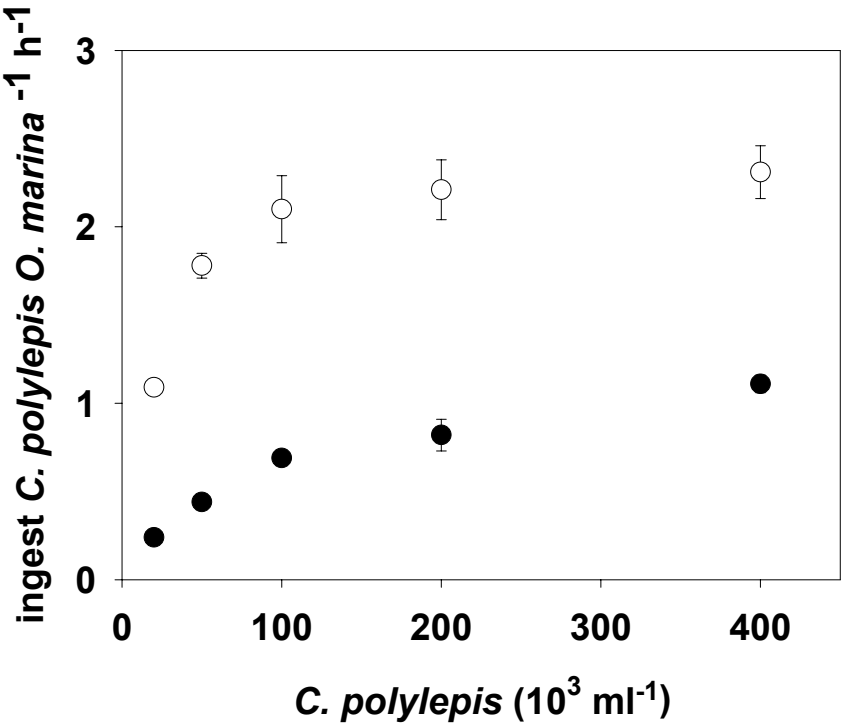



**Fig. 6:** Initial ingestion rate of *O. marina* (fluorescence counts of ingested algae after 2 h of incubation) as a function of initial food concentration when fed with the toxic clone B1511 (closed circles) or the non-toxic clone B11 (open circles). Data points represent triplicate mean ± 1SD.

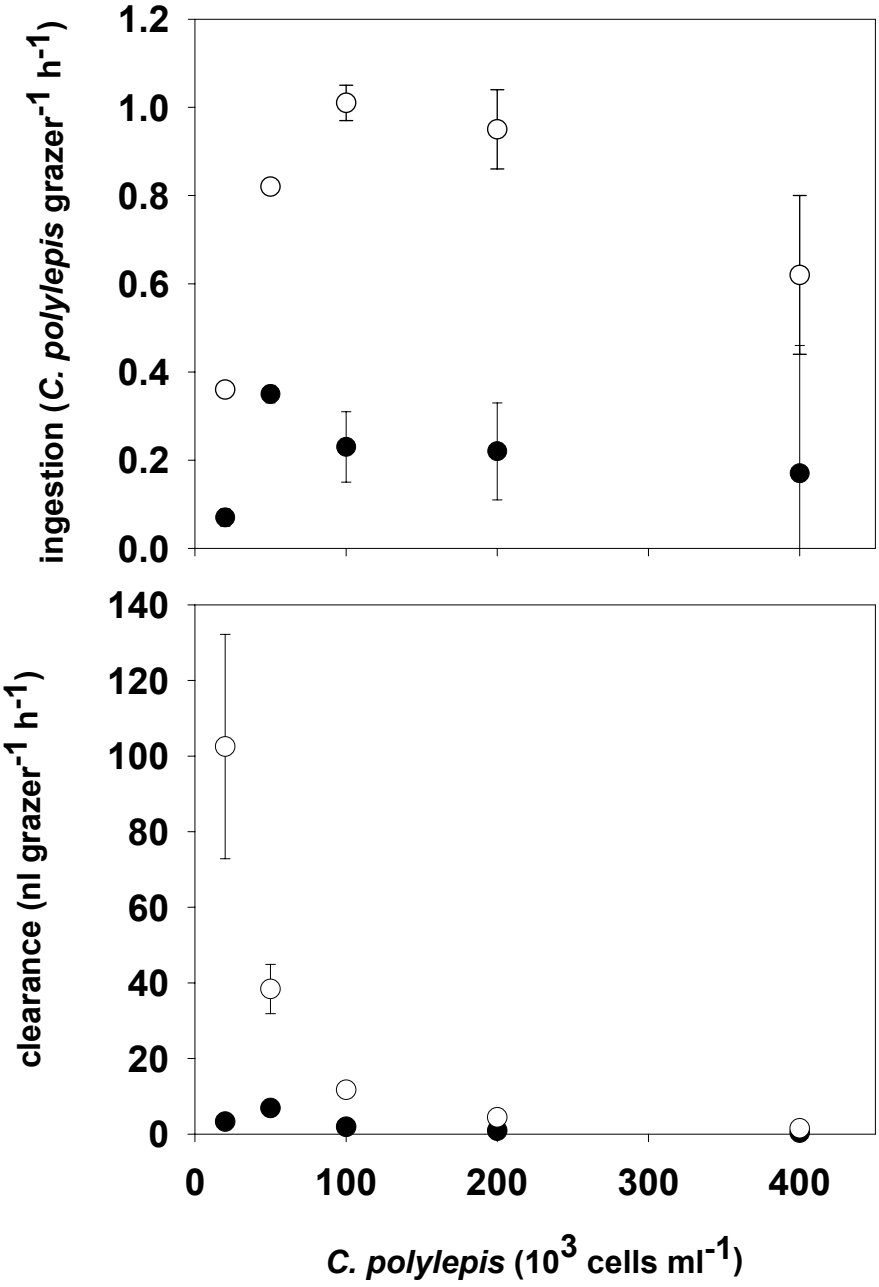



**Fig. 7:** Ingestion and clearance of *O. marina* as a function of initial food concentration, calculated according to Frost (1972) for a 48 h incubation period. Data points represent triplicate mean ± 1SD. A: Ingestion of *O. marina* when fed with the toxic clone B1511 (closed circles) or the non-toxic clone B11 (open circles). B: Clearance of *O. marina* when fed with the toxic clone B1511 (closed circles) or the non-toxic clone B11 (open circles).

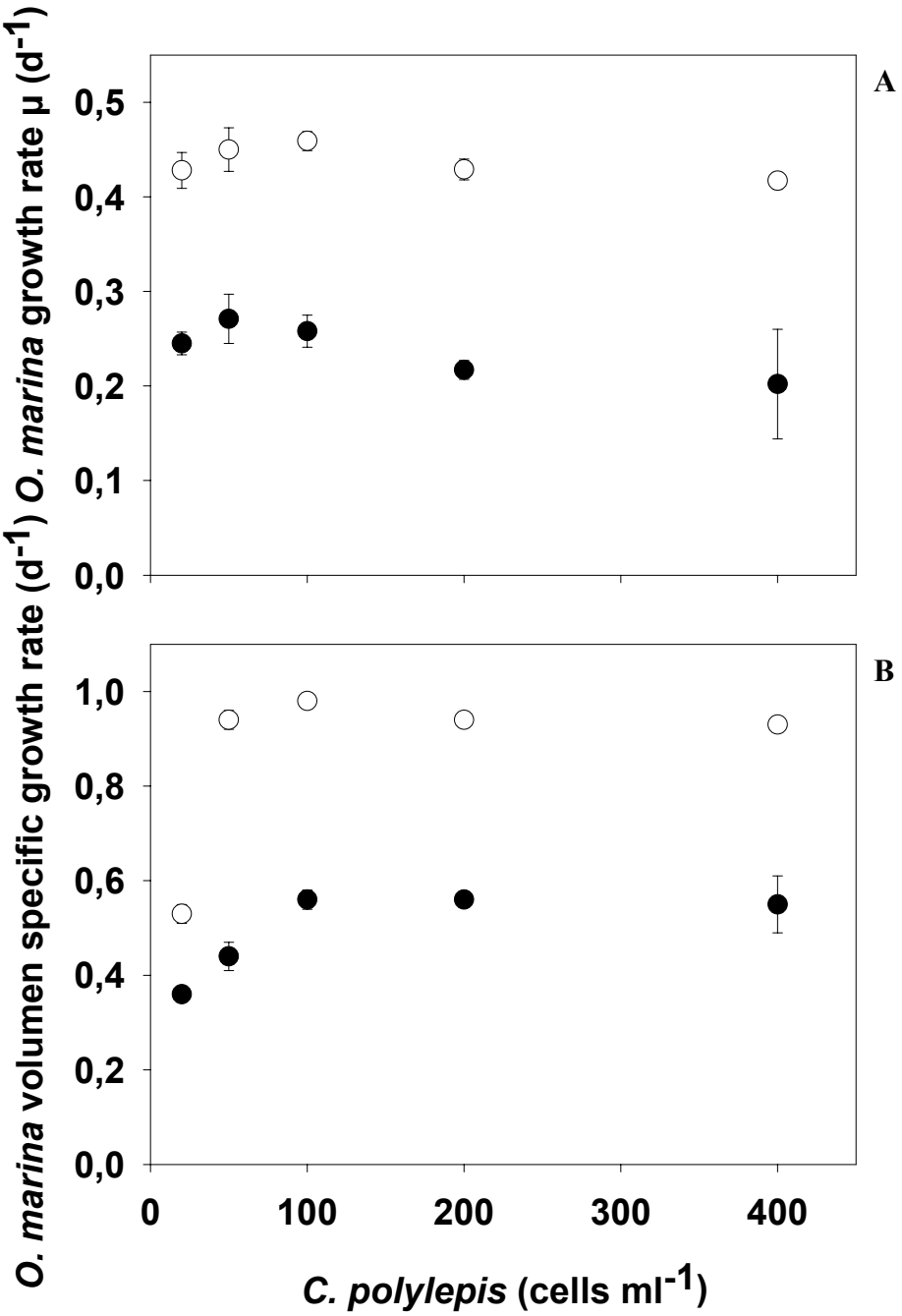



**Fig. 8:** Growth of *O. marina* as a function of initial food concentration when fed with the toxic clone B1511 (closed circles) or the non-toxic clone B11 (open circles). Data point represent triplicate mean ± 1SD. A: Division rate of *O. marina* calculated from the increment of cell numbers. B: Growth rate calculated from the increment of total *O. marina* cell volume.

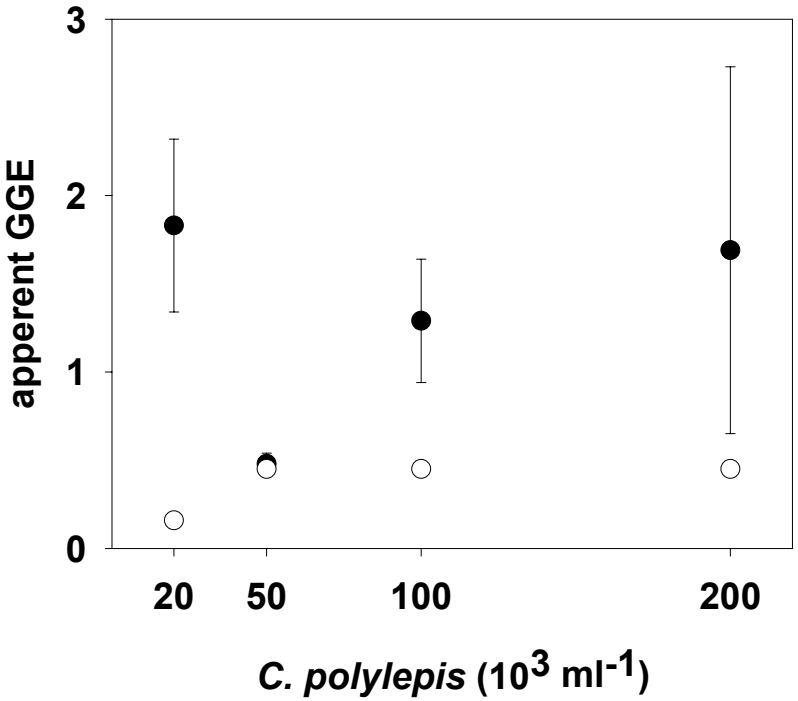



**Fig. 9:** Apparent gross growth efficiency (aGGE) of *O. marina* as a function of initial food concentration when fed with the toxic clone B1511 (closed circles) or the non-toxic clone B11 (open circles). Data point represent triplicate mean ± 1SD.

## *2.5 Publication III:* Cell cycle dependent toxin production of the ichthyotoxic prymnesiophyte *Chrysochromulina polylepis.*

Erik Eschbach *[1,2], Uwe John[1], Marcus Reckermann[3], Bente Edvardsen[4] and Linda K. Medlin[1]

1 Alfred-Wegener-Institute for Polar and Marine Research, Section of Biological Oceanography, Bremerhaven, Germany, Germany.
2 present address: University Stuttgart, Biological Institute, Stuttgart
3 University Kiel, Research and Technology Center West Coast, Büsum, Germany.
4 Norwegian Institute for Water Research (NIVA), Oslo, Norway.

***Abstract***

Cultures of the ichtyotoxic prymnesiophyceae *Chrysochromulina polylepis* (Manton) were synchronized under exponential growth with a 14:10h light dark cycle. The synchronization was induced for two cell division yielding 63% for the first division after 48 h and 68% of synchronized cells for the second division after 72h. The G1 phase occupied approximately 19-22h, the S phase around 4h and G2+M lasted between 1 and 2 hours. The cell concentration and chlorophyll *a* concentration increased in a stepwise pattern as typical for synchronized eukaryotic cells. Whereas the cell concentration increased during the light period, chl *a* increased during the dark. The chl *a* cell quota increased parallel with the cell size to a maximum at the end of the light phase and subsequently decreased until the end of the dark period. Analysis of the toxicity of *C. polylepis* was conducted using a erythrocyte lysis assay (ELA). In which fish erythrocytes were incubated with cells of *C. polylepsis* under standard conditions and cellular toxin content was measured as haemolytic activity and stated as percentage of lysed erythrocytes. The toxicity of *C. polylepis* was induced by light and increased during the first hours of the light phase, indicating that the biosynthesis of the lytic compounds are apparently promoted by light-dependent events during the cell cycle.

***Introduction***

In May 1988 the marine flagellate *Chrysochromulina polylepis* Manton et Parke (Prymnesiophyceae) caused a devastating toxic bloom in the Kattegat and Skagerrak Straits connecting the Baltic with the North Sea. This was surprising, because before this bloom, *C. polylepis* was assumed to be non toxic (Manton and Parke, 1962) and blooms of this species in the affected coastal regions had not been previously recorded. The 1988 bloom, whose definite cause still remains unknown, was exceptional for several other reasons. It covered an area of approximately 75.000 square kilometers and reached cell densities of 10 x $10^7$ cells per litre in its later stages (Dahl et al., 1989). During the peak of the bloom it was essentially monospecific consisting of *C. polylepis* as the completely dominating algal species. It exhibited strong toxicity to many marine animals and macro algae leading to severe ecological damage to wild biota and to high economic losses of fish farms along the Swedish and Norwegian coasts (Rosenberg et al., 1988, Nielsen et al., 1990, Skjoldal & Dundas 1991). After this huge bloom, however, *Chrysochromulina blooms* (not only the species *C. polylepis*) have been repeatedly observed, but these have seldom been monospecific or toxic to wild biota (Tangen & Brieby, 1988, Tangen 1989, Knipschildt 1992, Hansen et al., 1995, review by Edvardsen & Paasche 1998). The last bloom causing large economical losses occurred in 1991 in the Lofoten Archipelago off the Norwegian coast. It was dominated by the species *C. leadbeateri* and killed large amounts of farmed salmonids (Aune et al., 1992, Johnsen et al., 1999).

Generally, toxins produced by *C. polylepis* are non-selective, interfering mainly with membrane function, and thus may affect organisms ranging from protozoa to fish. Their chemical structures are not fully elucidated, although Yasumoto et al. (1990) described them as lipids and/or fatty acids. However, John et al. (2002a) have shown in a comparative approach of a toxic and non-toxic clone of *C. polylepis* that substances as lipids or fatty acids were rather unlikely involved in the ichtyotoxicity of *C. polylepis*. Several parameters affect the growth and toxicity of microalgae (White 1978, Boyer et al., 1987, Ogata et al., 1987, Anderson 1990). Unbalanced nutrient conditions with high nitrogen and limiting phosphorous content, as they exist today in many coastal regions due to human activity, seem to play an especially important role in the manifestation of algal toxicity (Johannsson & Granéli, 1998). For *Chrysochromulina polylepis* Dahl et al. (1989) suggested that phosphorus deficiency may have led to increased toxicity in the 1988 bloom. Using this potent weapon all competitors and predators could have been efficiently eliminated, leading - together with favorable

meteorological and hydrographical conditions - finally to uncontrolled exponential growth and a massive monospecific bloom (Granéli et al., 1993). John et al. (2002a) have shown that grazers, *Oxyrrhis marina*, was inhibited from feeding when grown together with the toxic clone of *C. polylepis*. Thus, it seems that inhibition of competitors is a viable strategy for this microalga to achieve bloom proportions. Clear experimental evidence shows that phosphorus and nitrogen limitations increase the toxicity of *C. polylepis* (Edvardsen et al., 1990, Edvardsen et al., 1996). Salinity also has an important influence on the degree of the toxicity of *C. polylepis* (Edvardsen et al., 1996). The fact that toxicity is much weaker at lower salinity was used, as a mitigation strategy by towing fish pens into brackish waters (Lindahl et al., 1990). Furthermore toxicity of *C. polylepis* is strongly dependent on the growth phase of the algal population and the pH of culture medium (Schmidt and Hansen 2001). Under non-limiting nutrient conditions, toxicity is highest in the mid exponential growth phase, whereas in phosphorus-limited cultures, cells remain highly toxic in stationary phase (Edvardsen et al., 1996, Moestrup & Arlstad, 1993). The effects of nutrient limitation must be carefully interpreted because one may see simply toxin accumulation in the cells because cell division has been arrested or retarded so that a true increase in toxin production is not seen (Taroncher-Oldenburg et al., 1997, John et al. 2002b).

In order to delve more deeply into the factors that make species toxic or that regulate their toxicity, it is necessary to identify which factors induce the toxin production and its regulation. This can be achieved with cell cycle analysis with synchronised cultures. Two recently published studies show that in a pelagic and in a benthic dinoflagellate, toxin production was coupled to a defined time period within their cell cycle. *Alexandrium fundyense* (causing Paralytic Shellfish Poisoning, PSP) produced saxitoxins in a ten hour period within G1 phase, with slight time shifts in the production of the different saxitoxin derivatives (Taroncher-Oldenburg et al., 1997). For *Prorocentrum lima* (causing Diarrhetic Shellfish Poisoning, DSP) "morning toxins" produced in G1 and "afternoon toxins" synthesised later during S and G2 phases were discovered among the different DTX toxin derivatives (Pan et al., 1999). Thus toxin biosynthesis in these dinoflagellates is not continuous, but follows a strict time schedule coupled to their cell cycle and was light induced. In phototropic organisms, light plays an important role in inducing diurnal physiological processes, e.g, cell division, nutrient assimilation, toxin production, onset of sexual reproduction just to name a few (Taroncher-Oldenburg et al., 1997, Sweeney, 1987).

The aim of the present work was to investigate toxin production in *C. polylepis* to find out, if it is coupled to a defined time period within the cell cycle as in the dinoflagellates. Our experimental approach comprised synchronisation of batch cultures of the *C. polylepis* strain B1511, which was clonally derived from the 1988 bloom (Edvardsen and Paasche 1992). Our next focus was on the qualitative analysis of toxicity using haemolysis as a proxy for toxicity in *C. polylepis*, to find out if there is a correlation between toxin biosynthesis and cell cycle phases and if toxin production was light induced.

## *Material & Methods*

### *Cultures*

The experiments were conducted with two haploid clones of *Chrysochromulina polylepis* Manton et Parke, named B1511 and B11. Both clones were isolated by Bente Edvardsen from a toxic strain B1, which was isolated from the Oslo Fjord (Norway). Each clone represents one of the two different cell types of *C. polylepis* described in detail by Paasche et al. (1990), Edvardsen and Paasche (1992), Edvardsen and Vaulot (1996), and Edvardsen and Medlin (1998). Briefly, both cell types are virtually indistinguishable with light microscopy, but electron microscopy observation has shown differences in the fine structure of the organic scales covering the cells. Based on the original description by Manton and Parke (1962), one cell type was termed authetic (α), whereas the second type was termed alternate (β) (Edvardsen and Paasche 1992). The clone B1511 consists of authentic cells, whereas the clone B11 contained only cells of the alternate type (Edvardsen and Vaulot 1996). Both clones were grown in axenic batch cultures in IMR ½ medium (Eppley et al. 1967), supplemented with selenite (Dahl et al. 1989) under controlled conditions of 15° C with artificial light of 45 µmol $m^{-2}$ $s^{-1}$ and a light/dark cycle of 14:10.

### *Synchronisation conditions and sampling procedure*

For synchronisation, algal cells in exponential growth phases were sequentially inoculated in 50 mL, 500 mL, 1 L, 5 L and finally 10 L to reach a final concentration of 1 x 105 $mL^{-1}$in each transfer. The light-dark regime, however, has been used to synchronize the cultures. Five and ten L cultures were gently bubbled with sterile filtered air to provide them with sufficient $CO_2$ and to achieve a homogenous distribution in the culture. Sampling was done from three parallell cultures during exponential growth phase and beginning of

stationary growth phase throughout a culture period of 72 h at 2 h intervals starting at a cell concen-tration of about 4 x $10^5$ cells $mL^{-1}$.

The stationary phase of the culture starts when growth rate begins to cease, which is in the 2d derivative point of the curve, in this case about day 5,5. When the values in fig. 2 are log transformed the slope will decline in this point. That some of the cells have reached stationary phase during the experiment is clear also from the calculations of μ (Table 1b). This may explain the prolonged G1 phase after 36 h and possibly the reduced toxicity after 50 h.

Samples were collected from 10 L cultures via a silicon tube with an inner diameter of 3 mm by gently applying a vacuum created by pulling a 50 mL syringe. Samples were immediately stored on ice and after determination of the cell numbers processed according to their respective analytical destina-tion. During the dark periods samples were collected in nearly complete darkness to avoid disturbance of synchronous growth and algal metabolism. The degree of synchronisation of *C. polylepis* cultures was calculated using the algorithm of Engelberg (1961), which makes use of the cell growth pattern during a cell doubling period.

*Determination of cell concentrations and cell sizes*

To determine the cell concentration 2 mL samples were diluted in 18 mL of sterile sea water pre-cooled to 15°C. Cell numbers were counted with a Coulter Multisizer II (Coulter Electronics, Krefeld, Germany) equipped with a 100 μm aperture. Cells were counted within a size window of 5 to 12 μm, which excluded background particles present in the sea water. Cell size profiles were calculated with Coulter Multisizer Software.

*Chlorophyll determination*

Samples for chlorophyll a (chl *a*) determination were prepared by filtering 5 mL algae culture in triplicate onto glass microfibre filters GF/F (Whatman International Ltd., Maidstone, UK) and extracting these with 10 mL 90% acetone at -20°C. Chlorophyll a concentrations were determined with a fluorometer (Gamma Analysen Technik GmbH, type 10AK, Bremerhaven, Germany) using an excitation wavelength of 435 nm and measuring fluorescence at 670 nm. Fluorescence of other pigments was subtracted by measuring the samples additionally after acidification with 1 N HCl. Chlorophyll a concentration was determined with the formula (Arar & Collin 1992).

chl a [ng mL -1] = Fm / (Fm - 1) x (F0 - Fa) x Kx x (VolEx / VolS)

where

Fm = acidification coefficient of chl *a* standard (= 2.18), F0 = relative fluorescence before acidification, FA = relative fluorescence after acidification, Kx = calibration factor (= 2.48), VolEx = extract volume (= 10 ml), VolS = sample volume (= 5 ml).

*Cell cycle analysis*

Twenty mL culture samples were fixed with 0.25% glutaraldehyde (GA), stained with 5 µM SytoxGreen (Molecular Probes, Leyden, The Netherlands) and subsequently analysed for DNA fluorescence using a FACS Vantage flow cytometer (Becton and Dickinson, San José, CA) equipped with an Innova Enterprise II 621 laser as previously described (Eschbach et al., 2001a). In brief at least 10.000 cells were analysed per sample at a pressure of 1 psi. Dot plots and histograms were created with WinMDI 2.8 software (Joseph Trotter, The Scripps Research Institute, La Jolla, CA). Cell cycle analysis was achieved with Multicycle software (Phoenix Flow Systems, San Diego, California). The number of cells in a certain cell cycle phase were calculated as percent of the total cell number in the sample. Duration of single cell cycle phases was determined based on the algorithm developed by Beck (1978) for synchronised cell cultures. Duration of G1, S and G2+M was calculated for two consecutive complete cell cycles of *C. polylepis*.

*Toxicity determination*

The toxicity of *Chrysochromulina polylepis* was determined with an improved version of the erythrocyte lysis assay (ELA) as previously described (Eschbach et al., 2001b). Its basic principle is the lysis of carp erythrocytes, because of the membrane perturbating activity of the *C. polylepis'* toxin, and the subsequent photo-metrical measure-ment of released cellular content at 414 nm wavelength. We performed two types of toxin measurements: (1.) 4.8 x $10^6$ cells were sampled every two hours to measure haemolytic activity throughout the 72 h sampling period, (2.) 6.4 x $10^6$ cells were sampled at 16:00 every day throughout the sampling period (= hours 24, 48 and 72), to measure toxin kinetics. Sample volumes needed for toxin measurements were calculated based on cell counts at the sampling time point. Samples were centrifuged for 15 min at 3.200 g at 4°C in 15 mL tubes (Sarstedt, Nümbrecht, Germany) to yield cell pellets of 0.4, 0.8 or 1.6 x $10^6$ cells per tube, depending on the cell

concentration in the culture at the time of sampling. Pellets were frozen immediately and stored at -20°C until use.

16:00 was chosen as the time to sample cells for the toxin kinetics, because toxic activity of synchronised *C. polylepis* cultures was found to be high at this time point from previous experiments. Kinetics were necessary to determine the optimal incubation time ($IT_{Opt}$) of algal extracts with erythrocytes in the ELA, which was found to vary in different experiments from 12 to 20 hours. $IT_{Opt}$ is defined as the incubation time where lysis can be measured very sensitively and at the same time with a high resolution of extracts with different toxic activities. Different toxicity of algal extracts was simulated in these measurements by preparing dilutions of algal extracts of 0.1, 0.2, 0.3, 0.4 and 0.5 x $10^5$ cells $mL^{-1}$ (= final concentration in the ELA). Lytic activity of the diluted extracts was measured in 4 h intervals throughout 24 hours and used as a proxy for toxicity. $IT_{Opt}$ was then used as the standard incubation time to determine lytic activity of the samples taken every two hours during the 72 hour sampling period.

Lytic measurements with the ELA were performed in triplicate according to Eschbach et al. (2001b). Lytic activity was calculated as percent of lysis of carp erythrocytes, by comparing absorbances mediated by the lytic activity of algal extracts (final concentration in the ELA = 5 x $10^5$ algal cells $mL^{-1}$, except for the kinetic experiments mentioned above) with absorbances obtained from completely lysed erythrocytes (final concentration in the ELA = 5 x $10^6$ erythrocytes $mL^{-1}$), which was defined as 100% lysis. Toxin production (µTox) was determined with the formula according to Anderson et al. (1990):

$$\mu Tox\ [h^{-1}] = \ln(HA1 / HA0) / t1 - t0$$

where
Haemolytic activities (HA1 and HA0) are expressed as % lysis of erythrocytes at sampling times t1 and t0, respectively

However we calculated µTox not on the basis of toxin content per mLof culture volume, but on the basis of toxin content of a defined and constant number of cells (= 5 x $10^5$ algal cells $mL^{-1}$).

***Results***

The typical growth of *C. polylepis* strain B1511 in batch cultures under synchronising conditions is depicted in Fig. 1. Given an inoculum of 1 x $10^5$ cells $mL^{-1}$, the cells reached early exponential growth phase after four days in the 10 L cultures. Maximum cell densities of about 3.5 x $10^6$ cells $mL^{-1}$was reached within 10 days after the first innoculation. There was no prolonged stationary phase, but a rapid break down of the *C. polylepis* population after reaching their cell maximum. Sampling was started at a cell density of approximately 4 x $10^5$ cells $mL^{-1}$over a 72 hour period indicated in Fig. 1. All results described below originate from three independently sampled 10 L batch cultures of *C. polylepis* growing under identical synchronising conditions.

*Synchronous growth and chlorophyll a synthesis*

*Chrysochromulina polylepis* cultures showed a stepwise increase of both cell number (N) and cellular chlorophyll *a* (chl *a*) concentrations during the 72 h sampling period (Fig. 2A). This is because of the experimental cultivation procedure, which produced 57.7% synchronisation of the algal population during the first 24 hours of the sampling period as calculated according to the algorithm suggested by Engelberg (1961). Afterwards synchronisation increased in steps of approximately 5% yielding 63.0% after 48 h and 68.1% after 72 h, respectively.

Increases of chl *a* and cell number were temporally staggered, with chl *a* increasing during the light periods and cell number increasing during dark periods. Increases in cellular chl *a* content started after the dark to light switch and continued throughout the entire light period. Likewise a decrease in, chl *a* content occurred after a light to dark switch and continued throughout the entire dark period (Fig. 2B). During the first 24 hours of the sampling period increase in cell numbers started and finished in the same dark period. During the next two 24 h periods it extended two and finally four hours into the light period (Fig. 2A).

Growth rates expressed as divisions per day show that the growth rate of *C. polylepis* decreased over time from 0.82 to 0.65 to 0.61 for the three day experiment. Cell sizes (equivalent spherical diameter) varied periodically during the 72 hour sampling period from maximum values of 8.3 μm to minimum values of 6.7 μm (Fig. 2D). Towards the end of the

sampling period cells became smaller, as indicated by a maximum cell size of only 7.5 µm on day three of the sampling period.

*Cell cycle analysis*

Flow cytometric (FC) determination of the DNA content of *C. polylepis* revealed successive cell cycle phases typical for eukaryotic cells (Fig. 2C). We obtained single and distinct peaks for G1, S and G2+M phases for each cell cycle (G2 and M phases cannot be distinguished by FC, because cells in these phases contain the same DNA amount).
Duration of the cell cycle phases was determined according to Beck (1978) using the 6 to 48 hour interval in Fig. 2C for the first and the 30 to 72 hour interval for the second complete cycle. G1 phases occupied most of the cell cycle of *C. polylepis* with 19.6 ± 0.3 and 22.0 ± 0.6 h in the first and the second complete cycle, respectively (Table 1). S phases lasted 4.3 ± 0.9 and 4.1 ± 0.1 h and G2+M phases took 1.0 ± 0.7 and 1.6 ± 0.8 h of time. Total cell cycle time increased slightly from 24.8 ± 1.9 to 27.8 ± 1.6 h during the 72 h sampling period. The percentage of the single phases, except for G1, remained constant within the range of the standard deviations..

DNA synthesis (S) always started two hours before and was completed by the end of the dark periods (Fig. 2C). During the first 48 hours of the sampling period a peak of DNA synthesis appeared after two hours in the dark. The last S phase peak was shifted by four hours within the third dark period. Cell division (G2+M) started and was completed during the dark periods of the first 48 hours of the sampling period. During this period mitosis peaks followed peaks of DNA synthesis with a delay of four hours. At the end of the sampling period cell division extended into the light period and the delay between S and G2+M peaks was reduced to two hours. An increase in the number of cells in S and G2+M phases is always synonymous with a decrease in the number of cells in G1 phase. During the first 48 hours of the sampling period nearly all cells gathered in G1 by the end of the dark period. A delay of 4 hours was obvious after the third dark period.

*Toxin analysis*

A time period of 16 hours was chosen as the optimal incubation time ($IT_{Opt}$ indicated in Fig. 3a) to measure very sensitively and with high resolution the cellular lytic capacity of the *C. polylepis* extracts sampled over 72 hours. As depicted in Fig. 2E the cellular lytic capacity varied periodically throughout the sampling period, with minimum levels by the end

of the dark periods. Immediately after the dark cellular lytic capacity increased throughout the entire light period and dropped down again during the subsequent dark period. After 48 hours this rhythmic pattern was lost and toxin values obtained varied irregularly, despite the cell synchonisation being maintained. Differences in the lytic kinetics of the toxic B1511 and the non-toxic clone B11 of *C. polylepis* were dramatic between 12 and 16 hours incubation time (Fig. 4), which fits well with the chosen $IT_{Opt}$ of t 16 h.

***Discussion***

Toxic algal blooms affecting human and animal health cause increasing ecological and economic impact on a world wide scale (Okaichi 1989, Hallegraef 1993, Dahl et al. 1998, Edvardsen & Paasche 1998). To develop efficient mitigation strategies, a comprehensive understanding of the formation of algal blooms and the reasons for their toxicity is required. Research on this topic, however, is complicated if algal species like *C. polylepis* bloom with irregular frequency and toxicity and, as long as the true mechanisms that trigger toxin production are not known. The 1988 bloom of *C. polylepis* drastically showed that this species can be highly toxic, although it was originally described as harmless (Manton & Parke, 1962). Thus, other species might harbor a toxic potential, which is unknown up to now, but which may appear once the conditions are favorable for the expression of that trait. As outlined in the introduction many factors have been shown experimentally to influence algal toxicity. Synchronized cultures are essential to boost the signal from the cells as to how toxin production is induced and be regulated. Important insights into the role that the cell cycle plays in algal toxicity is discussed by Taroncher-Oldenburg et al. (1997), Pan et al. (1999), and John et al. (2002b) who showed that toxin production in dinoflagellates varied throughout the cell cycle and was coupled to defined time periods. With this present work we have shown for the first time that this is also true for *Chrysochromulina polylepis*, s a bloom forming, ichthyotoxic representative of the division Haptophyta.

*Synchronization and growth*

Establishment of synchronous growth conditions was an essential element for the analysis of the *C. polylepis* strain B1511 a clonal descendant of the toxic 1988 bloom. Synchronization was necessary to allow conclusive cell cycle analysis and was needed to follow the temporal changes of toxin synthesis throughout the three day sampling period. In the past, many methodological methods have been employed to achieve synchronisation of

microalgal cultures. Among them the block-release approaches uses metabolic inhibitors (van Dolah et al., 1998, Ng et al., 1999) or light deprivation techniques (Taroncher-Oldenburg et al., 1997, Pan et al., 1999).

For *C. polylepis* it was not necessary to employ a block-release. The sequential inoculation of increasingly larger culture volumes with cells from early exponential growth phase and especially the 14:10 hours light-dark regime led to high levels of synchronisation. A favoring factor was the high growth rate (μ) that can be reached by *C. polylepis* populations. Under routine culture conditions growth rates are ca. 0.5 division per day (data not shown). Under optimal growth conditions *C. polylepis* can reach >1.0 divisions per day (Throndsen et al., 1995). In our experiments *C. polylepis* reached maximum growth rates of 0,82 in the early exponential growth phase. High growth rates represent an experimental advantage compared, e. g., to dinoflagellates, which because of their larger cell size and genome sizes have extended generation times and are not as easy to fit into a 24 hour diel photoperiod (Taroncher-Oldenburg et al., 1997, Pan et al., 1999, John et al. 2002b).

We observed an increase of synchronisation with time from initially approximately 60% to finally approximately 70%. This is also in congruence with Engelberg´s considerations showing that in general, some synchronisation is achieved whenever a cell culture moves towards or away from the exponential growth phase. Indeed we found that the growth rate of the *C. polylepis* cultures decreased from initially 0,82 to 0.61 by the end of the 72 hour sampling period. Synchronous growth was indicated by the step wise increase of cell numbers and chlorophyll *a* (chl *a*) content following the common scheme of cell division during the dark and chloroplast division and chl a synthesis during the light period (Fig. 2A) This was also shown in the dinoflagellate studies of Trancher-Oldenburg (1997) and Pan et al. (1999). Growth rates determined with both methods coincided 100% and showed the successive decrease in the divisons per day from 0,82 to 0.65 to finally 0.61. This on one hand may be indicative of the limited capacity of the batch cultures used, which cannot provide sustaining nutrients, $CO_2$ or light for the exponentially growing algae population. However the continuous aeration of our cultures prevented $CO_2$ limitation or broad pH changes .It is also unlikely that the medium used in our cultures was exhausted of nutrients during our short experimental time frame. IMR is very high nutrient enriched medium, Schmidt and Hansen (2001) calculated that their batch cultures of *C. polylepis* grown on the comparable F/2 medium used approximately 10-20% of available nutrients in their medium, yet at the end of their time course of 2-3 weeks, the cells has lost their toxicity. In fact they had lost their

toxicity when only 2% of the nutrients were exhausted. Thus, an alternative explanation must be sought for the loss of toxicity when cell density is high and nutrients are not limiting.

Another effect of batch cultures can be unbalanced growth, where high metabolic needs cannot be compensated by nutrient uptake over extended periods of time, because of the exceedly high growth rates of the cells. As the cells divide rapidly, there is a natural reduction in the chl a content per cell (Fig. 2B) and a decrease in the mean cell size (Fig. 2D)

*Cell cycle analysis*

Synchronized cultures of *C. polylepis* was analysed with flow cytometry (Edvardsen & Vaulot 1996). Cell cycle analysis is usually accomplished by quantifying the amount of DNA in fixed cells (Grey et al., 1990). We used an improved fixation method based on the use of glutaraldehyde (Eschbach et al. 2001a).

Our results show (Fig. 2C) that *C. polylepis* proceeds through the typical phases of a eukaryotic cell cycle consisting of mitosis (M phase), during which cells divide into two daughter cells, followed by a metabolically very active phase called "gap one" (G1 phase). After the G1 phase cells prepare for the next cell division by doubling their DNA content in a distinct phase of DNA synthesis (S phase). S and M phases are separated by another gap phase called "gap two" (G2 phase). As summarised in Table 1 and illustrated in Fig. 5, DNA synthesis in *C. polylepis* takes place duirng a discrete time period of ca. four hours following a G1 phase occupying ca. 20 hours, which is almost 80% of the total generation time and precedes the short time period of only one to two hours in which mitosis takes place.

Table 1 also shows that the second complete cell cycle lasted longer by three hours compared to the first cycle, but with the standard deviation taken into account, it is likely that this is not statistically significant. However, if each part of the cell cycle is examined, it appears that there is a significant difference in the length of the G1 phase. This might illustrate that culture conditions of the batch culture became unbalanced with increasing cell densities by the later stages of the experiment. Other reports support the finding that lengthening of the generation time accompanies decreasing growth rates and that is because of the expansion of a single cell cycle phase, e. g., the G1 phase (Olsen & Chisholm, 1986, Micheli et al., 1996), but this could also accompanied by a proportional increase of the duration of all phases of the cell cycle, a behaviour that has, e. g., been observed for the primitive eukaryote *Saccharomyces cerevisiae* (Rivin & Fangman, 1990). We did not observe this latter trend in our experiments. At the beginning of our experiments *C. polylepis* cultures were in balanced growth and S and G2+M phases were passed through during the dark period

(Fig. 2C). This is typical for most other microalgal species, although exceptions of this rule do exist, e. g., dark adapted species (Pan & Cembella, 1998). During the light period all *C. polylepis* cells gathered in G1 during which they grew and physiological activities were performed, like chlorophyll and toxin synthesis. After the second cell cycle, there appears to be a slight temporal shift of the cell cycle phases during the dark period. This might be caused by the forced rapid growth (from normally 0.5 to 0.82 division per day), which could not be sustained and which might provoke a change or shift in the cell cycle (Taroncher-Oldenburg et al., 1997, Pan & Cembella, 1998)

*Toxin biosynthesis*

Quantitative toxin analysis of *C. polylepis* is hampered by the fact that its ichthyotoxic substances are chemically unknown. Although Yasumoto et al. (1990) suggested a galactolipid similar to haemolysin-2 of *Amphidinium carteri* as the main component and polyunsaturated fatty acids as additional substances contributing to the lethal effects towards fish, but John et al. (2002a) have shown that lipids and polyunsaturated fatty acids are not likely to be the causative toxic substances because of their the identical lipid and fatty acid composition in an toxic and non toxic clone of *C. polylepis*. Also, a standard substance is still missing to permit quantitative chemical analysis, e.g., by HPLC-MS. Because these substances are known to cause lysis of cells, which is the mechanism that damages the gills of fish (Skjoldal & Dundas, 1991), the erythrocyte lysis assay (ELA) can be employed as a proxy to estimate potential toxic activity. B1511 and B11 show different heamolytic effects or kinetics, and the differences in the haemolytic capacities of the two species can be assumed to be related to toxicity of the former. The ELA is an acknowledged assay to determine haemolytic substances (Johansson et al., 1998) and has recently been improved for more sensitivity and high sample throughput (Eschbach et al., 2001b) and can be used as a proxy for ichthyotoxic estimates. A relative quantification is not possible with this assay, however, since it is not possible to calculate amounts of the toxin, i.e., pg toxin per cell or per pg protein, as it is possible for chemically well characterised toxins, e.g., saxitoxins and dinophysistoxins, only toxic activity. For these substances toxin profiles, i.e., different derivatives of one group of toxin, can be determined giving a clue to possible transformation mechanisms and possible biosynthetic pathways (Taroncher-Oldenburg et al., 1997, Pan et al., 1999). This cannot be achieved with the ELA, which determines only the total haemolytic effect of *C. polylepis* extracts, which may consist of several substances with lytic activity as mentioned above. Advantages of the ELA, however, are that it can determine haemolytic

substances, although their chemical nature is unclear and that these substances (with the improved assay) can be detected very sensitively down to dilutions of single algal cells (Eschbach et al., 2001b).

Cellular toxin content was measured here as haemolytic activity of a constant number of *C. polylepis* cells incubated with a constant number of fish erythrocytes. Therefore changes in the measured activity throughout the sampling period are representative for average differences in the haemolytic content of the single algal cell

As it has been reported for dinoflagellates (Taroncher-Oldenburg et al., 1997, Pan et al., 1999) lytic activity (toxicity) of *C. polylepis* also appears to be induced by light and is discontinuous. As can be seen in Fig. 2E haemolytic levels increased throughout the entire G1 phase until the beginning of the S phase leading to an intracellular accumulation of the toxin (illustrated by the color shift in Fig. 5) that dropped down again throughout the remainder of the cell cycle.

This rhythmic behavior for the lytic activity lasted, however, only for the first 48 hours of the sampling period, thereafter lytic activity of the cells showed large variations resulting in an arrhythmic course of the haemolysis curve (Fig. 2E). This cannot be explained by a decrease of synchronicity, because the percentage of cells entrained in the synchonisation has been shown to increase throughout the entire sampling period.A small shift in the duration of the G1 phase of the cell cycle appears to have taken place (Fig. 2C and Table 1) leading to a G2+M phase forced into the light period. The cultures entered stationary phase after 36 h which may explain the decrease in toxicity. A decline in toxicity to *Artemia* nauplii entering stationary phase was recorded in nutrient repleated cultures of *C. polylepis* (Edvardsen et al. 1996). As discussed above, we assume that it is unlikely that nutrients have become limiting in our batch cultures. John et al (2002b) have shown that the growth rate of the dinoflagellate grazer, *Oxyrrhis marina*, was clear negatively affected because it avoided ingestion of the toxic clone B1511 but was never inhibited from feeding by increasing cell concentrations of the toxic clone. (B1511). The toxin might affect other organisms only at very short signalling distances. Schmid and Hansen (2001) also reported that at high cell concentrations of *C. polylepis*, toxiciy is diminshed.

Our flow cytometric analysis showed that *C. polylepis* proceeds through a typical eukaryotic cell cycle with a distinct DNA synthesis phase. Toxin production (increase in lytic activity per cell) of *C. polylepis*, as a representative of the division Prymnesiophyta, is can be

studied with synchonised cutlures like it has been shown before for species of the division Dinophyta (Taroncher-Oldenburg et al., 1997, Pan et al., 1999).

Apparently fundamentally different mechanisms can become operative in toxin production. For dinoflagellates it was reported, e. g., that during slow growth under limiting conditions (nutrients, light) certain cell cycle phases are temporarily expanded (Olson & Chisholm 1986, Micheli et al. 1996). If toxins are produced during such a phase of the cell cycle this leads to an increase in intracellular toxin concentration.. For *C. polylepis* it can be deduced from our cell cycle data that the heamolytic activity is light indueced, even that after the third day, hemolysis is no longer light induced and therefore probably uncoupled from the cell cycle. The growth rate is reduced and the toxin relevant G1 phase is expanded

Moreover, the analysis of toxin biosynthesis and its regulation on the gene and protein level must definitely be pushed forward, to understand the way in which phytoplankton as an essential component of the food web is turned into a harmful danger with far reaching negative consequences for ecosystems and economies. Because we could prove here that toxin biosynthesis of *C. polylepis* is switched on in a narrow time window at the beginning of the G1 phase of the cell cycle, this will be possible now with directed molecular biological approaches and has already been initiated (Eschbach et al., 2000).

Acknowledgements: This research was supported as part of the BMBF TEPS project 03F0244. The tehcnical assistance of Helga Mehl is gratefully acknowledged.

***References***

Arar EJ, Collins GB (1992) In vitro determination of chlorophyll a and phaeophytin a in marine and freshwater phytoplankton by fluorescence. Method 445.0. In: USEPA, Methods for the determination of chemical substances in marine and estuarine environmental samples. EPA Cincinnati OH

Anderson D. M. 1990. Toxin variability in Alexandrium species. In: Toxic marine phytoplankton, Proc. 4th Int. Conf. pp. 41-51. Elsevier, New York.
Anderson D. M., Kulis D. M., Sullivan J. J., Hall S. & Lee S. 1990. Dynamics and physiology of saxitoxin production by the dinoflagellates Alexandrium spp. Mar. Biol. 104: 511-524.

Aune T., Skulberg O. M. & Underdal B. 1992. A toxic phytoflagellate bloom of Chrysochromulina cf. leadbeateri in coastal waters in the north of Norway, May-June 1991. Ambio 21: 471-474.Beck H. P. 1978. A new analytical method for determining duration of phases, rate of DNA synthesis and degree of synchronization from flow-cytometric data on synchronized cell populations. Cell Tissue Kinet. 11: 139-148.

Boyer G. L., Sullivan J. J., Andersen R. J., Harrison P. J. & Taylor F. J. R. 1987. Effects of nutrient limitation on toxin production and composition in the marine dinoflagellate Protogonyaulax tamarensis. Mar. Biol. 96: 123-128.

Dahl E., Edvardsen B. & Eikrem W. 1998. Chrysochromulina blooms in the Skagerrak after 1988. In: Harmful Algae (Ed. by B. Reguera, J. Blanco, M. Fernandez & T. Wyatt), pp. 104-105. GRAFISANT, Santiago de Compostela, Spain.

Dahl E., Lindahl O., Paasche E. & Throndsen J. 1988. The Chrysochromulina polylepis bloom in Scandinavian waters during spring 1988. In: Novel phytoplankton blooms: causes and impacts of recurrent brown tides and other unusual blooms (Ed. by E. M. Cosper, M. Bricelj & E. J. Carpenter), pp. 383-405. Springer, Berlin.

Edvardsen B. & Paasche E. 1998. Bloom dynamics and physiology of Prymnesium and Chrysochromulina. In: Physiological Ecology of Harmful Algal Blooms (Ed. by D. M. Anderson, A. D. Cembella & G. M. Hallegraeff), pp. 193-208. Springer Verlag, Heidelberg, Germany.

Edvardsen B., Larsen A., Kristiansen S. & Paasche E. The effect of growth stage, salinity, temperature and nutrient limitation on the toxicity of *Chrysochromulina polylepis* (Prymnesiophyceae). Marine Biology, submitted.

Edvardsen B., Moy F. & Paasche E. 1990. Hemolytic activity in extracts of *Chrysochromulina polylepis* grown at different levels of selenite and phosphate. In: Toxic Marine Phytoplankton (Ed. by E. Granéli, B. Sundström, L. Edler & D. M. Anderson), pp. 284-289. Elsevier, New York.

Engelberg J. 1961. A method of measuring the degree of synchronization of cell populations. Exp. Cell Res. 23: 218-227.

Eschbach E., John U., Reckermann M. & Medlin L. K. 2000. Variation in toxin production of synchronized cultures of *Chrysochromulina polylepis* (Prymnesiophyta). J. Phycol. 36: 20.

Eschbach E., Reckermann M., John U., Medlin L. K. 2001a. A simple and highly efficient fixation method for *Chrysochromulina polylepis* (Prymnesiophytes) for analytical flow cytometry. Cytometry 44: 126-132.

Eschbach E., Scharsack J. P., John U. & Medlin L. K. 2001b. Improved erythrocyte lysis assay in microtitre plates for sensitive detection and efficient measurement of haemolytic compounds from ichthyotoxic algae. J. Appl. Toxicol. 21: 513-519.

Granéli E., Paasche E. & Maestrini S. Y. 1993. Three years after the *Chrysochromulina polylepis* bloom in Scandinavian waters in 1988: some conclusions of recent research and monitoring. In: Toxic Phytoplankton Blooms in the Sea (Ed. by T. J. Smayda & Y. Shimizu), pp. 23-32. Elsevier, Amsterdam.

Grey J. W., Dolbeare F. & Pallavicini M. G. 1990. Quantitative cell cycle analysis. In: Flow cytometry and sorting, 2nd edition, pp. 445-467. Wiley-Liss, New York.

Hallegraef G. M. 1993. A review of harmful algae blooms and their apparent global increase. Phycologia 32: 79-99.

Johansson N. & Granéli E. 1998. Toxicity of *Chrysochromulina polylepis* cells growing in different N:P ratios. In: Harmful Algae (Ed. by B. Reguera, J. Blanco, M. L. Fernández & T. Wyatt), pp. 329-330. GRAFISANT, Santiago de Compostela.

John, U., Tillmann, U. and Medlin, L.K. 2002a. A comparative approach to study inhibition of grazing and lipid composition of a toxic and non-toxic clone of *Chrysochromulina polylepis* (Prymnesiophyceae), Harmful Algae, in press.

John, U., Quilliam, M.A., Medlin, L. and Cembella, C. 2002b. Spirolide production and photoperiod-dependent growth of the marine dinoflagellate *Alexandrium ostenfeldii*, Proceedings of the IXth International Conference on Harmful Microalgae, G.M. Hallegraeff, S.I. Blackburn, C.J. Bolch & R.J. Lewis, eds., IOC-UNESCO, Paris, pp. 299-302.

Johnsen G., Dallökken R., Eikrem W., Legrand C., Aure J. & Skjoldal H. R. 1999. Eco-physiology, bio-optics and toxicity of the ichthyotoxic *Chrysochromulina leadbeateri* (Prymnesiophyceae). J. Phycol. 35: 1465-1476.

Karentz D. 1983. Patterns of DNA synthesis and cell division in marine dinoflagellates. J. Protozool. 30: 581-588.

Knipschildt F. 1992. A new Chrysochromulina outbreak and fish kills in Danish coastal waters. Harmful Algae News 2: 2.

Hansen P. J., Nielsen T. G. & Kaas H. 1995. Distribution and growth of protists and mesozooplankton during a bloom of Chrysochromulina spp. (Prymnesiopyceae, Prymnesiales). Phycologia 34: 409-416.

Lindahl O. & Dahl, E. 1990. On the development of the Chrysochromulina polylepis bloom in the Skagerrak in May-June 1988. In: Toxic Marine Phytoplankton (Ed. by E. Granéli, B. Sundström, L. Edler & D. M. Anderson), pp. 189-194. Elsevier, New York.

Manton I. & Parke M. 1962. Preliminary observations on scales and their mode of origin in Chrysochromulina polylepis sp. nov.. J. Mar. Biol. Ass. U. K. 42: 565-578.

Micheli C., Magaletti E. & Chimenz C. 1996. Effect of stress on the growth and cell cycle of dinoflagellates. In: Harmful and Toxic Algal Blooms (Ed. by T. Yasumoto, Y. Oshima and Y. Fukuyo), pp. 317-319. Sendai Kyodo Printing Co. Ltd., Sendai, Japan.

Moestrup O. & Arlstad S. 1993. The toxicity of Chrysochromulina and Prymnesium in relation to salinity and growth phase. 6th international conference on toxic marine phytoplankton, Nantes, Ifremer.

Ng C. K. F., Lam C. M. C., Yeung P. K. K. & Wong J. T. Y. 1999. Flow cytometric analysis of nocodazole-induced cell-cycle arrest in the pennate diatom Phaeodactylum tricornutum Bohlin. J.Appl. Phycol. 10: 569-572.

Nielsen A. & Richardson K. 1988. Chrysochromulina polylepis bloom in Danish, Swedish and Norwegian waters, May-June 1988: an analysis of extent , effects and causes. In: European Economic Community Workshop on Eutrofication, Brussels, 26-28 October 1988, 12 pp.

Ogata T., Kodama M. & Ishimaru T. 1987. Toxin production in the dinoflagellate Protogonyaulax tamarensis. Toxicon 25: 923-928.

Okaichi T. 1989. Red tide problems in the Seto Inland Sea, Japan. In: Red Tides: Biology, Environmental Science and Toxicology (Ed. by T. Okaichi, D. M. Anderson & T. Nemoto), pp. 137-142. Elsevier Science Publishing, New York.

Olson R. J. & Chisholm S. W. 1986. Effects of light and nitrogen limitation on the cell cycle of the dinoflagellate Amphidinium carteri. Journal of Plankton Research, 8: 785-793.

Pan Y. & Cembella A. D. 1998. Flow cytometric determination of cell cycles and growth rates of Prorocentrum spp. In: Harmful microalgae (Ed. by B. Reguera, J. Blanco, M. L. Fernandez & T. Wyatt), pp. 173-176.

Pan Y., Cembella A. D. & Quilliam M. A. 1999. Cell cycle and toxin production in the benthic dinoflagellate Prorocentrum lima. Mar. Biol. 134: 541-549.

Rivin C. J. & Fangman W. L. 1980. Cell cycle phase expansion in N-limited cultures of Saccharomyces cerevisiae. J. Cell Biol., 85: 96-107.

Rosenberg R., Lindahl O. & Blanck H. 1988. Silent spring in the sea. Ambio 17: 289-290.

Schmidt, L.E., Hansen, P.J., 2001. Allelopathy in the prymnesiophyte *Chrysochromulina polylepis*: effect of cell concentration, growth phase and pH. Mar. Ecol. Prog. Ser. 216, 67-81.

Skjoldal H. R. & Dundas I. 1991. The Chrysochromulina polylepis bloom in the Skagerrak and the Kattegat in May-June 1988: Environmental conditions, possible causes and effects. In: ICES Cooperative Research Report No. 175, 59 pp.

Tangen K. & Brieby A. 1988. A new outbreak of Chrysochromulina polylepis. Red Tide Newsletter 1: 4-5.

Tangen, K. 1989. Algal blooms in Norway in 1989. Red Tide newsletter 2: 2-3.

Taroncher-Oldenburg G., Kulis D. M. & Anderson D. M. 1997. Toxin variability during the cell cycle of the dinoflagellate Alexandrium fundyense. Limnol. Oceanogr. 42: 1178-1188.

Throndsen J., Larsen J. & O. Moestrup. 1995. Toxic algae: toxicity of Chrysochromulina with new ultrastructural information on C. polylepis. In: Algae, Environment and Human Affairs (Ed. by W. Wiessner, E. Schnepf & R. C. Starr), pp. 201-222. Biopress Limited, Bristol.

van Dolah F. M., Leighfield T. A. & Sugg L. M. 1998. Mechanisms regulating the cell cycle in two dinoflagellate species, Gambierdiscus toxicus and Amphidinium operculatum. In: Harmful Algae (Ed. by B. Reguera, J. Blanco, M. Fernandez & T. Wyatt), pp. 156-159. GRAFISANT, Santiago de Compostela, Spain.

White A. W. 1978. Salinity effects on growth and toxin content of Gonyaulax excavata, a marine dinoflagellate causing paralytic shellfish poisoning. J. Phycol. 14: 475-479.

Yasumoto T., Underdal B., Aune T., Hormazabal V., Skulberg O. M. & Oshima Y. 1990. Screening for hemolytic and ichthyotoxic components of Chrysochromulina polylepis and Gyrodinium aureolum from Norwegian coastal waters. In: Toxic Marine Phytoplankton (Ed. by E. Granéli, B. Sundström, L. Edler & D. M. Anderson), pp. 436-440. Elsevier, New York.

Table 1: Two complete cell cycles were calculated according to Beck (1978) using the time intervals from 6 to 48 and from 30 to 72 hours, respectively (numbers in brackets). Results are expressed in absolute numbers (h) and as percent (%) of the total duration (total) of a complete cell cycle. Means and standard deviations (SD) are calculated from three independent 10 l batch cultures. G1 = gap one, S = DNA synthesis, G2+M = gap two and mitosis phase.

| | **1st complete cycle [6-48 h]** | | | | **2nd complete cycle [30-72 h]** | | | |
|---|---|---|---|---|---|---|---|---|
| | **[h]** | | **%** | | **[h]** | | **%** | |
| | **mean** | **SD** | **mean** | **SD** | **mean** | **SD** | **mean** | **SD** |
| **G1** | 19.6 | 0.3 | 78.7 | 1.1 | 22.0 | 0.6 | 79.3 | 2.2 |
| **S** | 4.3 | 0.9 | 17.1 | 3.5 | 4.1 | 0.1 | 14.8 | 0.5 |
| **G2+M** | 1.0 | 0.7 | 4.1 | 2.6 | 1.6 | 0.8 | 5.8 | 2.9 |
| **total** | 24.8 | 1.9 | 100 | 7.2 | 27.8 | 1.6 | 100 | 5.6 |

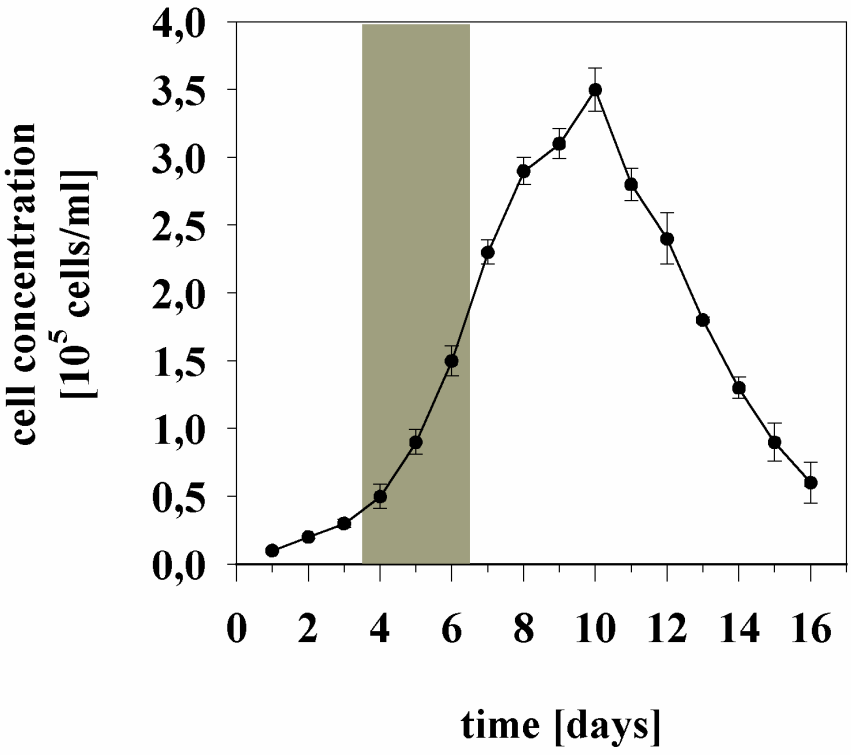



Fig. 1: Growth curve of synchronized *C. polylepis* B1511 showing the development of the algal populations (y-axis) over a period of 16 days (x-axis) in batch cultures. The rectangular frame indicates the sampling period of 72 hours. Data points represent the means of three independent cultures + standard deviation.

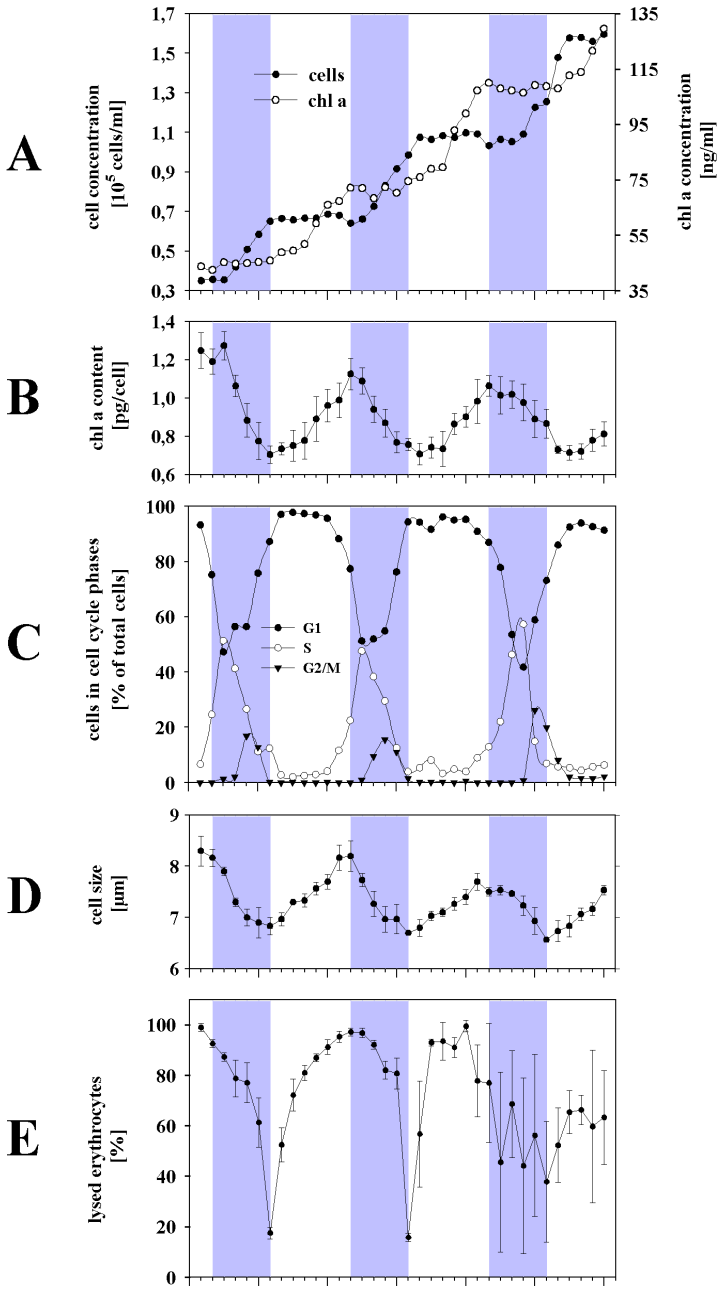



Fig. 2: Analytical data determined from three synchronised batch cultures of C. polylepis B1511 during a 72 hour sampling period (x-axis): A = algal growth calculated from cell numbers and chl a concentrations, B = chl a content per cell (derived from data in A), C = percentage of cells in G1, S and G2/M phase of the cell cycle, D = cell size distribution (equivalent spherical diameters), E = toxin content of algal cells expressed as erythrocyte lysis. All data points represent the mean of three independent measurements. Error bars were left out in curves with more than one curve for clarity. Grey areas indicate the 10 hours dark periods. Cells = cell concentration, chl a = chlorophyll a concentration, G1 = gap one, S = DNA synthesis and G2/M = gap two and mitosis phases

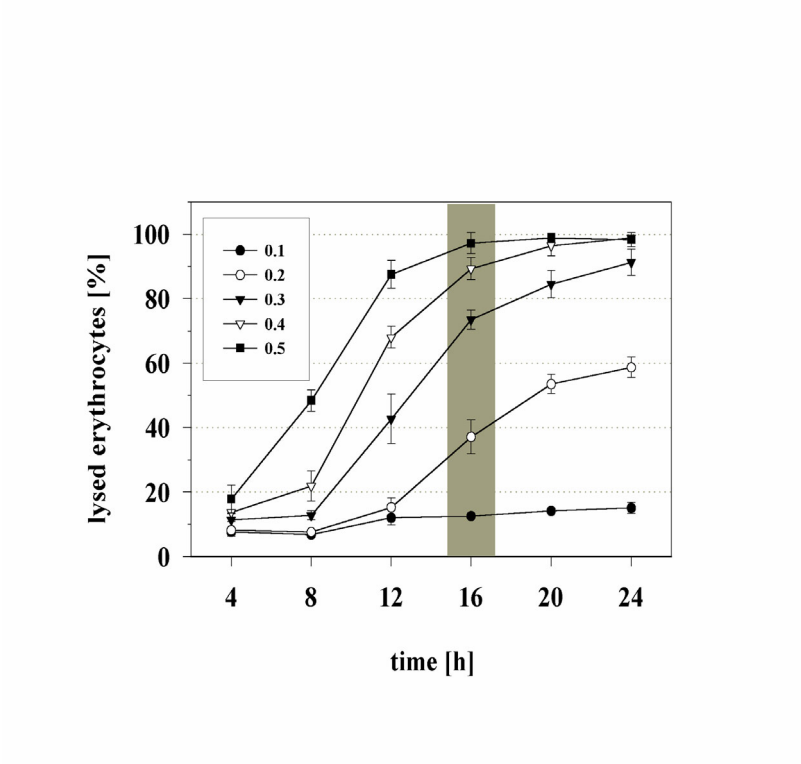



Fig. 3: Toxin kinetics for determination of the optimal incubation time (ITOpt) of the toxic *Chrysochromulina polylepis* clone B1511 extracts with erythrocytes in the ELA. A: Different toxicity of extracts of the toxic clone B15ll was simulated in these measurements by preparing dilutions of algal extracts of 0.1, 0.2, 0.3, 0.4 and 0.5 x 105 cells mL -1(legend). Haemolytic activity (y-axis) of the diluted extracts was measured in 4 h intervals throughout 24 hours (x-axis). 16 hours were chosen as ITOpt (highlighted in grey) and was used as the standard incubation time to measure all extracts taken over the 72 hour sampling period.

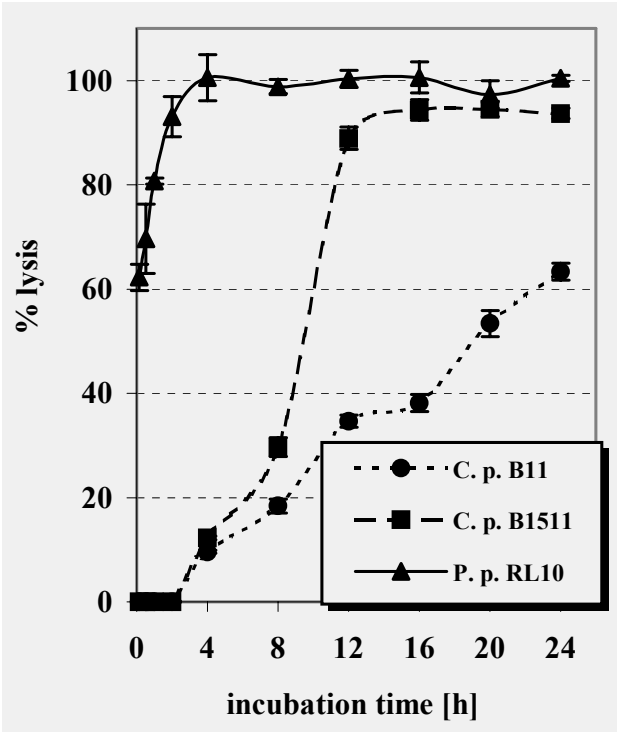



Fig.4: Toxin kinetics for determination of the optimal incubation time (ITOpt) of algae extracts with erythrocytes in the ELA Different haemolytic activities exhibited by the toxic clone B1511, the non-toxic clone B11 of *C. polylepis* and *Prymnesium parvum* (P.p. RL10) used as control. Haemolytic activity (y-axis) of the extracts was measured in 4 h intervals throughout 24 hours (x-axis). 16 hours were chosen as ITOpt for *C. polylepis*.

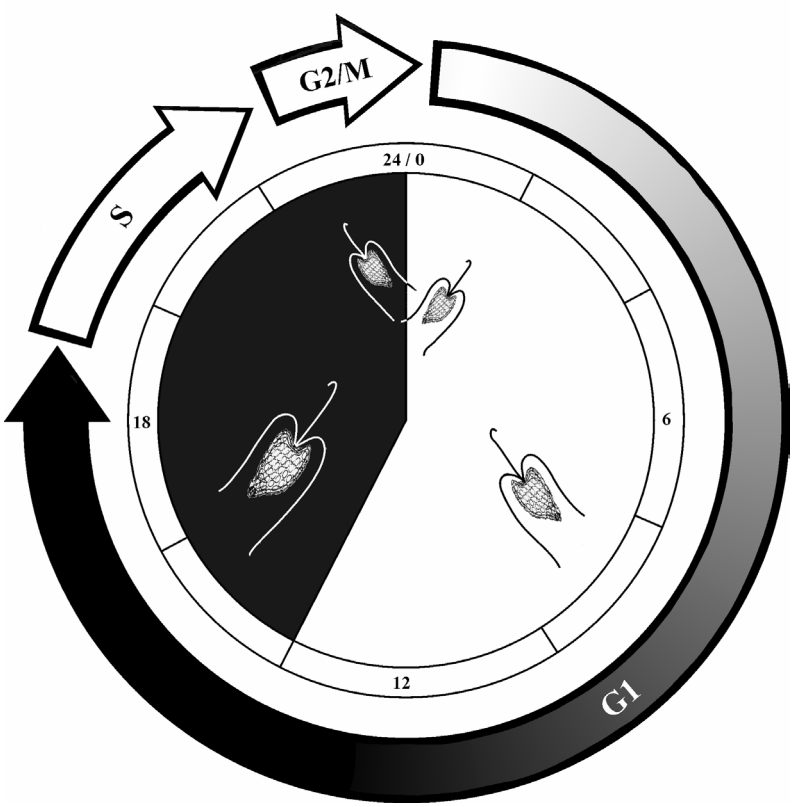



Fig. 5: Schematic representation summarising the events determined during the cell cycle of *C. polylepis* in a 24 hour diel photoperiod: Inner circle = 14 : 10 hour light-dark regime and cell growth; middle circle = 24 hour diel photoperiod; outer circle = cell cycle phases with arrow lengths indicating the duration of the respective cell cycle phases. G1 = gap one (colour shift within the arrow symbolising intracellular toxin accumulation), S = DNA synthesis, G2/M = gap two and mitosis phases.

## *2.6. Publication IV:* SPIROLIDE PRODUCTION AND PHOTOPERIOD-DEPENDENT GROWTH OF THE MARINE DINOFLAGELLATE *ALEXANDRIUM OSTENFELDII.*

Uwe John[1], Michael A. Quilliam[2], Linda Medlin[1] and Allan D. Cembella[2]

[1]Alfred-Wegener-Institut, Postfach 120161, D-27570 Bremerhaven, Germany;
[2]Institute for Marine Biosciences, National Research Council of Canada, 1411 Oxford St., Halifax, Nova Scotia, Canada

***ABSTRACT***

The effects of physiological status on spirolide production were studied in nutrient-replete batch cultures of a toxic strain of the dinoflagellate *Alexandrium ostenfeldii*. Although complete cell synchronisation was not achieved by dark adaptation, the concentration of motile vegetative cells apparently increased in the light and decreased in the dark. The concentration of extracted chlorophyll *a* followed the same trend as the cell concentration, with no apparent shift in the amount of chlorophyll *a* per cell in relation to the light/dark (L/D) phase. Analysis of spirolides by liquid chromatography coupled with mass spectrometry (LC-MS) showed that the toxin profile did not vary significantly over the L/D cycle, and consisted primarily of a des-methyl-C derivative (>90% molar), with minor constituents C, C3, D, D3 and des-methyl-D. The total spirolide concentration per unit culture volume was directly related to the concentration of cells and chlorophyll *a*, but there was a dramatic increase in cell quota of spirolides at the beginning of the dark phase and a corresponding decrease in the light. The biosynthesis of these polyketide-derived metabolites is apparently governed by light-dependent events during the cell division cycle.

## *INTRODUCTION*

The marine dinoflagellate *Alexandrium ostenfeldii* (Paulsen) Balech *et* Tangen has been recently identified as the source of toxic spirolides [1]. These potent macrocyclic imines were first isolated and characterised from shellfish viscera [2,3], and later identified in the plankton from Nova Scotia, Canada [4,5]. New rapid and highly sensitive methods to quantify spirolides in only few plankton cells by liquid chromatography-mass spectrometry (LC-MS) [6] have been applied to the analysis of a spirolide-producing *A. ostenfeldii* clone [1].

The biosynthesis of other toxic metabolites by *Alexandrium* spp. is known to be regulated by a complex interplay of environmental and intrinsic genetic factors (reviewed by [7]). Typically, changes in environmental variables, such as light, salinity, turbulence, temperature, and macronutrients, influence the cell quota of toxin ($Q_t$), either by direct effect or via a feedback interaction with cell growth rate. As the cell divides, $Q_t$ is partitioned between the daughter cells [7,8]. The synthesis of PSP toxins in *Alexandrium* occurs during vegetative growth in the G1 phase of the cell cycle [8], thus any prolongation of G1 phase (decrease in growth rate [$\mu$]) may result in higher $Q_t$ even if the rate of toxin synthesis is constant. Physiological studies on dinoflagellate production of tetrahydropurine neurotoxins (e.g., saxitoxin derivatives) [7] and polyether toxins [9] have generally indicated that the toxin composition is characteristic of the strain, and that the toxin profile is rather refractory to change [10,11], except under extreme environmental stress.

In photoautotrophic dinoflagellates, the photoperiod influences many diurnal physiological processes, including cell division, nutrient assimilation, vertical migration and bioluminescence rhythms. The direct dependence of cellular processes on light/dark (L/D) cycles can be exploited to phase or synchronise the cell division cycle. Dark-induced synchronisation followed by entrainment on a defined L/D cycle has been previously used to study the cascade of events involved in toxin production in the dinoflagellates *Alexandrium fundyense* [8] and *Prorocentrum lima* [12].

There are few studies on the effects of photoperiod and cell division cycle events on the production and accumulation of polyketide-derived metabolites. We attempted to use dark-induced synchronisation of *A. ostenfeldii* cultures to determine the effects of photoperiod on the cell quota of spirolides through successive cycles of cell division. Such studies are a prerequisite to establish the links between toxin biosynthesis and discrete stages of the cell division cycle. Furthermore, these data can be used to determine the optimum photoperiod for maximum growth and to quantify the effects of light induction on other cellular processes, such as chlorophyll synthesis.

## *MATERIALS AND METHODS*

Experiments were conducted on a clonal isolate of *Alexandrium ostenfeldii* (AOSH1) from Ship Harbour, Nova Scotia in unialgal batch cultures using aseptic techniques. Stock cultures (1.0 L) in exponential growth phase were inoculated into 12 L of L1 growth medium in triplicate 15 L Belco glass carboys. Cultures were grown with gentle aeration to maintain homogeneity at 15±1 °C under a 14:10 light/dark (L/D) photocycle at an ambient photon flux density of 260 μmol $m^{-2}$ $s^{-1}$. After 106 h of dark adaptation, culture samples were collected by sterile syringe at 2 h intervals throughout three L/D cycles for measurements of chlorophyll *a* (extracted and *in vivo*), cell number, cell size and spirolide concentration. During the dark period, samples were collected under red light (<0.1 μmol $m^{-2}$ $s^{-1}$) to avoid photo-induction.

Growth of cultures prior to dark adaptation was monitored by optical microscopic counts (125X). During the experiment, cell concentrations were determined using a Coulter Counter (Multisizer II). *In vivo* chlorophyll *a* fluorescence of whole cultures (10 ml) was measured by fluorometry (Turner Designs Model 10). Particulate chlorophyll samples were filtered (Whatman GF/C) and extracted in darkness with 90% acetone (72 h) at –20 $^{o}$C for quantitation by fluorometry.

Spirolides were analysed from three culture fractions: cells, filtrate (cell free medium), and whole culture. Duplicate samples of whole culture were filtered through 0.5 ml spin-cartridges (Millipore Ultrafree-MC, 0.45 μm) by centrifugation at 500 x *g*. The filtered cells were extracted by spin-filtration with 1 ml of 100% methanol [5]. Extracellular spirolides in the cell free medium were determined by direct injection of the filtrate. Spirolides were analysed by liquid-chromatography with ion-spray mass spectrometry (LC-MS) (PE-SCIEX API-III) [6] using purified standards.

## *RESULTS*

After inoculation of stock cultures into fresh growth medium, *A. ostenfeldii* cells remained in lag phase for one week. Dark adaptation for 106 h was initiated after Day 8, when the mean cell concentration had reached 800 ± 150 ($n$=3) cells $ml^{-1}$. During dark adaptation, the mean cell concentration declined substantially to 468 ± 109 cells $ml^{-1}$. After transfer to the 14:10 L/D cycle, the cell concentration oscillated with the photoperiod, decreasing in the dark and increasing in the light phase (Fig. 1). This variation in cell concentration between the light

and the dark phase was maintained throughout the experiment, for three L/D cycles. Non-motile cells, resembling pellicular cysts, accumulated on the bottom of the culture vessel, particularly during the dark phase. Based upon cell counts of motile vegetative cells alone, the net growth rate, calculated from T=0 to the end of the experiment, was low ($\mu = 0.18$ div. $d^{-1}$).

The concentration of particulate chlorophyll *a* (ng $ml^{-1}$) in the cultures exhibited the same trend as the cell concentration (Fig. 2). As for the cell concentration, the amplitude of the oscillation in chlorophyll *a* between the light and dark phases increased with each successive L/D cycle through the experiment. There was no apparent shift in chlorophyll *a* per cell related to the L/D phases.

Analyses by LC-MS showed that the sum of spirolides extracted from the cellular fraction, plus that found in the cell-free culture medium, was similar to that extracted from the whole culture. Leakage or excretion of spirolides from healthy vegetative cells accounted for <3% of the total spirolide content of the *A. ostenfeldii* cultures. Total spirolide concentration per unit culture volume (whole culture) fluctuated in response to the L/D cycle, similar to the pattern exhibited by the cell number and chlorophyll *a* concentrations (Fig. 3). Spirolide levels in the culture peaked at the end of the light phase and plummeted by as much as 20% early upon entry into darkness. However, in contrast to the pattern of cellular chlorophyll *a*, there was a dramatic increase in cell quota of spirolides at the beginning of the dark phase, peaking by the middle of the dark phase, and a corresponding decrease in the light (Fig. 3). The variation in the cell quota of spirolides over the last two L/D cycles was >50%, when the increase was calculated from the middle of the light period to the maximum in the dark. Variation in total concentration of chlorophyll *a* and spirolides over the L/D cycle was not due to cell size differences; mean cell diameter in the light was 27.9 (± 0.4 s.d.)µm, compared to 26.5 (± 0.6 s.d.)µm in darkness.

The spirolide profile of this isolate was very stable, and no substantial variations were noted in response to the photoperiod. Des-methyl-C comprised >90% of the total toxin on a molar basis, whereas derivatives C, C3 and des-methyl-D were minor components.

## DISCUSSION

We report here the first evidence that extrinsic environmental factors, specifically photoperiod, can influence the rate of production and cell quota of macrocyclic imines in marine dinoflagellates. Toxin production in dinoflagellates is also known to be subject to

genetic regulation [7,8,9], but physiological mechanisms and regulatory control of the biosynthetic pathways of toxin production are poorly understood.

*Cell Growth and Photoperiodic Events*

Compared with other *Alexandrium* isolates, this *A. ostenfeldii* strain is fastidious and less robust in mass culture. Even under recently optimised growth, reducing the light from 250 to 70-100 μmol $m^{-2}$ $s^{-1}$, cells appear healthy, but growth rates remain <0.2 div. $d^{-1}$ (A. Cembella, unpublished data). Long term acclimation to higher than optimal light intensity followed by prolonged dark exposure to achieve cell synchronisation may account for the apparent decline in cell numbers in darkness and the subsequent low net growth rate.
To reduce the deleterious effects of turbulence on cell growth, diffuse aeration was supplied at a level only sufficient to maintain roughly homogeneous distribution of motile cells and to minimise sedimentation. Prior to the experiment, samples were drawn simultaneously from several locations within the carboy to confirm homogeneity. The inlet port for cell sampling was situated several centimetres off the bottom of the carboy to ensure that motile (and presumably cycling) cells were primarily selected. The phasing of the cell concentration with the photoperiod, following a pattern of increasing cell concentration in the light and decreasing in the dark phase could be correlated with the vertical migration of motile cells. Higher deposition of dead cells, cell debris and pellicular cysts on the bottom during the dark period, and regeneration of vegetative cells from pellicular cysts in the light, might also account for the apparent growth kinetics. Nevertheless, visual observations confirmed that turbulence was sufficient to prevent layer formation of motile cells even in the dark. This phenomenon of vertical migration and encystment (pellicular cyst formation) during the dark phase has been described for *Alexandrium taylori* [14]. In this species, pellicular cysts give rise to motile cells at the beginning of the light phase, indicating that excystment and encystment may be controlled by light and regulated via the cell cycle.

For *A. ostenfeldii*, the rate of chlorophyll *a* production was approximately in balance with the cell division rate, as evidenced by the coupled oscillation in the total amount of chlorophyll *a* and cell numbers in the culture over the L/D cycles. If the cells were synchronised, chlorophyll *a* concentration should increase in the same stepwise manner as the cell concentration (see [13]). For *Prorocentrum lima*, Pan *et al*. [13] showed a L/D period-dependent increase and decrease of chlorophyll *a* cell quota, at least in the period before the

cultures became asynchronous. For *A. ostenfeldii*, there was no shift in the chlorophyll *a* per cell in relation to the L/D phases. This might be due to the low cell division rate and/or to poor synchronisation.

Numbers of motile cells collected were insufficient for statistically valid identification of the cell cycle phases using nuclear DNA staining and flow cytometry. Since we were unable to attain a high level of cell synchronisation with *A. ostenfeldii* via dark acclimation, we did not observe the typical pattern of stepwise increases in cell concentrations, as for *A. fundyense* [8] and *Prorocentrum* spp. [13].

*Production of Spirolides*

The dramatic increase in total spirolide per cell at the beginning of the dark period and the decrease during the light periods showed that spirolide biosynthesis is affected by light-dependent metabolic events. The >50% increase in the cell quota of spirolides after the L/D shift through several photocycles indicates a coupling of spirolide production to the photoperiod and cell cycle. By comparison, in *Prorocentrum lima*, the cell quota of the polyketide-derived DSP toxins increased in the light, but also extended through several phases of the cell cycle [12]. In contrast, although PSP toxin production by *A. fundyense* occurred in the light, synthesis was restricted to the G1 phase [8].

The transition of a fraction of the motile vegetative cells to pellicular cysts and the formation of dead cells may account for the variation in total spirolide concentration per unit culture volume. Pellicular cysts are a temporary quiescent stage produced through ecdysis of vegetative cells [14]. Since pellicular cysts are arrested in $G_o$-phase, maintaining only basal metabolism, these recurrent cells should have approximately the same cell quota of spirolides as vegetative cells before ecdysis. The maximal cell quota observed primarily from motile vegetative cells at the end of the dark phase is explicable as net spirolide production if this period also represents the late mitotic phases G2+M, just prior to cytokinesis.

The consistently low concentration of spirolide found in the medium (<3% of total spirolide of the whole culture) tends to indicate that leakage and excretion of spirolides from healthy vegetative cells, pellicular cysts and cell debris is not an important cycling mechanism. There is some preliminary evidence (M. Quilliam, unpublished data) that spirolides may be somewhat unstable in water at pH >5, although the decomposition rates in buffered seawater are unknown. Thus although it is conceivable that decomposition could account for low ambient spirolide levels in the medium, this is counter-indicated by the

relative consistency in the spirolide profile (major derivative des-methyl-C) found in both the cellular fraction and the medium.

It is still unclear if spirolide biosynthesis is directly light-dependent, or if biosynthesis, intracellular transport and excretion are indirectly mediated via the effects of light on enzymes and other functional metabolites. In any case, the apparent lack of any photoperiod-dependent shift in spirolide composition indicates that the cascade of events leading to biosynthesis of the various spirolide analogues is on a time-scale shorter than that of the sampling intervals. By comparison, in *Prorocentrum lima,* the production of DTX4 derivatives was initiated in G1 phase and continued into S phase, whereas other derivatives, such as OA and DTX1, were produced later in S and G2 phases [12].

This study has provided significant insights into the light-dependence of spirolide production, but little information is available on the biosynthesis of polyketide-derived metabolites by dinoflagellates. Further effort will be directed towards the use of cell synchronisation techniques coupled with studies of gene expression of putative biosynthetic genes for spirolides.

**ACKNOWLEDGEMENTS**

We thank N. Lewis (IMB, NRC) for technical assistance and W. Hardstaff (IMB, NRC) for help with spirolide analysis. This publication is NRC (Canada) No. 42335. The work was supported by BMBF TEPS (Project No. 03F0161).

**REFERENCES**

1. A.D. Cembella, N.I. Lewis and M.A. Quilliam, Phycologia 39, 67-74 (2000).
2. T. Hu, J.M. Curtis, J.A. Walter, J.L.C. Wright, Chem. Soc., Chem. Commun., 2159-2161 (1995).
3. T. Hu, J.M. Curtis, J.A. Walter, J.L.C. Wright, Tetrahed. Lett. 37, 7671-7674 (1996).
4. A.D. Cembella, M.A. Quilliam, N.I. Lewis, A.G. Bauder and J.L.C. Wright, in: Harmful Algae, B. Reguera, J. Blanco, M.L. Fernández, T. Wyatt, eds., (Xunta de Galicia and IOC-UNESCO, Santiago de Compostela), pp. 481-484 (1998).
5. A.D. Cembella, N.I. Lewis and M.A. Quilliam, Nat. Tox. 8, 1-10 (2000).
6. M.A. Quilliam, A. Cembella, D. Richard and S. Hancock, J. AOAC Int., in press.
7. A.D. Cembella, in: Physiological Ecology of Harmful Algal Blooms, NATO ASI Series, Vol. 41, (Springer-Verlag, Heidelberg), 381-403 (1998).
8. G. Taroncher-Oldenburg, D.M. Kulis and D.M. Anderson, Limnol. Oceanogr. 42, 1178-1188 (1997).
9. J.L.C. Wright and A.D. Cembella, in: Physiological Ecology of Harmful Algal Blooms, NATO ASI Series, Vol. 41, (Springer-Verlag, Heidelberg), pp. 427-451 (1998).
10. D.M. Anderson, D.M. Kulis, J.J. Sullivan, S. Hall and C. Lee, Mar. Biol.104, 511-524 (1990).
11. J.P. Parkhill and A.D. Cembella, J. Plank. Res. 21, 939-955 (1999).
12. Y. Pan, A.D. Cembella and M.A. Quilliam, Mar. Biol. 134, 541-549 (1999).
13. Y. Pan and A.D. Cembella, in: Harmful Algae, B. Reguera, J. Blanco, M.L. Fernández and T. Wyatt, eds., (Xunta de Galicia and IOC-UNESCO, Santiago de Compostela), pp. 173-176 (1998).
14. E. Garcés, M. Delgado, M. Masó, and J. Camp, J. Phycol. 34, 880-887 (1998)

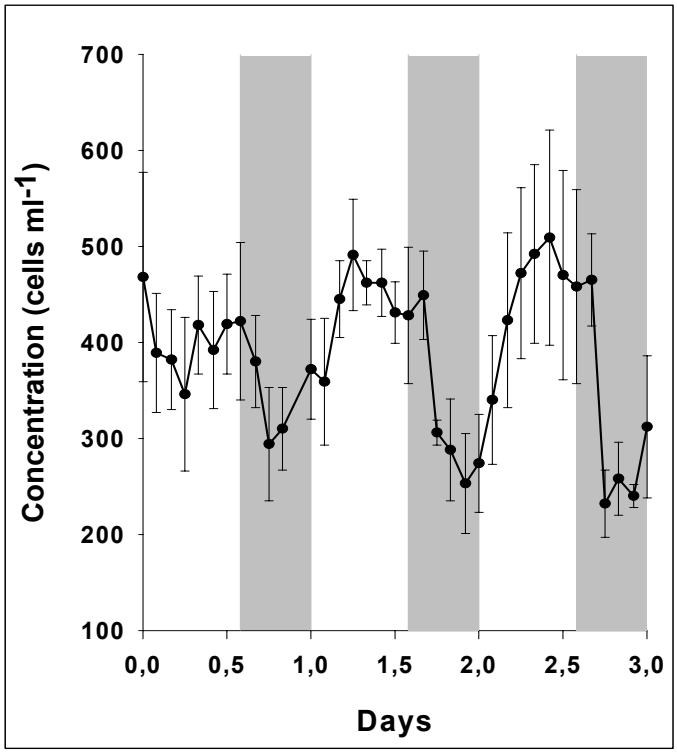



Fig. 1. Variation in cell concentration of *A. ostenfeldii* AOSH1 over several photocycles. Dark bars denote the darkness periods.

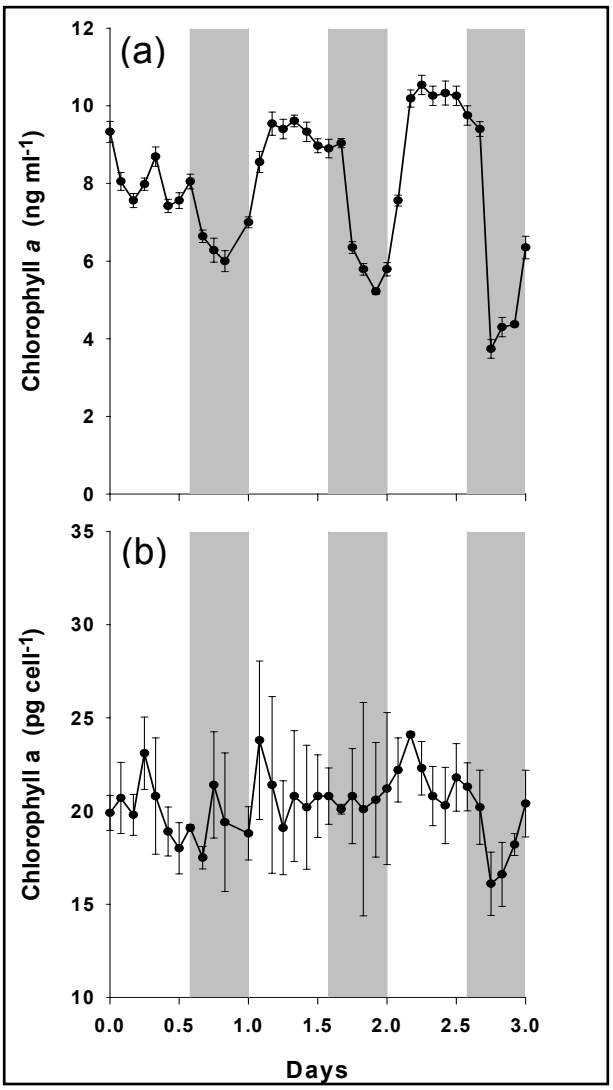



Fig. 2. Variation in chlorophyll *a* per unit culture volume (a) and per cell (b) over several photocycles. Note different scaling of the Y-axes.

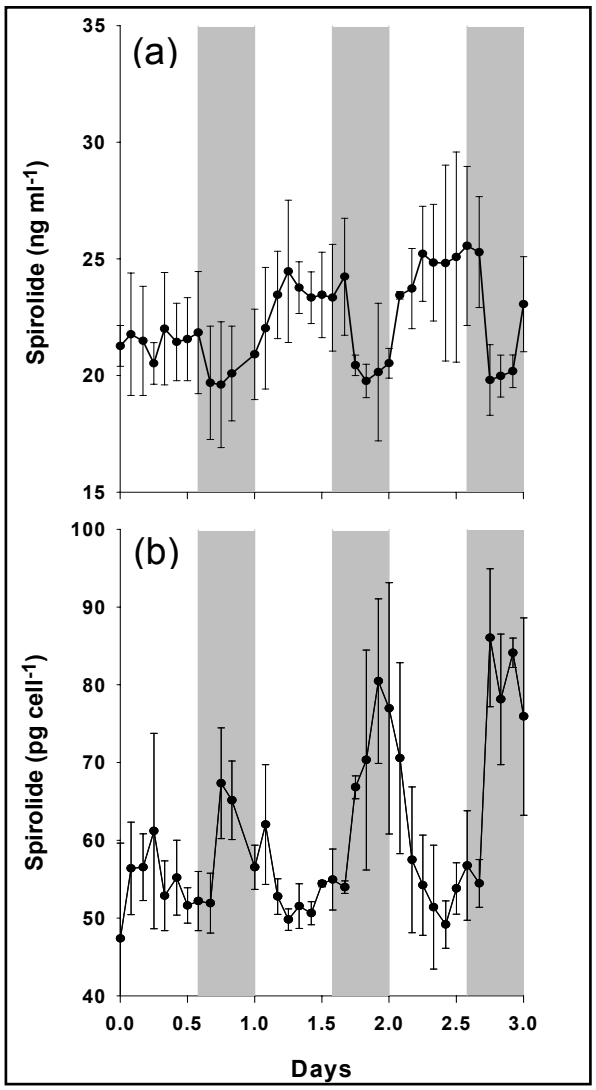



Fig. 3. Variation in total spirolide concentration per unit culture volume (a) and per cell (b) over **several** photocycles. Note different scaling of the Y-axes. X-axes.

# *2.7* Publication V: THE APPLICATION OF A MOLECULAR CLOCK BASED ON MOLECULAR SEQUENCES AND THE FOSSIL RECORD TO EXPLAIN BIOGEOGRAPHIC DISTRIBUTIONS WITHIN THE *ALEXANDRIUM TAMARENSE* "SPECIES COMPLEX" (DINOPHYCEAE)

*Uwe John*
Alfred-Wegener-Institut für Polar- und Meeresforschung, Am Handelshafen 12, D-27570 Bremerhaven, Germany

*Robert A. Fensome*
Natural Resources Canada, Geological Survey of Canada (Atlantic), Bedford Institute of Oceanography, P.O. Box 1006, Dartmouth, Nova Scotia, Canada B2Y 4A2

*and*

*Linda K. Medlin*[1]
Alfred-Wegener-Institut für Polar- und Meeresforschung, Am Handelshafen 12, D-27570 Bremerhaven, Germany

1 Correspondance to: L.Medlin. E-mail: lmedlin@awi-bremerhaven.de

*Abstract*

The cosmopolitan dinoflagellate genus *Alexandrium,* and especially the *A. tamarense* species complex, contain both toxic and non-toxic strains. An understanding of their evolution and paleogeography is a necessary precursor to unravelling the development and spread of toxic forms. The inclusion of more strains into the existing phylogenetic trees of the *Alexandrium tamarense* species complex from LSU rDNA sequences has confirmed that geographic distribution is consistent with the molecular clades, but not with the three morphologically-defined species that constitute the complex. In addition, a new clade has been discovered, representing Mediterranean non-toxic strains. The dinoflagellates fossil record was used to calibrate a molecular clock: key dates used in this calibration are the origins of the Peridiniales (estimated at 190 Ma), Gonyaulacaceae (180 Ma) and Ceratiaceae (145 Ma). Based on the data set analyzed, the origin of the genus *Alexandrium* was estimated to be around Late Cretaceous (77 Ma) with its earliest possible origination in the mid Cretaceous (119 Ma). The *A. tamarense* species complex potentially diverged around the early Neogene (23 Ma), with a possible first appearance in the late Paleogene (45 Ma). A paleobiogeographic scenario for *Alexandrium* is based on: 1) the calculated possible ages of origination for the genus and its constituent groups; 2) paleogeographic events determined by plate movements, changing ocean configurations and currents, as well as climatic fluctuations, and 3) the present geographic distribution of the various clades of the *Alexandrium tamarense* species complex.

Key index words: *Alexandrium tamarense*/*Alexandrium catenella*/*Alexandrium fundyense* species complex, biogeography, dinocysts, dinoflagellates, evolution, harmful algal blooms, molecular clock, phylogeny, toxic algae.

*INTRODUCTION*

*Alexandrium* is a much-studied goniodomacean dinoflagellate genus that currently contains 29 species, nine of which are known to produce paralytic shellfish poisoning (PSP) toxins (Balech 1995). Harmful algal blooms (HABs) involving these organisms are responsible for a wide variety of environmental and public health problems (Graneli et al. 1990; Hallegraeff 1993) and have a world-wide occurrence. Moreover, for reasons yet to be explained fully, such blooms appear to be increasing in frequency, intensity and distribution (Hallegraeff 1993, 1995).

The genus *Alexandrium* is subdivided primarily on the basis of differences of shape of particular plates, the presence or absence of a ventral pore, ornamentation in a few species, plus cell size, shape and chain formation (Balech 1995). Within the genus *Alexandrium, A. tamarense, A. fundyense* and *A. catenella* comprise a closely-related cosmopolitan toxigenic grouping of morphology-based species ("morphospecies"), the "*Alexandrium tamarense*" species complex, that play a prominent role in HABs. Individual morphospecies are identified by differences in cell shape and in the geometry of the apical pore complex (APC), by the presence (in *A. tamarense*) or absence (in *A. catenella*/*A. fundyense*) of a ventral pore on the apical plate (1'), and by the tendency to form chains (in *A. catenella*) or not (in *A. tamarense*/*A. fundyense*). Although the tabulational differences are sometimes very slight they remain consistent in cultures, aberrant individuals being very rare (Taylor 1975).

Phylogenetic studies of the *Alexandrium tamarense* species complex, based on 18S rDNA (Scholin 1993), the D1/D2 region of 28S rDNA (Scholin et al. 1994; Medlin et al. 1998; Higman et al. 2001) and ITS sequences (Adachi et al. 1996a), have yielded results that contrast with the conventional morphological approach. These studies have identified strains within the *A. tamarense* species complex that are distributed geographically rather than by morphospecies. Indeed, several of the ribotypes contain specimens that would be assignable to each of the three morphospecies of the *A. tamarense* species complex (Scholin et al. 1995). Thus, at least for molecular phylogenetic purposes, the three morphospecies are generally referred to collectively as the *A. tamarense* species complex.

Within the *A. tamarense* species complex, five different ribotypes/geographic clades have been previously identified: western European (WE), North American (NA), temperate Asian (TA), Tasmanian (TASM), and tropical Asian (TROP) clades. The NA, TA, and TROP clades consist only of toxic strains, whereas the WE and TASM clades are exclusively non-toxic. A new Mediterranean non-toxic clade (ME) is reported here for the first time.

Many dinoflagellate species produce zygotic cysts as part of their sexual cycle, some of which (about 13-16%) are fossilizable (Head, 1996). This fossil record, even though incomplete, yields important information that can be used to calibrate the timing of divergences in the lineage leading to *Alexandrium*. Although biological and biogeochemical evidence suggests an origin for the dinoflagellate lineage dating back to the late Proterozoic, which ended 545 million years ago (Ma), the earliest fossils confidently determined to be dinoflagellates date from about 240 Ma (Fensome et al. 1996, 1999). At around this time, dinoflagellates appear to have diverged in a true radiation event (Fensome et al. 1996). *Alexandrium* belongs to the family Goniodomaceae, within the order Gonyaulacales. The order Gonyaulacales appeared in the Late Triassic (about 200-210 Ma), but no confirmed members of the Goniodomaceae predate the Cretaceous - about 140 Ma (Fensome et al. 1993; 1996), and no fossils attributable to the genus *Alexandrium* have ever been recognized. However, fossil cyst-based genera, such as *Dinopterygium* and *Xiphophoridium,* reflect a tabulation very similar to that of *Alexandrium*, and first appear in the Albian age of the Cretaceous period, about 105 Ma. This date can therefore be used to provide some constraint on possible estimated dates for the divergence of *Alexandrium*-like morphotypes.

Unfortunately, only a few species that produce fossilizable cysts have been sequenced, most sequences deriving from species with no fossil record. However, molecular data can be used to reconstruct the phylogenetic relationships of recent organisms, and those organisms with a fossil record can be used to calibrate a molecular clock that can be used to extrapolate to potential divergence times of taxa lacking a fossil record. Certain biases exist in calculating a molecular clock: they are (1) the potential inaccuracy of fossil dates, (2) the possible misalignment of sequence data, (3) the algorithm chosen for tree construction, (4) unequal rates of evolution between lineages, and (5) unequal rates of evolution within a lineage through time. Software packages are available to correct for biases 2 and 3. Lintree (Takezaki et al. 1995) checks the molecular clock constancy for the given data set to eliminate quickly for slowly evolving sequences. Using Lintree, the rate of evolution is linearized to average the rate of evolution through time and between lineages. Thus, although a universal molecular clock may not exist and base substitution rates probably vary within lineages and genes (Ayala 2000) by correcting some biases, it is possible to use molecular data to estimate organism divergence times. However the fossil dates will always be underestimates because they record the first appearance of a taxon and not its molecular divergence. Hence, all molecular clocks underestimate divergence times.

The main objective of this study is to use information from both molecular sequences and the fossil record to construct a molecular clock and thus model the historical biogeography of *Alexandrium* and the *A. tamarense* species complex. An integral part of this objective is the

development of an evolutionary scenario for the distribution of the *Alexandrium tamarense* species complex that is consistent with paleoceanographic regimes, paleoclimates, and the present geographic distribution of the molecularly-identified clades. In the course of this study, we have also analyzed several new strains of *Alexandrium* with respect to their phylogenetic relationships within the genus *Alexandrium*.

*MATERIAL AND METHODS*

*Strains and culturing conditions*. For DNA extraction, unialgal strains of various taxa were cultured (Table 1). Cultures were grown in 500 ml Erlenmeyer flasks in IMR/2 growth medium (Eppley et al. 1967), supplemented with 10 nM selenite (for *Alexandrium tamarense*, *A. catenella*, *A. fundyense*, *A. pseudogoniaulax*, *A. taylorii*, and *A. minutum*), or in K medium (Keller et al. 1987) for *A. ostenfeldii*. All cultures were maintained at 15 $^{o}$C in a controlled growth chamber with a 14:10h light:dark photocycle, at a photon flux density of 150 µmol $m^{-2}$ $s^{-1}$, except for *A. ostenfeldii* (90 µmol $m^{-2}$ $s^{-1}$).

*DNA extraction, amplification of rRNA genes and sequencing.* DNA extractions were made from 500 mL of culture in logarithmic growth phase with a 3% CTAB (hexadecyltrimethylammonium bromide) procedure (Doyle and Doyle 1990). Thereafter, the DNA was treated with 10 µL RNase A (10 mg $mL^{-1}$) (QIAGEN, Hilden, Germany) for 30 min incubated at RT, followed by a 90 min incubation in a thermoshaker at 37°C with 20 µL of proteinase K (10 mg $mL^{-1}$), and purified using phenol:chloroform extraction with alcohol precipitation. DNA concentration was measured spectrophotometrically at 260 nm, and integrity was verified by agarose-gel electrophoresis. Polymerase chain reaction (PCR) conditions for amplifying the SSU rDNA gene and the D1/D2 region of the LSU rDNA gene follow the methodologies of Medlin et al. (1988), and Scholin et al. (1994), respectively. Three PCR products of amplified SSU genes and LSU D1/D2 regions, respectively, were pooled, purified, and then sequenced using the Long Read kit (Biozym, Hessisch Oldendorf, Germany) on a LiCor 4000L automatic sequencer (MWG, Ebersberg, Germany). Sequence alignment was done with CLUSTAL X software, and improved by eye for the SSU and LSU sequences. Full alignments for both genes can be obtained from the authors upon request.

*Sequence Analyses.* The data set for the D1/D2 region of the LSU rDNA contained 70 taxa and 635 unambiguously aligned bp out of 720 bases and was rooted using *Prorocentrum minimum* as an outgroup. Hierarchical Likelihood Ratio Tests (hLRTs) were performed using Modeltest

Version 3.(Posada and Crandall 1998; 2001) to determine the best model out of 56 different models of evolution that best fit the data for the Maximum Likelihood (ML) analysis.

ML phylogenies were reconstructed with PAUP* 4.0b8 (Swofford 1998) constrained with the following Modeltest parameters. The model selected for the LSU rDNA data set was General Time Reversible model with a gamma distribution (GTR+G) with base frequencies of A = 0.2486, C = 0.1706, G = 0.2586, T = 0.3222; base substitution rates of (G T = 1.0000, A C = 0.8472, A G = 1.8546, A T = 0.8128, C G = 0.5084, C T = 2.8610, G T = 1.0000; proportion of invariable sites I = 0; and gamma distribution shape parameter = 0.5980.

For the SSU rDNA sequence data set containing 34 taxa and 1751 bp, we used *Tetrahymena thermophila* as an outgroup. hLRTs gave GTR model allowing for invariant sites and a gamma distribution (GTR+I+G) as the model that fit best the data set. The ML-tree calculation was constrained using base frequencies of A = 0.2781, C = 0.1803, G = 0.2477, T = 0.2939; base substitution rates of: A C = 1.0000, A G = 2.2697, A T = 1.0000, C G = 1.0000, C T = 4.5862, G T = 1.0000; proportion of invariable sites I = 0.2239; and a gamma distribution shape parameter = 0.6120. Bootstrap values (Felsenstein 1985) were generated for the Maximum Parsimony (MP) and with Neighbor –Joining (NJ) analyses using the ML settings for the distance analysis with 500 replicates for LSU analysis and 1000 replicates for SSU analysis, respectively. For the SSU data set, 572 sites were informative for the MP analysis resulting in a tree with a length of 1970 steps, a 0.6122 CI index and 0.7318 RI index. For the LSU data set, 324 sites were informative for the MP analysis resulting in a tree with a length of 1114 steps, a 0.6266 CI index and 0.8930 RI index.

The phylogenetic relationships of the dinoflagellates in general and species of the genus *Alexandrium* in particular were also determined by Bayesian inference (BI) (Huelsenbeck and Ronquist 2001; Huelsenbeck et al. 2001) using the SSU rDNA and the D1/D2 region of the LSU rDNA data sets, respectively. The advantages of BI are that it is relatively fast, even when large data sets are used, and it generates probabilistic measures of tree strength, which gives posterior probabilities (PP) for phylogenetic stability (Huelsenbeck et al. 2001 and references therein). These values are more straightforward to interpret than bootstrap values, because they can be taken as the probability that the topology of a tree is correct and represents the best estimated phylogeny. The BI settings for the SSU rDNA sequence data set were GTR+G+I with base frequencies estimated and 1.2 x $10^6$ Markov chain Monte Carlo (MCMC) generations and four simultaneous MCMC chains, for the LSU rDNA GTR+G with base frequencies estimated and 1.5 x $10^6$ MCMC generations and four simultaneous MCMC chains, respectively. The analysis was done using MrBayes (http://morphbank.ebc.uu.se/mrbayes/).

To estimate the approximate divergence times of species and clades with molecular data within the data set, a linearized tree was constructed under the assumption of a molecular clock. Lintree constructed an NJ tree with the pairwise distance option of TrN+G, allowing for variable base substitution rates and a gamma distribution. The data set was tested with the two-cluster test, which examines the equality of the average substitution rate for two clusters that are created by each node in the tree. Sequences that evolved significantly (at 1% level) faster or slower compared to the average rate were eliminated from the data set. Because elimination of sequences from the data set affects tree topology, NJ trees and two-cluster tests were repeated iteratively until a data set was obtained with nearly all taxa evolving within a Poisson distribution rate of evolution. Some fast or slow evolving taxa can be retained in the data set if their inclusion is critical for the tree topology and for the analyses (Takezaki et al. 1995). Thereafter, a linearized tree for a given topology was constructed for the remaining sequences after using the two-cluster test.

A regression of first appearance dates of the genus *Alexandrium* and the *A. tamarense* species complex from fossil occurrences (Ma) against branch lengths (distance) of taxa and strains in the linearized tree was performed. The average possible age for the undated nodes was estimated by multiplying the length of its average branch by the regression coefficient. The earliest possible age of the undated nodes is taken from the upper 95% confidence limit given the distance of its average branch (Hillis et al. 1996).

## *RESULTS*

### *Phylogeny of Alexandrium*.

Starting with 67 dinoflagellate SSU rDNA sequences, 33 were eliminated because they evolved too fast or too slow at $p > 0.05$ level according to the two cluster test (Takezaki et al. 1995): the resulting SSU tree is shown in Fig. 1. Dinoflagellate phylogenies constructed with all available sequences can be found in Edvardsen et al. (2003). Our ML-tree generated from 34 sequences used *Tetrahymena thermophila* and two *Perkinsus* strains as closest outgroups to the dinoflagellates. The remaining 31 sequences, belonging to several species of dinoflagellates, were used to analyze the phylogenetic relationship of *Alexandrium* to other dinoflagellates. If *Perkinsus* remains intermediate between apicomplexans and dinoflagellates (Litaker et al. 1999), then *Noctiluca scintillans* is the earliest derived extant dinoflagellate species, diverging before the thecate Peridiniphycidae. In this data set, the Peridiniales, represented by species of *Peridinium*, diverge before the Gonyaulacales. Within the Gonyaulacales, the subfamily Gonyaulacoideae of

the family Gonyaulacaceae, represented by species of *Gonyaulax*, diverged first, followed by *Protoceratium*, a gonyaulacacean of the subfamily Cribroperidinioideae; next was the Ceratiaceae represented by *Ceratium*, then the Pyrocystaceae represented by *Pyrocystis*, and lastly the Goniodomaceae represented by *Alexandrium*.

Of species of *Alexandrium* examined to date, *A. taylorii* appears to be the earliest to diverge. Thereafter, species diverge into two clusters. The first cluster consists of *A. margalefii, A. ostenfeldii* and the *A. minutum/lusitanicum* species complex. Within the second cluster, the first species to diverge is *A. tamiyavinichii*, with well-supported bootstrap and PP values, then the *Alexandrium tamarense* species complex. The two SSU sequences of the new Mediterranean clade fall into the *A. tamarense* species complex. Again, the SSU sequences of the *Alexandrium tamarense* species complex do not support the monophyletic nature of the three morphospecies. In contrast to the LSU sequence analysis (see below), no geographic clades were differentiated because the rate of evolution in the SSU gene is much slower than that of the D1/D2 region of the LSU gene.

The analysis of the LSU rDNA data set provides better resolution of the *A. tamarense* species complex (Fig. 2). The simplest measure of evolutionary distance in molecular phylogenetics is the number of base differences per species. We have calculated nucleotide differences among strains of the *Alexandrium* species complex. An alignment of 635 bp of 22 *Alexandrium* strains shows that the number of different nucleotides among the sequences of the *A. tamarense* species complex varies from 12 for the WE clade, to over 15 for NA clade sequences, to 19 for the new Mediterranean clade (ME), and to 29 for the TA clade. However, the sequence of CU13 strain, formerly designated by Scholin et al. (1994) as the TROP clade, contained 46 nucleotides that distinguish it from the *A. tamarense* species complex. Of these 46 nucleotides, 39 were shared with *A. tamiyavanichii*, which is distinguished from CU13 by 27 nucleotides, and 66 nucleotides from to the *A. tamarense* species complex. In contrast, there were only two nucleotide differences observed between *A. concavum* and *A. affine*. The distance matrix calculated from these base differences shows that, within each geographic clade or ribotype, the distance ranged from 0.006 to 0.024. However, between clades or ribotypes the average distance was 0.103 and ranged from 0.078 between WE to NA to 0.165 between TA to ME. The TROP clade showed an average distance to the remaining members of the species complex of 0.182 and to TA of 0.192. Within the TROP clade, the distance between CU13 and *A. tamiyavanichii* was 0.113, whereas the distance between *A. concavum* and *A. affine* was only 0.009.

The phylogenetic analysis of the D1/D2 region of the LSU rDNA shows *A. taylorii* as the first divergence in *Alexandrium*. Thereafter, *A. margalefii* diverges, followed by a split into the *A.*

*minutum/lusitanicum* species complex with *A. pseudogoniaulax* and a cluster consisting of *A. ostenfeldii* and *A. andersonii* (TCO2). The final divergence in the tree is between the *A. affin*e, *Alexandrium tamiyavanichii*, and the *A. tamarense* species complex. In this latter cluster, we see the expected differentiation of the species complex into geographic clades as previously described by Scholin et al. (1995), Medlin et al. (1998), and Higman et al. (2001). All clades and their branching order within the species complex were well supported by bootstrap values (MP/NJ) and posterior probabilities (PP), except for the tropical Asian clade (TROP), which now consists of the strain CU13 of the *A. tamarense* species complex and the species *A. tamiyavanichii*. There is no bootstrap support or posterior probabilities for the position of this clade, and in this analysis it falls unsupported prior to the divergence of the geographic clades of the *A. tamarense* species complex. Analyses using different models resulted in trees in which CU13 and *A. tamiyavanichii* diverge before *A. affine* (data not shown). The major species complex diverges in two clusters, the first cluster containing the non-toxic WE clade and the toxic TA clade and, within it, an early divergence of the Tasmanian strain ATBB01. The second cluster, which diverges slightly after the first one, consists of the toxic NA clade, to which the Orkney Islands (Scotland) isolates belong. The NA clade is sister to the four sequences of our new non-toxic Mediterranean clade (ME).
*Linearized Tree*. As mentioned, from an original data set of 67 dinoflagellate sequences, we eliminated 33 because their rate of evolution did not fall within a Poisson distribution (Takezaki et al. 1995). Nevertheless, three taxa were retained in the data set even though their SSU sequences evolved too fast. This is because their inclusion helped to produce a tree topology similar to that of the LSU rDNA tree as well as to the evolutionary tree produced by Fensome *et al.* (1993) from morphological data. *Perkinsus* was too fast but was used as outgroup in the ML analysis. *Pyrocystis* evolved too fast with respect to *Alexandrium*. Within *Alexandrium*, *A. margalefii* evolved too fast. However, all other clusters evolved at the same average speed and were used for molecular clock calculation. The topology of the linearized TrN+G NJ tree (Fig. 3A) compared well with the ML tree (Fig. 1), there being only slight differences. *Noctiluca, Peridinium*, and *Protoceratium* collapsed to a polytomy. Also, *Ceratium* and *Pyrocystis* could not be separated with this analysis. The topology of the linearized tree is in accord with the classification of Fensome *et al.* (1993) (Fig. 3B).

*Fossil dates plotted on the geological time scale.*

Times of origin for extant families, genera, and species were obtained from the plots and charts of Fensome et al. (1996, 1999) and Williams et al. (1998, 1999). Based on fossil evidence, the divergence between gonyaulacaleans and peridinialeans (the two principal orders of thecate

dinoflagellates found as fossils) appears to have occurred early in the Jurassic, about 190 Ma. Hence, we use this date for the origin of the Peridiniales. The order Gonyaulacales, as defined by Fensome et al. (1993), included the atypical rhaetogonyaulacineans, whose range extends back into the late Triassic, to about 210 Ma. However, gonyaulacaleans with a typical gonyaulacacean tabulation first appear around the Early/Mid Jurassic boundary, about 180 Ma, a date we thus use for the divergence node of *Gonyaulax spinifera* in our linearized tree (Fig 3A and C). For the family Ceratiaceae, Riding et al. (2000) reported the dinoflagellate *Muderongia simplex* from the late Kimmeridgian *rotunda* Zone (about 145 Ma), which is thus used to date the divergence of the Ceratiaceae. These three dates were plotted onto a geological time scale (Fig. 3C), with black arrows showing their position in the linearized tree (Fig. 3A). We used first appearance dates of taxa of higher rank (orders and families) to calibrate our tree, because their first appearance dates are less ambiguous than those of taxa of lower rank.

*Calibration of the molecular clock*.

Linearized branch lengths were regressed against the three fossil dates to calculate a molecular clock according to the method described by Hillis et al. (1996). As already noted, ages derived from the fossil record represent the latest date for an event and are underestimates. We used the dates mentioned above: 190 Ma for the Peridiniales; 180 Ma for the Gonyaulacaceae; and 145 Ma for the Ceratiaceae. The molecular clock thus constructed was then used to extrapolate dates for the nodes of unfossilized taxa, e.g., *Alexandrium* and its species. The average time of origin for the genus *Alexandrium* (77 Ma) and the *Alexandrium tamarense* species complex (23 Ma) was calculated from the average branch lengths of each group, respectively. The earliest possible origin of the genus (119 Ma) and the species complex (45 Ma) was calculated from the upper 95% confidence limit, given the lengths of the average branch of each group respectively.

*DISCUSSION*

We have used the SSU rDNA analysis to investigate relationships within the genus *Alexandrium* because, using this marker, the resolution between major species is appropriate for the comparisons needed. The D1/D2 region of the LSU rDNA is useful only when finer resolution between strains is needed, because it evolves at a much higher rate. Our phylogenetic analysis of the SSU of rDNA sequences was consistent with those of previous studies (Saunders et al. 1997; Walsh et al. 1998, Litaker et al. 1999, Saldarriaga et al. 2001, Edvardsen et al. 2003). Our analysis

also generally agreed with the conventional classification of dinoflagellates by Fensome et al. (1993). The SSU rDNA tree shows that the Goniodomaceae was one of the last families to diverge within the Gonyaulacales, that *Alexandrium* is monophyletic, supported by high bootstrap and posterior probability values, and that there is a clear differentiation of species (or species complexes) within the genus. However, the two subgenera of *Alexandrium*, *Alexandrium* subgenus *Alexandrium* (in which the first apical homologue - *1' – contacts the apical pore complex - apc) and *Alexandrium* subgenus *Gessnerium* (in which *1' does not contact the apc) form no clear groups in our phylogenetic trees. *Alexandrium taylorii*, *A. margalefii* and, in the case of the LSU analysis, *A. pseudogoniaulax,* all representatives of subgenus *Gessnerium,* formed no distinct group. Instead *Alexandrium pseudogoniaulax* is a sister group of *A. minutum* and *A. lusitanicum*, both members of subgenus *Alexandrium*. We suggest that more species and isolates of subgenus *Gessnerium* should be analyzed in future studies to clarify the phylogenetic status of the two subgenera. The close relationship between *Alexandrium ostenfeldii* and the *A. lusitanicum/minutum* species complex was unexpected, because of their different sizes and morphologies. Also, the *A. tamarense* species complex shares a last common ancestor with *A. tamiyavanichii*, which was also its sister taxon in the LSU tree. Unfortunately, no sequences were obtained from the TROP clade nor from *A. affine*. Hence, for the latter species, for which sequences were obtained from our SSU data set, its order of divergence with respect to *A. tamiyavanichii* and the CU13 strain could not be clarified (see the discussion below on the resolution in the LSU tree). The sequences of the new Mediterranean clade fall as expected within the *A. tamarense* species complex.

The phylogenetic analysis of the LSU rDNA gene of the *Alexandrium* sequences confirms earlier reports (Scholin et al. 1994; Medlin et al. 1998; Adachi et al. 1996a) that the *A. tamarense* species complex is separated into distinct geographic clades. These are the NA, TA, WE, and ME clades, not the three morphotypes (*A. tamarense*, *A. catenella*, and *A. fundyense*). Hence, of the 29 species that Balech (1995) included in *Alexandrium*, some may not be truly distinct species (Taylor and Fukuyo, 1998).

The LSU rDNA sequences of the four isolates from the Mediterranean Sea form a sister group to the North American clade within the *A. tamarense* species complex, with well supported bootstrap and posterior probability values. Also the nucleotide differences and the distance values of these sequences, compared to the sequences within the other geographic clades support their recognition as a new clade in the tree. This may not be the last discovery of a new ribotype within the *A. tamarense* species complex: reports of the *A. tamarense* species complex from the Southern Hemisphere indicate that they are part of the NA clade (reviewed by Taylor 1987b; Gayoso 2001;

Lilly et al. 2002). However, in order to determine whether these new isolates are indigenous or introduced by human activity through ballast water or shellfish stocks (Scholin et al. 1995), they will have to be analyzed by the more recently available molecular probes (Adachi et al. 1996b; Scholin et al. 1997, John et al. 2003). As an example, the strains BAHME215, 217, and 222 have been isolated from the Spanish coast and their sequences group together with ALcatHK1, ALcatHK2 and ALexcat1 isolates from Hong Kong Harbour. This result could indicate that the Spanish isolates have been introduced by human activity.

Earlier studies have shown that the TROP clade represents the ancestral population of the *A. tamarense* species complex (Scholin et al. 1994; Medlin et al. 1998). Adachi et al. (1996a) have suggested that the isolates of the TROP clade might be a different species, because the distance values of the ITS region between isolates of the TROP clade and the NA clade is greater than those between the other clades. Similar relationships of the distance values were obtained in our analysis. Among the NA, ME, WE, and TA clades, the average distance was 0.103, but 0.192 between TROP and the other species complex clades. The distance between CU13 and *A. tamiyavanichii* was 0.09. However, *A. tamiyavanichii* is morphologically clearly different from the *A. tamarense* morphotype (Balech 1995), so a misidentification of CU 13 is unlikely. Therefore we suggest that at this cladogenesis, the *A. tamarense* morphotype appeared and that CU 13 and *A. tamiyavanichii* diverged from a common ancestral taxon, which likely bore the *A. tamarense* morphotype because the CU13 strain bears that morphotype. However, the position of the branch of CU13 and *A. tamiyavanichii* had no bootstrap and posterior probability support. Higman et al. (2001) and Usup et al. (2002) used the NJ method to construct a phylogenetic tree and showed that the TROP clade diverged before *A. affine*. Higman et al. (2001) suggested that these results were obtained because they had only one representative of each in their analysis, and the analytical method used might have affected the outcome as well. Unfortunately, no bootstrap values were presented in their analysis, which makes an interpretation of their results difficult. But the analysis of Usup et al. (2002) shows a strong support of boostrap values for *A. affine* to be the sister group of *A. tamarense* species complex. However, in future studies more sequences of the TROP clade and *A. tamiyavanichii* should be included in the analysis to clarify the position and identity of the true sister group of the *A. tamarense* species complex.

*Alexandrium affine* and *A. concavum* cluster together, bootstrap values and the posterior probability supporting their position as sister to the *A. tamarense* species complex (see above), with the TROP clade either diverging before or after them depending of the evolutionary model used. Based on morphological features, *A. affine* and *A. concavum* should diverge before the TROP clade, as shown in Fig. 2 and by Scholin et al. (1994), Adachi et al. (1996a) and Medlin et

al. (1998). Balech (1995) considered the position of *A. concavum* to be uncertain. Despite its exceptionally large size, it is difficult to study because of its delicate theca. Even its biology is poorly understood: it is one of the rarest oceanic *Alexandrium* species. If it has not been misidentified based on the small distance value of 0.006, the divergence between *A. concavum* and *A. affine* must have occurred very recently.

In generating the linearized tree, 33 taxa were excluded from the data set because the evolution rates of their SSU rDNA gene were too fast. Our final SSU rDNA data set for phylogenetic study of the dinoflagellates and for the calibration of a molecular clock included 34 taxa. Similar problems, with large variation in the substitution rate of rDNA genes has been previously shown for foraminifera (Pawlowski et al. 1997). The rDNA of planktonic foraminifera evolves 50 to 100 times faster than those of the benthic foraminifera. There are two hypotheses that might explain these differences in DNA substitution rates: the generation time effect hypothesis (Li et al. 1996); and the metabolic rate hypothesis (Martin 1995). These factors might be responsible for the acceleration of the evolution rate in the planktonic versus the benthic foraminifera. Pawlowski et al. (1997) assumed that a higher reproduction rate, shorter generation time, more exposure to solar UV radiation, and changes in the DNA replication or DNA repair mechanism have resulted in a higher mutation rate for the planktonic foraminifera. Benthic, planktonic, parasitic, and endosymbiontic species were among the 67 dinoflagellate taxa that were initially used in the two cluster test (Takezaki et al. 1995). These species exhibited variable generation times and metabolisms, with some being autotrophic, some mixotrophic, and others heterotrophic. Any of these factors might have resulted in a high variance in evolutionary rates among the sequences, and similar explanations to those invoked for foraminifera may also be applicable to dinoflagellates.

Our molecular clock is only a hypothetical model to investigate the biogeographic distribution of the *A. tamarense* ribotypes, because the relationships among the geographic clades exhibit vicariant events rather than dispersal events. We estimate that the average age of the genus *Alexandrium* is 77 Ma (Late Cretaceous), and no earlier than 119 Ma (mid Cretaceous); these dates do not conflict with the 105 Ma date for the closest dinoflagellates with similar tabulation and fossilizable cysts. At 120 Ma, climate and water temperature were much warmer than today. Shallow seas covered much of the continental areas, with sea levels up to 200 m higher than today. These continental areas were arranged such that there was a global circum-equatorial current within the Tethys Ocean (Scotese 1991; Marincovich et al. 1990). Between 65 Ma and 55 Ma, two catastrophic events affected global biodiversity: the end Cretaceous mass extinction event (65 Ma); and the Late Paleocene thermal maximum (55 Ma), with a deep-sea temperature

increase of 5 - 6°C that killed benthic foraminifera and apparently caused planktonic microalgae, including dinoflagellates to proliferate (Crouch et al. 2001; Zachos et al. 2001). In the early Paleogene (40-65 Ma), the ocean basins were significantly re-arranged as Tethys closed, new oceans opened, resulting in lowered sea level and a cooler seasaonal global climate. Permanent polar ice sheets formed (Bice et al. 2000; Zachos et al. 2001), and the length of global coastlines and the area of continental shelves both increased. Coastal regions became more heterogeneous in topological, hydrodynamic and climatic conditions, thus promoting regional differences (Scotese 1997).

Under these mid Cenozoic conditions, *Alexandrium* likely diverged into several taxa (Fig. 1, 3A). The *A. tamarense* species complex diverged probably around the early Neogene (23 Ma), but no earlier than the late Paleogene (45 Ma). A global distribution of planktonic species was possible through the eastern Indian Ocean, Tethys and the Pacific Ocean, with counter currents for anti-clockwise distributions. At 36 Ma, the Tasmania-Antarctica and Drake passages opened, forming the Antarctic Circumpolar Current (ACC) and intensifying conditions favorable for the build up of increasing Antarctic ice sheets and ocean fertility (Zachos et al. 2001 and references therein). When the Tethys Ocean closed, populations became isolated in various ocean basins. This regionalizing effect was enhanced when, from about 3-13 Ma, the Isthmus of Panama was uplifted, cutting of the tropical Pacific-Atlantic connection and reorganizing Northern Hemisphere ocean circulation. As a result, surface waters cooled through North Atlantic deep water formation, which could have increased precipitation of the Northern Hemisphere and promoted glaciation after 2.5-3 Ma (Haug and Tiedemann 1998). These geological events likely lead to allopatric speciation of global planktonic populations.

Given mid Cenozoic paleoclimatic and geological changes, we propose the following scenario to explain the modern distribution of the strains within the *Alexandrium tamarense* species complex. Our scenario starts with a globally distributed ancestral population (Fig. 4A,B), which diverges first into eastern and western Pacific populations (Fig. 4C,D) as a response to a relatively short but deep glacial maximum around 23 Ma (Paul et al. 2000). The eastern Pacific population was connected to Atlantic populations through the Central American Seaway and its counter currents, whereas the western Pacific population was connected to eastern Atlantic populations through Tethys (Fig. 4C, D). The heterogeneous climatic and oceanic conditions between 40-65 Ma likely promoted phenotypic and genetic differentiation within the *A. tamarense* species complex. When the Tethys Ocean closed, the western Pacific population diverged into TA (yellow stars in Fig. 4E) and WE clades (black stars in Fig. 4E). As the Isthmus of Panama uplifted, ancestral populations in the sub-tropical Atlantic (white stars in Fig. 4E) were

separated from those in the eastern Pacific (NA clade: orange stars in Fig 4E). The closing of Tethys, the formation of the Mediterranean Sea, and the uplift of the Panama Isthmus created significant changes in circulation and paleoclimate (Haug and Tiedemann 1998). Around 5 Ma, the Mediterranean Sea dried up and was subsequently refilled by tropical and sub-tropical Atlantic water with sub-tropical Atlantic *A. tamarense* populations. Eventually, indigenous sub-tropical Atlantic populations became extinct because of unfavorable environmental conditions, leaving relict populations, the ME clade (white stars in Fig. 4F), in the Mediterranean. Relict populations of the ancient sister group of the *A. tamarense* species complex can be found in tropical waters (red stars Fig. 4F) although, as already discussed, the precise species identification of this sister group is still under debate.

Scholin et al. (1998) reported an isolate from the Kamchatka Peninsula that has a TA/NA intermediate genotype, an observation that may support the initial east/west separation in the Pacific. As suggested by Scholin et al. (1995), the North American east coast population may have originated from an ancestral population from the west coast. Veron (1995) stated that as the Panama Isthmus was emplaced, northern Pacific waters were drawn into the North Atlantic. Thus, Pacific populations may have migrated through the Bering Strait into the Arctic Ocean and the Labrador Sea. Alternatively, as Medlin et al. (1997) noted, migration may have been via the Fram Strait and Greenland currents, with later dispersal via the Gulf Stream; this scenario also explains the occurrence of the NA clade along the Scottish coast. The possibility of human introduction of the Scottish occurrence has been discussed (Higman et al. 2001), but was discounted by Medlin et al. (1997) because of the high number of base substitutions within and between the Scottish isolates. We assume that the relationships uncovered in the LSU rDNA tree show speciation in progress and represent allopatric vicariant populations. Fig 4F shows the idealized distribution of the *A. tamarense* species complex populations. In recent times, populations from different geographic clades have been introduced into new areas via ballast water or shell fish stocks exchange, often into areas where *Alexandrium* populations had never been previously reported (Scholin 1995; Hallegraeff 1998). More intensive examinations of sediment material has uncovered the presence of cysts (Taylor pers. comm.)

The *Alexandrium tamarense* morphotype can be found in all ribotypes, and the ribotypes are not fully reproductively isolated: they can still interbreed, even if with lower zygote survival rates (Sako et al. 1990). Based on current data, it is difficult to offer an explanation as to why the three different morphotypes are found in the two toxic ribotypes, whereas the non-toxic ribotypes contain only the *A. tamarense* morphotype. We suggest that the *A. tamarense* morphotype, which is characterized by, for example, the presence of a ventral pore on the first apical plate, is

plesiomorphic. The tendency in the *A. catenella* morphotype, for example, to form chains may be an apomorphic feature; this tendency is represented in the TA and NA clades. The *A. fundyense* morphotype, in which a ventral pore is lacking, is only present in the NA clade; thus, this morphotype is probably apomorphic. Both the *A. catenella* and *A. fundyense* morphotypes may indicate an ongoing speciation process. The results at least show that morphological features used to discriminate *A. fundyense* delineate a biologically meaningful clade within the species complex. However, not even these features make an unambiguous identification of the NA clade possible, because this clade also includes *A. tamarense* and *A. catenella* morphotypes. In further studies, taxonomists might examine isolates from the different clades of the *A. tamarense* species complex to seek new morphological features that might reflect the different ribotypes. However, such features may not be obvious since cryptic speciation appears to be common in unicellular organisms (De Vargas et al. 1999, Medlin et al. 1995).

The observation that ribotypes of *Alexandrium*, rather than morphotypes, reflect geographic areas is not new. Cembella et al. (1988) was the first to discuss the distinction between *A. tamarense* and *A. catanella* and, since then, much effort has been made to understand the geographic and genetic distribution of the *A. tamarense* species complex. Our knowledge of the species complex today results primarily from the work of Scholin (1998). Our discovery of a new ribotype emphasizes that ideas concerning the evolution and distribution of forms within the genus have to be reconsidered continuously. The development of a molecular clock using data from the fossil record helps to predict when groups may have diverged, and offers a new hypothesis to explain the extant distribution of clades within the *Alexandrium tamarense* species complex. It has also helped to elucidate evolutionary relationships among *Alexandrium* species recovered in our phylogenetic analyses.

*Acknowledgements*: The work was supported in part by research funds to LKM from BMBF TEPS 03F0161. We thank the individuals listed in Table 1 for supplying us with cultures and Drs Wiebe H.C.F. Kooistra and Malte Elbrächter for fruitful discussions of an earlier version of the manuscript. We are grateful to Drs Martin Head and André Rochon for informal reviews of the manuscript. We would also like to thank Dr. Alberto Garcia Sáez for introducing MrBayes software. This is Geological Survey of Canada Contribution no. 2002166.

*Reference List*

Adachi, M., Sako, Y. and Ishida, Y. 1996a. Analysis of *Alexandrium* (Dinophyceae) species using sequences of the 5.8S ribosomal DNA and internal transcribed spacer regions. *J. Phycol*. 32:424-32.

Adachi, M., Sako, Y. and Ishida, Y. 1996b. Identification of the toxic dinoflagellates *Alexandrium catenella* and *A. tamarense* (Dinophyceae) using DNA probes and whole-cell hybridization. *J. Phycol*. 32:1049-52.

Adachi, M., Sako, Y. and Ishida, Y. 1997. Restriction fragment length polymorphism of ribosomal DNA internal transcribed spacer and 5.8S regions in Japanese *Alexandrium* species (Dinophyceae). *J. Phycol*. 33:440.

Ayala, F.J. 2000. Neutralism and selectionism: the molecular clock. *Gene* 261:27-33.

Balech, E. 1995. The Genus *Alexandrium* Halim (Dinoflagellata). Sherkin Island Marine Station, Sherkin Island Marine Station, Ireland.

Bice, K. L., Scotese, C. R., Seidov, D. and Barron, E. J. 2000. Quantifying the role of geographic change in Cenozoic ocean heat transport using uncoupled atmosphere and ocean models. *Palaeogeogr., Palaeoclimatol., Palaeoecol*. 161(3-4):295-310.

Cembella, A.D., Taylor, F.J.R. and Therriault, J.C. 1988. Cladistic analysis of electrophoretic variants within the toxic dinoflagellate genus *Protogonyaulax* . *Bot. Mar*. 31:39-51.

Crouch, E.M., Heilmann-Clausen, C., Brinkhuis, H., Morgans, H.E.G. and Rogers, K.M. 2001. Global dinoflagellate event associated with the late Paleocene thermal maximum. *Geology* 29:315–318.

Destombe, C., Cembella, A.D., Murphy, C.A. and Ragan, M.A. 1992. Nucleotide sequence of the 18S ribosomal RNA genes from the marine dinoflagellate *Alexandrium tamarense* (Gonyaulacales, Dinophyta). *Phycologia* 31:121-42.

deVargas, C., Norris, R., Zaninetti, L., Gibb, S. W. & Pawlowski, J. 1999. Molecular evidence of cryptic speciation in planktonic foraminifers and their relation to oceanic provinces *Proc. Natl. Acad. Sci. USA* 96**,** 2864-2868.

Doyle, J.J. and Doyle J.L. 1990. Isolation of plant DNA from fresh tissue. *Focus* 12:13-15.

Edvardsen, B., Shalchian-Tabrizi, K., Jakobsen, K., Medlin, L.K., Dahl, E., Brubak,S. and Paasche, E. 2003. Genetic variability and molecular phylogeny of *Dinophysis* species (Dinophyceae) from Norwegian waters inferred from single cell analyzes of ribosomal DNA. J. Phycol. 38.

Eppley, R.W., Holmes, R.W. and Strickland, J.D.H. 1967. Sinking rates of the marine phytoplankton measured with a fluorochrometer. *J. Exp. Mar. Biol. Ecol*. 1:191-208.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39:783-791.

Fensome, R.A., Taylor, F.J.R., Norris, G., Sarjeant, W.A.S., Wharton, D.I. and Williams, G.L. 1993. A classification of living and fossil dinoflagellates. *Micropaleontology*, Special Publication Number 7.

Fensome, R.A., Macrae, R.A., Moldowan, J.M., Taylor, F.J.R. and Williams, G.L. 1996. The early Mesozoic radiation of dinoflagellates. *Paleobiology* 22:329-338.

Fensome, R.A., Saldarriaga, J.F. and Taylor, F.J.R.M. 1999. Dinoflagellate phylogeny revisited: reconciling morphological and molecular based phylogenies. *Grana* 38:66-80.

Gayoso, A.M. 2001. Observations on *Alexandrium tamarense* (Lebour) Balech and other dinoflagellate populations in Golfo Nuevo, Patagonia (Argentinia). *J. Plankton Res*. 23:463-468.

Halim, Y. 1960: *Alexandrium minutum* nov. gen. nov. sp. dinoflagellé provocant des "eaux rouges". *Vie et Millieu*, 11: 102-105.

Hallegraeff, G.M. 1993. A review of harmful algal blooms and their apparent global increase. *Phycologia* 32:79-99.

Hallegraeff, G. M. 1995. Harmfull algal blooms: A global overview. *In* Hallegraeff, G. M., Anderson, D. M., and Cembella, A. D. [Eds.] Manual on Harmful Marine Microalgae, UNESCO, IOC Manual and Guides No. 33, pp. 1-22

Hallegraeff, G.M. 1998. Transport of toxic dinoflagellates via ships' ballast water: bioeconomic risk assessment and efficacy of possible ballast water management strategies. *Mar. Ecol. Prog. Ser*. 168:297-309.

Haug, G.H. and Tiedemann, R. 1998. Effect of the formation of the Isthmus of Panama on Atlantic Ocean thermohaline circulation. *Nature* 393: 673-676.

Head, M. 1996. Modern dinoflagellate cysts and their biological affinities. *In* Jansonius, J. & McGregor, D.C., [Eds.] Palynology: principles and applications. Dallas, USA., pp. 1197-1248

Higman, W.H., Stone, D.M. and Lewis, J.M. 2001. Sequence comparisons of toxic and non-toxic *Alexandrium tamarense* (Dinophyceae) isolates from UK waters. *Phycologia* 40:256-262.

Hillis, D. M., Moritz, C., and Mable, B. K. 1996. Molecular Systematics Sinauer Associates, Inc., Sunderland, Massachusetts U.S.A.

Huelsenbeck, J.P. and Ronquist, F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17:754-755.

Huelsenbeck, J.P., Ronquist, F., Nielsen, R. and Bollback, J.P. 2001. Evolution - Bayesian inference of phylogeny and its impact on evolutionary biology. *Science* 294:2310-2314.

John, U., Cembella, A. D., Hummert, C., Elbrächter, M., Groben, R. & Medlin, L. K. 2003. Discrimination of the toxigenic dinoflagellates *Alexandrium tamarense* and *Alexandrium ostenfeldii* in co-occurring natural populations from Scottish coastal waters. *Euro. J. Phycol*. In press.

Keller, M.D., Selvin, R.C., Claus, W. and Guillard, R.R.L. 1987. Media for the culture of oceanic ultraphytoplankton. *J. Phycol*. 23:633-638.

Li, W.H., Ellsworth, D.L., Krushka, J., Chang, B.H.J. and Hewett-Hemmett, D. 1996. Rates of nucleotide substitution in primates and rodents and the generation-time effect hypothesis. *Mol. Phylogenet. Evol*. 5:182-187.

Lilly, E., Taroncher-Oldenburg, G. and Anderson, D.M. 2002. The global biogeography of the genus *Alexandrium*. Xth International Conference on Harmful Algae, October 21-25 2002, St. Pete Beach, Florida, USA. Abstract pp. 171.

Litaker, R.W., Tester, P.A., Colorni, A., Levy, M.G. and Noga, E.J. 1999. The phylogenetic relationship of *Pfiesteria piscicida*, Cryptoperidiniopsoid sp., *Amyloodinoum ocellatum* and a *Pfiesteria*-like dinoflagellate to other dinoflagellates and apicomplexans. *J. Phycol*. 35:1379-1389.

Marincovich, L.Jr., Brouwers, E.M., Hopkins, D.M. and McKenna, M.C. 1990. Late Mesozoic and Cenozoic paleogeographic and paleaclimatic history of the Arctic Ocean Basin, based on shallow-water marine faunas and terrestial vertebrates. *In* Grantz, A., Johnson, L., & Sweeney, J.F., [Eds.] The Arctic Ocean region. The Geological Society of America, Boulder, Colorado, USA., pp. 403-426

Martin, A.P. 1995. Mitochondrial DNA sequence evolution in sharks - rates, patterns, and phylogenetic inferences. *Mol. Biol. & Evol*. 12:1114-1123.

Medlin, L.K., Lange, M., Barker, G.L.A. and Hayes, P.K. 1995. Can molecular techniques change our ideas about the species concept? *In* Joint, I., Falkowski, P., and Carr, N. [Eds.] NATO ASI Series - Molecular Ecology of Aquatic Microbes. Vol. 38 pp. 133-152

Medlin, L., Lange, M., Wellbrock, U., Donner, G., Elbrächter, M., Hummert, C. and Luckas, B. 1998. Sequence comparison links toxic European isolates of *Alexandrium tamarense* from the Orkney Islands to toxic north American stocks. *Europ. J. Protistol*. 34:329-335.

Paul, H.A., Zachos J.C., Flower B.P., Tripati A. 2000. Orbitally induced climate and geochemical variability across the Oligocene/Miocene boundary. *Paleoceanography* 15(5):471-485.

Pawlowski, J., Bolivar, I., Fahrni, J.F., Vargas, C.d., Gouy, M. and Zaninetti, L. 1997. Extreme Differences in Rates of Molecular Evolution of Foraminifera Revealed by Comparison of Ribosomal DNA sequences and the Fossil Record. *Mol. Biol. Evol*. 14:498-505.

Posada, D. and Crandall, K.A. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics Application Note* 14:818-18.

Posada, D. and Crandall, K.A. 2001. Selecting the Best-Fit Model of Nucleotide Substitution. *Syst. Biol*. 50:580-601.

Riding, J.B., Poulsen, N.E. and Bailey, D.A. 2000. A taxonomic study of the the dinoflagellate cyst *Muderongia simplex* Alberti 1961 and related species. *Palynology* 24:21-35.

Sako, Y., Kim, C.H., Ninomiya, H., Adachi, M. and Ishida, Y. 1990. Isozyme and cross analysis of mating populations in the *Alexandrium catenella/tamarense* species complex. *In*

Granéli, E., Sundström, B., Edler, L., & Anderson, D.M., [Eds.] Toxic Marine Phytoplankton Elsevier, New York, pp. 320-323

Saldarriaga, J.F., Taylor, F., Keeling, P.J. and Cavalier Smith, T. 2001. Dinoflagellate Nuclear SSU rRNA Phylogeny Suggests Multiple Plastid Losses and Replacements. *J. Mol. Evol*. 53: 204-213.

Saunders, G.W., Hill, D.R.A., Sexton, J.P. and Andersen, R.A. 1997. Small-subunit ribosomal RNA sequences from selected dinoflagellates: testing classical evolutionary hypotheses with molecular systematic methods. *In* Bhattacharya, [Eds.] Origins of Algae and their Plastids Springer, Wien, pp. 237-259

Scholin, C.A. (1993). Analysis of toxic and non-toxic Alexandrium (Dinophyceae) species using ribosomal RNA gene sequences. Ph.D. thesis, Massachusetts Institute of Technology/Woods Hole Oceanographic Institution, WHOI-93-08, 251 pp.

Scholin, C., Miller, P., Buck, K., Chavez, F., Harris, P., Haydock, P., Howard, J. and Cangelosi, G. 1997. Detection and quantification of *Pseudo-nitzschia australis* in cultured and natural populations using LSU rRNA-targeted probes. *Limnol. Oceanogr*.42:1265-1272.

Scholin, C.A. 1998. Morphological, genetic, and biogeographic relatioships of the toxic dinoflagellates *Alexandrium tamarense*, *A. catenella*, and *A. fundyense*. *In* Anderson, D.M., Cembella, A.D., & Hallegraeff, G.M., [Eds.] Physiological Ecology of Harmful Algal Blooms Springer-Verlag, Berlin, pp. 13-27

Scholin, C.A., Anderson, D.M. and Sogin, M.L. 1993. Two distinct small-subunit rRNA genes in the North American toxic dinoflagellate *Alexandrium fundyense* (Dinophyceae). *J. Phycol*. 29:209-16.

Scholin, C.A., Hallegraeff, G.M. and Anderson, D.M. 1995. Molecular evolution of the *Alexandrium tamarense* species complex (Dinophyceae)-dispersal in the North American and West Pacific regions. *Phycologia* 34:472-485.

Scholin, C.A., Herzog, M., Sogin, M. and Anderson, D.M. 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.

Scotese, C. R. 1991. Jurassic and Cretaceous plate tectonic reconstructions. *Palaeogeogr. Palaeoclimatol. Palaeoecol*. 87:493-501.

Scotese, C. R., 1997. Paleogeographic Atlas, PALEOMAP Progress Report 90-0497, Department of Geology, University of Texas at Arlington, Arlington, Texas, 45 pp.

Smayda, T.J. 1990. Novel and nuisance phytoplankton blooms in the sea: evidence for a global epidemic. *In* Graneli, E., Sundström, B., Edler, L., & Anderson, D.M., [Eds.] Toxic Marine Phytoplankton Elsevier, New York, pp. 29-40

Swofford, D. L. 1998. PAUP*. Phylogenetic analysis using Parimony (* and other methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Takezaki, N., Rzhetsky, A. and Nei, M. 1995. Phylogenetic Test of the Molecular Clock and Linearized Trees. *Mol. Bol. Evol*. 12:823-833.

Taylor, F.J.R. 1975. Taxonomic difficulties in red tide and paralytic shellfish poison studies: the 'Tamarensis complex' of *Gonyaulax*. *Environ. Lett.* 9:103-119.

Taylor, F. J. R. 1987a. The Biology of Dinoflagellates Blackwell, Oxford, UK.

Taylor, F. J. R. 1987b. General and marine ecosystems. *In* Taylor, F.J.R., [Eds.] The Biology of Dinoflagellates Blackwell, Oxford, pp. 399-502

Taylor, F.J.R. and Fukuyo, Y. 1998. The Neorotoxigenic Dinoflagellate Genus *Alexandrium* Halim: General Introduction. *In* Anderson, D.M., Cembella, A.D., & Hallegraeff, G.M., [Eds.] Physiological Ecology of Harmful Algal Blooms Springer, Berlin, pp. 3-12

Usup, G., Pin, L.C., Ahmad, A. and Teen, L.P. 2002. Phylogenetic relationship of *Alexandrium tamiyavanichii* (Dinophyceae) to other *Alexandrium* species based on ribosomal RNA gene sequences.*Harmful Algae* 1:59-68.

Veron, J.E.N. 1995. Corals in space and time. Comstock/Cornell, Itaker, U.S.A. 321 pp.

Walsh, D., Reeves, R.A., Saul, D.J., Gray, R.D., MacKenzie, L., Bergquist, P.R. and Bergquist, P.L. 1998. Heterogeneity of SSU and LSU rDNA sequences of *Alexandrium* species. *Biochem. Syst. Ecol*. 26:495-509.

Williams, G.L., Brinkhuis, H., Bujak, J.P., Damassa, S.P., Hochuli, P.A., De Verteuil, L. and Zevenboom, D., 1998. Dinoflagellate cysts. In: Appendix to: Hardenbol J., Thierry J., Farley M.B., Jacquin Th., de Graciansky P.-C. and Vail P.R. Mesozoic and Cenozoic sequence chronostratigraphic framework of European basins. In: de Graciansky, P.C., Hardenbol, Jacquin, T. and Vail, P.R., Mesozoic and Cenozoic sequence stratigraphy of European basins; *Society of Sedimentologists and Geologists, Special Publication*, 60: 764-765.

Williams, G.L., Fensome, R.A., Bujak, J.P. and Brinkhuis, H., 1999. Mesozoic-Cenozoic dinoflagellate cyst course, Urbino, Italy, May 17-22, 1999. (Unpublished course handbook)

Zachos, J., Pagani, M., Sloan, L., Thomas, E. and Billups, K. 2001. Trends, Rhythms, and Aberrations in Global Climate 65 Ma to Present. *Science* 292:686-693.

Table 1: List os strains used in this study

| Species | Strain or abbreviation as used in this study | Gene: SSU; *produced in this study | Gene: LSU; *produced in this study | Geographic clade | Geographic origin | Collector |
|---|---|---|---|---|---|---|
| *Alexandrium affine* (Inoue and Fukuyo) Balech | A.affine | | L38630 | | | |
| | Alexaffi | | AAU44935 | | | |
| *Alexandrium catenella* (Whedon and Kofoid) Balech | BAHME215 | | AJ535361* | TA | Tarragona (Spain) | M. Delgado |
| | BAHME217 | AJ535392* | AJ535362* | TA | Tarragona (Spain) | M. Delgado |
| | BAHME222 | | AJ535359* | TA | Tarragona (Spain) | M. Delgado |
| | ALexcat1 | | AF019408 | TA | | |
| | ALexcat3 | | AF042818 | TA | | |
| | ALcatHK1 | | AF118547 | TA | | |
| | ALcatHK2 | | AF118546 | TA | | |
| *Alexandrium concavum* (Gaarder) Balech | ALexconc | | AF032348 | | | |
| *Alexandrium fundyense* Balech | Alexfund | U09048 | | NA | | |
| *Alexandrium lusitanicum* Balech | A.lusita | | | | | |
| *Alexandrium margalefii* Balech | ALexmarg | U27498 | AF033531 | | | |
| *Alexandrium minutum* Halim | AL1T | | AJ535352* | | Gulf of Trieste (Italy) | A. Beran |
| | AL3T | AJ535388* | AJ535353* | | Gulf of Trieste (Italy) | A. Beran |
| | AL8T | | AJ535350* | | Gulf of Trieste (Italy) | A. Beran |
| | AL9T | | Aj535360* | | Gulf of Trieste (Italy) | A. Beran |
| | L20/2 | | AJ535351* | | Gulf of Trieste (Italy) | A. Beran |
| | Alexminu | U27500 | | | Gulf of Trieste (Italy) | A. Beran |
| | Alexmin1 | U27499 | | | | |
| *Alexandrium ostenfeldii* (Paulsen) Balech and Tangen | AOSH1 | | AJ535358* | | Nova Scotia (Canada) | A. Cembella |
| | Alexostf | U27500 | | | | |
| | K0324 | AJ535381* | AJ535363* | | Limfjord (Denmark) | P.J. Hansen |
| | K0287 | AJ535382* | AJ535356* | | Limfjord (Denmark) | P.J. Hansen |
| | BAHME136 | | AJ535357* | | Timaru (New Zealand) | N. Berkett |
| | Alexoste | | AF033533 | | | |
| *Alexandrium pseudogoniaulax* (Biecheler) Horiguchi, Yuki & Fukuyo | AP2T | | AJ535355* | | Gulf of Trieste (Italy) | A. Beran |
| *Alexandrium tamarense* (Lebour) Balech | Alextama | X54946 | AF033534 | | | |
| | Aletamar | AF022191 | | | | |
| | OF842332.4 | | AJ535364* | NA | Ofunata Bay (Japan) | Kodama |
| | AT-9 | | AJ535364* | NA | Ofunata Bay (Japan) | Kodama |
| | SZN01 | AJ535387* | AJ535368* | ME | Gulf of Naples (Italy) | M. Montresor |
| | SZN08 | | AJ535369* | ME | Gulf of Naples (Italy) | M. Montresor |
| | SZN19 | AJ535386* | AJ535370* | ME | Gulf of Naples (Italy) | M. Montresor |
| | SZN21 | | AJ535374* | ME | Gulf of Naples (Italy) | M. Montresor |
| | UW53 | | Higman et al. 2001 | WE | Belfast (Nord Ireland) | W. Higman |
| | UW42 | | Higman et al. 2001 | WE | Belfast (Nord Ireland) | W. Higman |
| | 31/4 | AJ535391* | Higman et al. 2001 | WE | Cork Harbour (Ireland) | W. Higman |
| | 31/9 | | Higman et al. 2001 | WE | Cork Harbour (Ireland) | W. Higman |
| *Alexandrium tamiyavanichii* Balech | Atamiy | AF113935 | AF174614 | | | |
| *Alexandrium taylorii* Balech | AY1T | AJ535390* | AJ535347* | | Lagoon of Marano (Italy) | A. Beran |
| | AY2T | AJ535385* | AJ535348* | | Lagoon of Marano (Italy) | A. Beran |
| | AY4T | AJ535389* | AJ535349* | | Lagoon of Marano (Italy) | A. Beran |
| *Ceratium fusus* (Ehrenberg) Dujardin | Cerafus2 | AF022153 | | | | |
| *Ceratium tenue* Ostenfeld et Schmidt | Certenue | AF022192 | | | | |
| *Gonyaulax spinifera* (Claparède et Lachmann) Diesing | Gonyspin | AF022155 | | | | |
| *Noctiluca scintillans* (Macartney) Kofoid et Swezy | Noct.ilu | AF022200 | | | | |
| *Peridinium bipes* Stein | Peri.bip | AF231805 | | | | |
| *Peridinium* sp. | Peridini | AF022199 | | | | |
| *Peridinium willei* Huitfelt-Kaas | Periwill | AF274272 | | | | |
| *Perkinsus marinus* | Perkmar | X75762 | | | | |
| *Perkinsus* sp. | Perksp | L07375 | | | | |
| *Prorocentum micans* Ehrenberg | PmicaM04 | | P385 Sylt | | | |
| *Prorocentum minimum* (Pavillard) Schiller | Prormin6 | | AF042813 | | | |
| *Protoceratium reticulatum* (Claparède et Lachmann) Bütschli | Protreti | AF274273 | | | | |
| *Pyrocystis lunula* (Schütt) Schütt | Pyrolunu | AF274274 | | | | |
| *Pyrocystis noctiluca* Murray ex Haeckel | Pyrocyst | AF022156 | | | | |
| *Tetrahymena thermophila* | Tetr.the | X56165 | | | | |

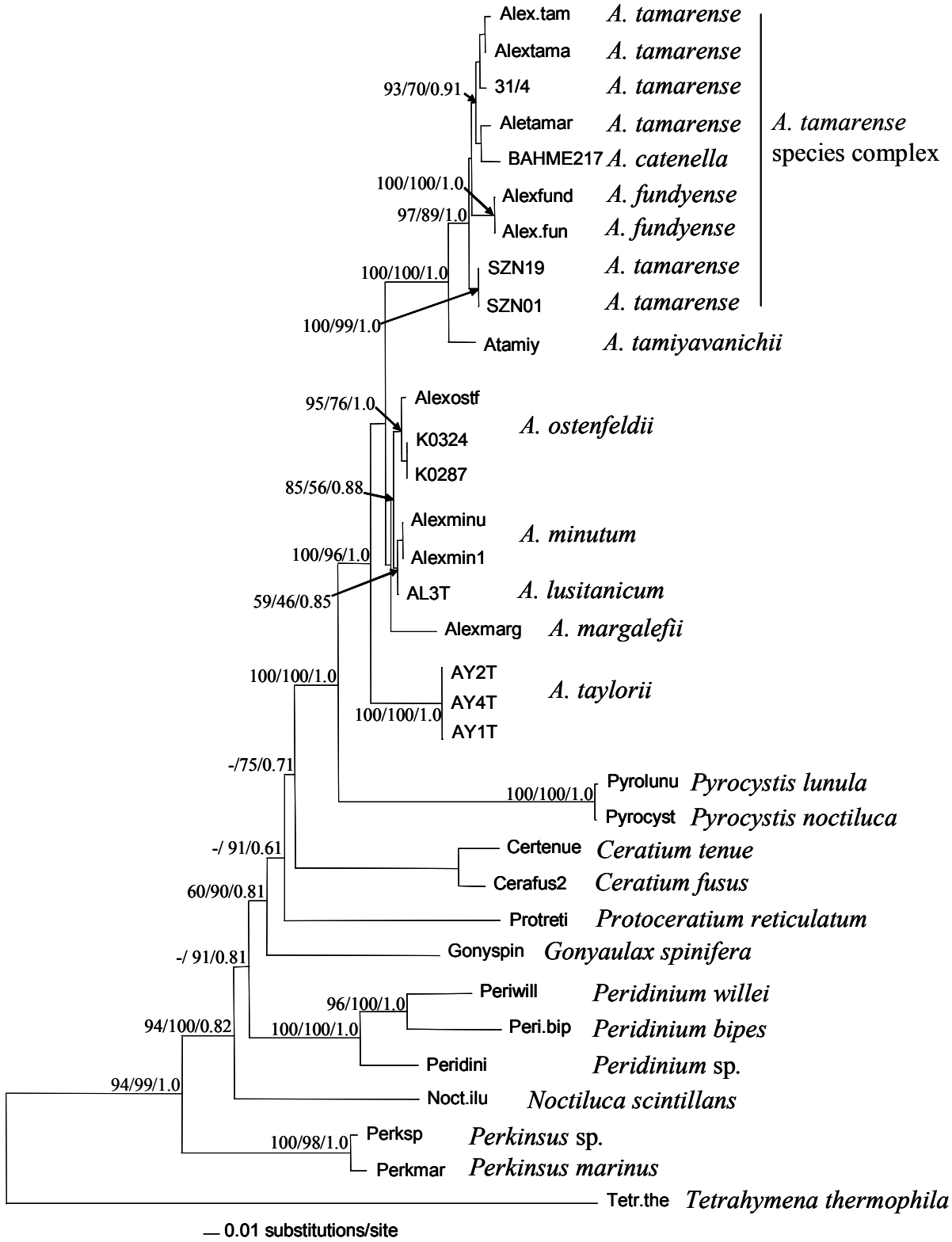
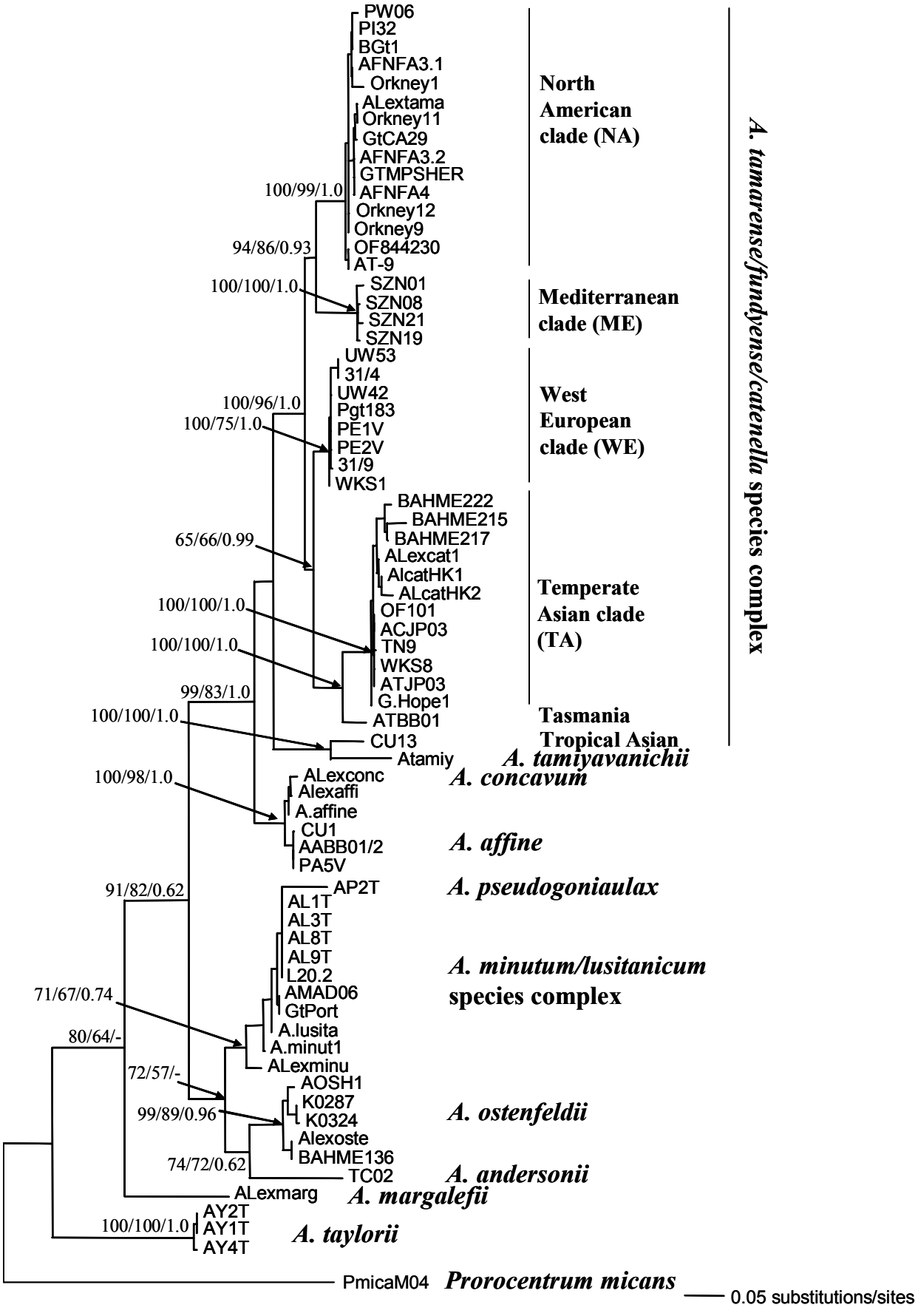

Alex.tam
A. tamarense
Alextama
A. tamarense
93/70/0.91
31/4
A. tamarense
Aletamar
A. tamarense
A. tamarense
species complex
BAHME217
A. catenella
100/100/1.0
Alexfund
A. fundyense
97/89/1.0
Alex.fun
A. fundyense
100/100/1.0
SZN19
A. tamarense
SZN01
A. tamarense
100/99/1.0
Atamiy
A. tamiyavanichii
95/76/1.0
Alexostf
A. ostenfeldii
K0324
K0287
85/56/0.88
Alexminu
A. minutum
100/96/1.0
Alexmin1
AL3T
A. lusitanicum
59/46/0.85
Alexmarg
A. margalefii
100/100/1.0
AY2T
AY4T
A. taylorii
100/100/1.0
AY1T
-/75/0.71
Pyrolunu
Pyrocystis lunula
100/100/1.0
Pyrocyst
Pyrocystis noctiluca
-/ 91/0.61
Certenue
Ceratium tenue
Cerafus2
Ceratium fusus
60/90/0.81
Protreti
Protoceratium reticulatum
Gonyspin
Gonyaulax spinifera
-/ 91/0.81
Periwill
Peridinium willei
96/100/1.0
94/100/0.82
Peri.bip
Peridinium bipes
100/100/1.0
Peridini
Peridinium sp.
94/99/1.0
Noct.ilu
Noctiluca scintillans
Perksp
Perkinsus sp.
100/98/1.0
Perkmar
Perkinsus marinus
Tetr.the
Tetrahymena thermophila
0.01 substitutions/site

Figure 1. (previous page) Maximum likelihood phylogenetic tree of 18S SSU rDNA sequences from dinoflagellates. *Tetrahymena thermophila* (a ciliate) was used as an outgroup. The tree is generated using PAUP 4.08b with a GTR+I+G model with a number of invariable positions = 0.2239 and a gamma shape = 0.6120. Sequences corresponding to strains that are not in Table 2 were taken from Destombe et al. (1992) and Scholin et al. (1993). Bootstrap values (>50%) from an MP/NJ analysis placed close to each node or arrow show the corresponding node. The BI tree was of similar topology, the third number noted at the branches are the posterior probabilities.

Figure 2. (next page) Maximum likelihood phylogenetic tree of representatives of the genus *Alexandrium* based on their sequences of the D1/D2 region of the LSU rDNA. *Prorocentrum micans* and *P. minimum* were used as outgroups. The tree was generated using PAUP 4.08b with a GTR+G model with a gamma shape = 0.5980. Sequences corresponding to strains that are not in Table 3 were taken from Scholin et al. (1994), Medlin et al. (1998) and Higman et al. (2001). Bootstrap values (>50%) from an MP/NJ analysis are placed close to each node or arrow show the corresponding node. The BI tree was of similar topology, the third number noted at the branches are the posterior probabilities.


PW06
PI32
BGt1
AFNFA3.1
Orkney1
ALextama
Orkney11
GtCA29
AFNFA3.2
GTMPSHER
AFNFA4
Orkney12
Orkney9
OF844230
AT-9
SZN01
SZN08
SZN21
SZN19
UW53
31/4
UW42
Pgt183
PE1V
PE2V
31/9
WKS1
BAHME222
BAHME215
BAHME217
ALexcat1
AlcatHK1
ALcatHK2
OF101
ACJP03
TN9
WKS8
ATJP03
G.Hope1
ATBB01
CU13
Atamiy
ALexconc
Alexaffi
A.affine
CU1
AABB01/2
PA5V
AP2T
AL1T
AL3T
AL8T
AL9T
L20.2
AMAD06
GtPort
A.lusita
A.minut1
ALexminu
AOSH1
K0287
K0324
Alexoste
BAHME136
TC02
ALexmarg
AY2T
AY1T
AY4T
PmicaM04
100/99/1.0
94/86/0.93
100/100/1.0
100/96/1.0
100/75/1.0
65/66/0.99
100/100/1.0
100/100/1.0
99/83/1.0
100/100/1.0
100/98/1.0
91/82/0.62
71/67/0.74
80/64/-
72/57/-
99/89/0.96
74/72/0.62
100/100/1.0
North American clade (NA)
Mediterranean clade (ME)
West European clade (WE)
Temperate Asian clade (TA)
Tasmania
Tropical Asian
A. tamarense/fundyense/catenella species complex
A. tamiyavanichii
A. concavum
A. affine
A. pseudogoniaulax
A. minutum/lusitanicum species complex
A. ostenfeldii
A. andersonii
A. margalefii
A. taylorii
Prorocentrum micans
0.05 substitutions/sites

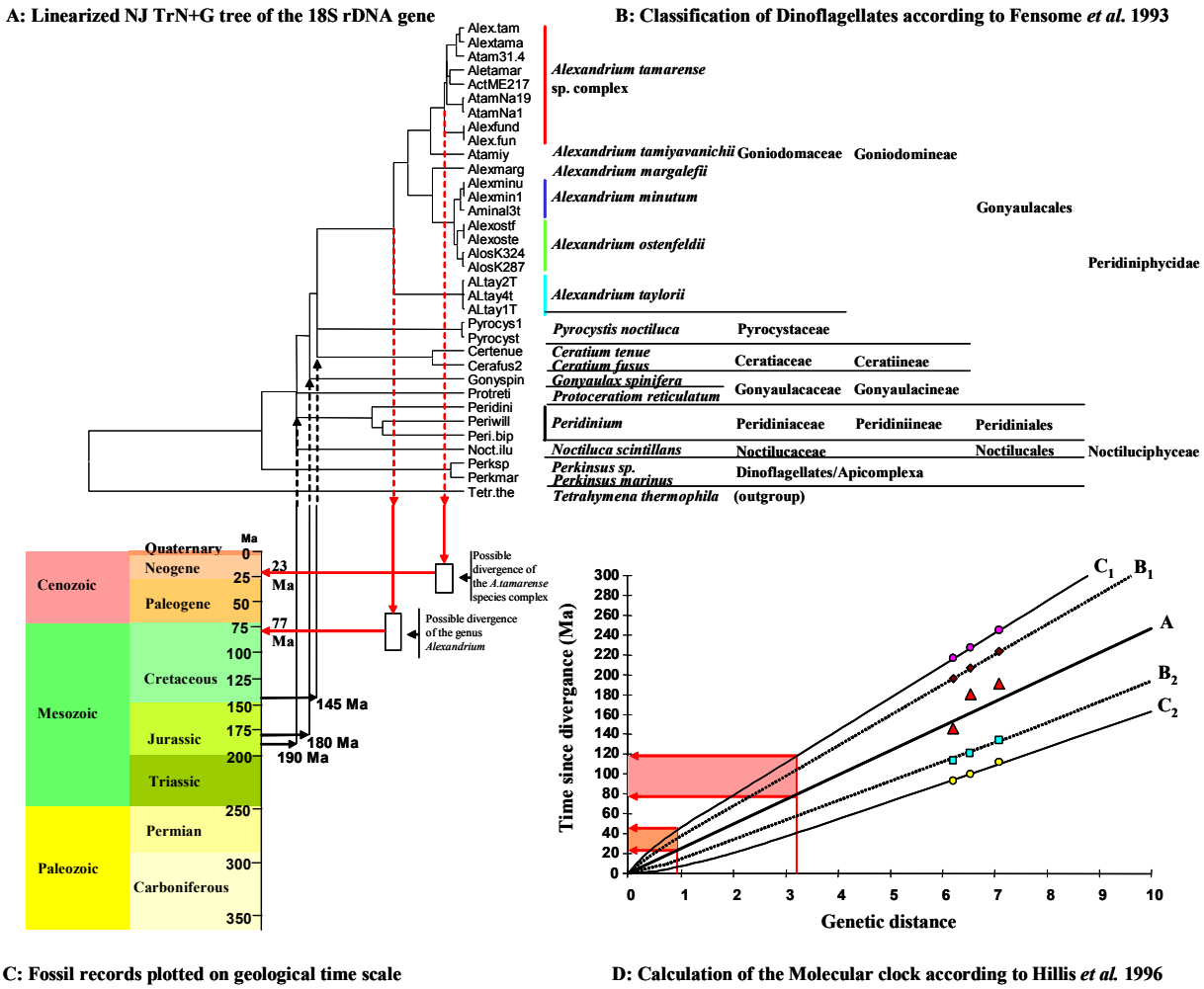



Figure 3. (next page) A: Linearized neighbor-joining tree constructed from the Tamura and Nei gamma distribution distances and from an unlinearized NJ tree generated using Lintree (Takezaki et al. 1995) of the SSU rDNA from dinoflagellates. Black arrows marks the fossil events in the linearized tree; the red arrow 1 shows the divergence of the genus *Alexandrium*; and red arrow 2 shows the divergence of the *A. tamarense* species complex. B: Systematic classification of dinoflagellates (Fensome et al. 1993). C: Fossil events and the calculated divergences of both the genus *Alexandrium* and the *A. tamarense* species complex plotted on a geological time scale. Boxes symbolize the variance in appearance dates: the y-axis shows the possible appearance from the lower 95% confidence ($B_2$) of regression line (A) to the earliest possible appearance ($C_1$) calculated using the molecular clock (D); the x-axis has no meaning. Black arrows show the fossil dates and demonstrate their position within the linearized tree (A); the red arrows connect the nodes of divergence of both the genus *Alexandrium* and the *A. tamarense* species complex with the geological time scale according to the calculated dates of the molecular clock. D: Molecular clock calibration for the linearized tree in A, from the SSU nuclear encoded rDNA gene from dinoflagellates. First appearance of the genus *Alexandrium* and the *A. tamarense* species complex were regressed against measured branch lengths from the linearized tree (A). For the molecular clock: A is the regression of estimated time since separation on sequence divergence of SSU rDNA in dinoflagellates, constrained through the origin. $B_1$ and $B_2$ are the bounds of the 95% confidence limits of the regression line. $C_1$ and $C_2$ are the bounds of the 95% confidence limits for a new predicted value of time given the lengths of an undated node. Arrows are shown the origin of the groups estimated from the molecular clock. Lower arrow shows the average age of the genus or the species complex respectively and the upper arrow shows the earliest possible time of origin based on the upper 95% confidence internal ($C_1$) of an undated node.

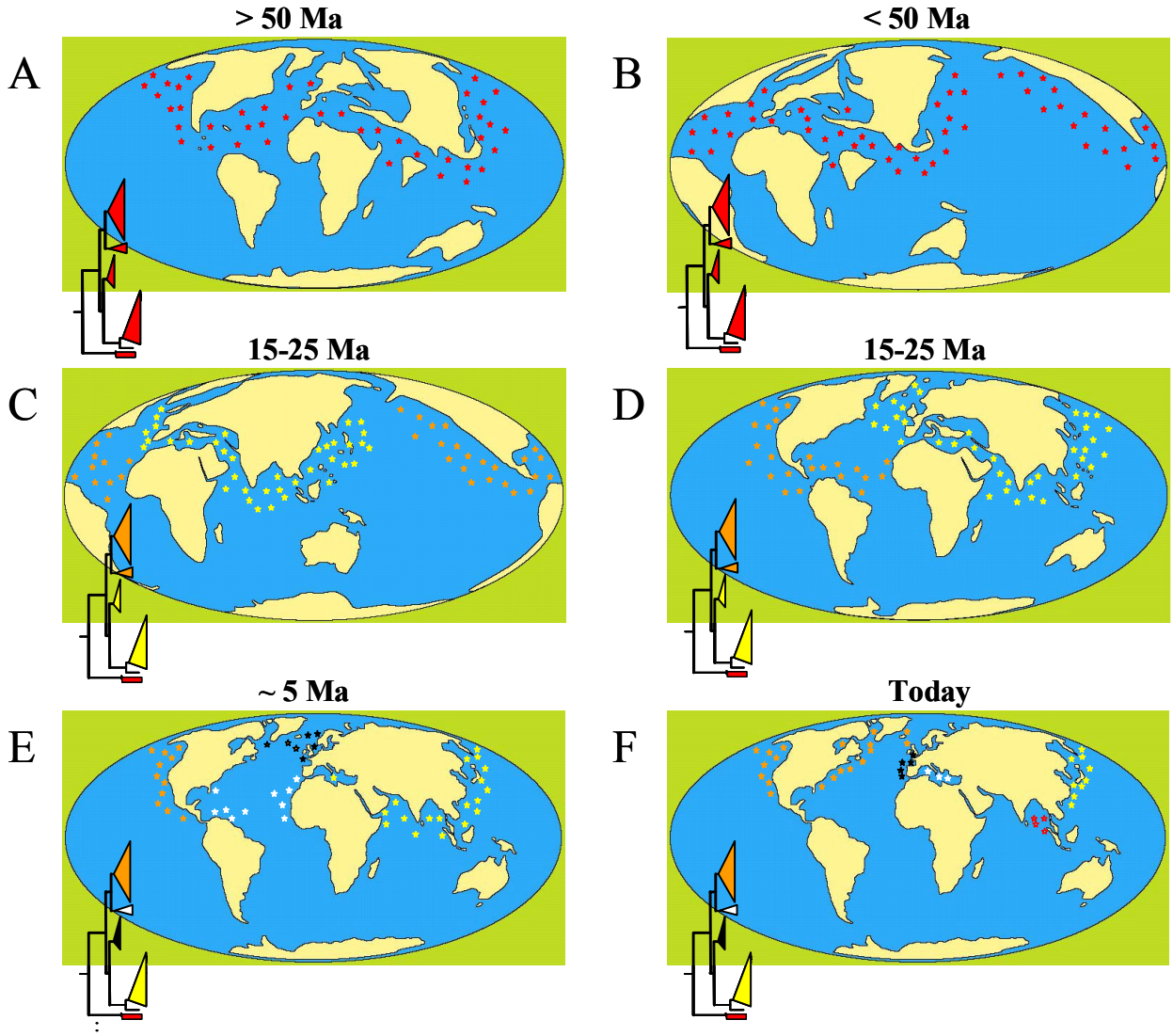



Figure 4. Maps showing hypothetical distributions of the populations of the *Alexandrium tamarense* species complex at specified times during the Cenozoic. Stars symbolize *A. tamarense* species complex distribution. Colors of stars correspond to the divergence stage of the *A. tamarense* population according to the modified tree inset of the D1/D2 region of the LSU rDNA phylogenetic tree (Fig. 2). Paleogeographic reconstructions after Scotese (1997).

.

## *2.8. Publication VI:* Development of specific rRNA probes and the application of Amplified Fragment Length Polymorphisms (AFLP) to analyse clades within the *Alexandrium tamarense* species complex.

UWE JOHN, LINDA MEDLIN, AND RENÉ GROBEN

Alfred Wegener Institute for Polar and Marine Research

Am Handelshafen 12, D-27570 Bremerhaven, Germany

Correspondance to: L. Medlin. E-mail: lmedlin@awi-bremerhaven.de

*Abstract*

The dinoflagellate genus *Alexandrium* (Halim) Balech and especially the *A. tamarense*/*fundyense*/*catenella* species complex are among the most prominent producers of paralytic shellfish poisoning (PSP) toxins. As there are toxic and non-toxic strains of these species occurring worldwide, a deeper insight into the phylogeny and population structure of the species complex and a reliable method to identify and characterize them is necessary. Recently published phylogenetic trees showing the original five ribotypes plus a new non-toxic ribotype from the Mediterranean Sea was used to develop specific molecular probes for the entire species complex and three different geographic clades (North America/Orkney Islands, Western Europe, Mediterranean Sea) which can be used to identify these strains even in mixed populations by DNA dot blot and *in situ* hybridization. These probes are a first step to the identification of *A. tamarense* in field samples and in the development of an early warning system for this species. For further genetic diversity studies, the fingerprint technique of Amplified Fragment Length Polymorphisms (AFLPs) was established for the species complex. General considerations of the use of this molecular marker type for phytoplankton are discussed as are the results for the analysis of *A. tamarense* populations. We could show that AFLP is a powerful method to investigate the genetic diversity inside a distinct geographic region or among the cells of an algal bloom, but it shows too much polymorphism to analyse the species complex as a whole because of its extensive genetic variability if single isolates from one location within a much broader geographic area are compared.

*Key index words*: AFLP, *Alexandrium catenella*, *Alexandrium fundyense, Alexandrium tamarense*, fingerprinting, harmful algal blooms, molecular probes, rRNA probes, toxic algae.

*Abbreviations*: AFLP, amplified fragment lengths polymorphism; HAB, harmful algal bloom; FISH, fluorescence *in situ* hybridization; ME, Mediterranean clade; NA, North American clade; TA, Temperate Asian clade; WE, Western European clade.

***Introduction***

Harmful algal blooms are a world wide occurring phenomenon, with an apparent global increase in frequency, intensity and geographic distribution. Among the several dozen reported toxigenic species, many belong to the genus *Alexandrium* (Halim) (Balech 1995). As a consequence, this genus, and especially the *A. tamarense/fundyense/catenella* species-complex has been extensively investigated in recent times because they produce paralytic shellfish toxins (Balech and Tangen 1985; Taylor 1984; Anderson and Wall 1978; Cembella 1988; Scholin et al. 1994). Paralytic shellfish poisoning (PSP) causes a wide variety of environmental and public health problems, e.g., through consumption of contaminated seafood (Graneli et al. 1990; Hallegraeff 1993).

Taxonomists use for morphological identification of these species e.g. the cell shape, the geometry of their apical pore complex (APC), the presence (*A. tamarense*) or absence (*A. catenella*/ *A. fundyense*) of a ventral pore on the apical plate (1‘), and whether the cells show a tendency for chain forming (*A. catenella*) or not (*A. tamarense*/ *A. fundyense*).

These morphological features are sometimes difficult to determine and might also be influenced by environmental factors and culture conditions (Masiga et al. 2000). Intermediate forms can also be found (Cembella and Taylor 1986; Granéli et al. 1990). For a better identification and to clarify the phylogenetic relationship between these species, biochemical and molecular biological methods have also been used, e.g., isozymes (Cembella et al. 1988) or toxin profiles (Cembella et al. 1987), but both of these phenotypic characteristics can be problematic for identification purposes because they are still influenced by environmental factors, which is not the case for DNA sequences. For the *A. tamarense* species complex the small subunit of the ribosomal gene (SSU rDNA) (Scholin et al. 1995), the large subunit (LSU rDNA) (Scholin et al. 1994; Medlin et al. 1998; Higman et al. 2001) and internal transcribed spacer (ITS) regions of the ribosomal RNA operon (Adachi et al. 1996a) have been analyzed, and each has shown that the phylogenetic relationships reflects geographic areas and not the morphotypes *A. tamarense*, *A. fundyense* and *A. catenella*. Recently John et al. (2002a, submitted) have shown that the species complex contains not five but six distinguishable ribotypes at the D1/D2 region of the 28S rRNA level. Besides the known toxic North American (NA) and Temperate Asian (TA) clade and the non-toxic Tropical Asian (TROP), Tasmanian (TASM), and West European (WE) clade they introduced a new non-toxic Mediterranean clade (ME).

The world wide increase in harmful algal blooms has lead to a growing number and intensity of mandatory monitoring programs, in which large numbers of samples are generated for cell analysis. A common problem found in phytoplankton field ecology is that the species of interest may be only a minor component of the planktonic community and that for an accurate species, strain or morphotype determination, individual cell characterization is necessary. Light microscopy has its limitations even when done by a specialized taxonomist, e.g., for the analysis of closely related species or strains of *Alexandrium* (John et al. 2002b, submitted). Molecular probes have been designed mostly on the basis of rRNA sequences, to detect higher groups and classes (Lange et al. 1996; Guillou et al. 1999; Simon et al. 2000), clades (Walsh et al. 1998; Guillou et al. 1999; Simon et al. 1997), genera (Lange et al. 1996) and species (Simon et al. 1997; Miller and Scholin 1998) of phytoplankton specifically. By using these probes in dot blot experiments (Guillou et al 1999) or with *in situ* hybridization followed by fluorescence microscopy (Simon et al 2000; John et al 2002b, submitted) or flow cytometry (Lange et al. 1996) it is possible to detect the species under investigation even in mixed assemblages and in field samples. For the *A. tamarense* species complex the development of probes is of great interest, because this provides a fast and efficient detection method of the identity and origin of certain isolates at the molecular level. Also, because two of the geographic clades are exclusively toxic and the others are exclusively non-toxic, probes for each geographic clades could be useful as a first step for a possible automated early warning system for toxic *A. tamarense* blooms. Probes for the different clades inside the species complex will help to identify and further characterize the different strains in European and American waters, and can also be used to track possible introductions from foreign waters.

Although the different geographic clades of the *Alexandrium tamarense* species complex are well distinguished by their sequences and by their RFLP patterns (Scholin and Anderson 1996; Scholin and Anderson 1994), their population structure and the development of their blooms has not been investigated at the genetic level because the rDNA and ITS sequences lack sufficient base substitutions to resolve differences at this level (Medlin et al. 1998; Scholin et al. 1994). Different types of molecular markers exist, that can overcome this limitation and are used to characterize phytoplankton at the level of individuals or closely related populations, e.g., RAPDs (Random Amplified Polymorphic DNAs; (Barker et al. 1994) or microsatellites (Rynearson and Armbrust 2000).

In this study, Amplified Fragment Length Polymorphisms (AFLPs) have been used to analyze the population structure of the *Alexandrium tamarense* species complex in more

detail than possible with rDNA sequences. The technique relies on the selective polymerase chain reaction (PCR)-amplification of restriction fragments from genomic DNA (Vos et al. 1995). AFLP has been used for taxonomy and population studies among others in fish (Seki et al. 1999; Felip et al. 2000), bacteria (Janssen et al. 1996), higher plants (Barrett and Kidwell 1998), fungi (Taylor 1987), and algae (Donaldson et al. 1998; Donaldson et al. 2000). AFLP combines the advantages of RAPDs (no sequence information necessary, large number of bands to search for polymorphisms, uses only small amounts of DNA) (Hauben et al. 1999) with those of RFLPs and microsatellites (high reproducibility). The AFLP technique involves several steps. First, genomic DNA is digested with specific endonuclease restriction enzyme, e.g., a rare cutting and a frequent cutting restriction enzyme. After digestion, restriction site specific adapters are ligated to the ends of the fragments. PCR amplification steps with primers of complementary sequences to the adapters and restriction sites and additional bases on the 3‘ end is carried out to amplify a specific subset of the total amount of fragments. The additional bases on the 3‘ primer end reduce the amount of resulting fragments by a factor of 4 (one base), by a factor of 16 (two bases) or by a factor of 64 (three bases), respectively, so that discrete bands can be separated and visualized by polyacrylamide gel electrophoresis (Vos et al. 1995; Hauben et al. 1999)**.**

***Material & Methods***

*Strains and culturing conditions*

A list of the algal strains used in this study is presented in Table 1. Unialgal cultures were grown in 500 mL Erlenmeyer flasks in IMR½ growth medium (Eppley et al. 1967), supplemented with 10 nM selenite (for *Alexandrium tamarense*, *A. catenella*, *A. fundyense*, A. *pseudogonyaulax*, *A. taylori*, *A. minutum* and *A. lusitanicum*), or in K medium (Keller et al. 1987) (for *A. affine* and *A. ostenfeldii*). All cultures were maintained at 15$^{o}$C in a controlled growth chamber on 14:10h light:dark photocycle, at a photon flux density of 150 µmol $m^{-2}$ $s^{-1}$, except for *A. ostenfeldii* (90 µmol $m^{-2}$ $s^{-1}$).

*DNA extraction and PCR amplification of rDNA genes.*

DNA extractions were made from 500 mL of culture in logarithmic growth phase using the PAN Plant kit (PAN Biotech, Aidenach, Germany) according to the manufacturer's instructions with minor modifications. Cultures were filtered onto 47 mm diameter, 3 µm pore-sized polycarbonate filters (Isopore, Millipore, Bedford, MA, USA). Afterwards, the

cells were washed from the filter into 1.5 mL reaction tubes with 400 µL preheated (65°C) lysis buffer. Thirty µL of proteinase K (10 mg $mL^{-1}$) were added, followed by 90 min incubation at 65°C in a thermoshaker. After cell lysis, 40 µL RNase A (10 mg $mL^{-1}$) were added and incubated at room temperature for 30 min. Extraction and cleaning of the genomic DNA took place on a silica membrane supplied with the kit. DNA concentration was measured spectrophotometrically at 260 nm, and DNA quality was verified by agarose-gel electrophoresis. Polymerase chain reaction (PCR) conditions for amplifying the SSU rDNA gene and the D1/D2 region of the LSU rDNA gene were chosen according to Medlin et al. (1988), and Scholin et al. (1994), respectively. All oligonucleotides (primers and probes) were supplied by MWG (Ebersberg, Germany) if not stated otherwise.

*Design of oligonucleotide probes*

Ribosomal RNA oligonucleotide probes were designed according to Simon et al. (2000) using the ARB (http://www.mikro.biologie.tu-muenchen.de/pub/ARB/) software package in combination with databases consisting of more than 450 published and unpublished algal 18S rRNA sequences, and 150 sequences of the variable D1/D2 region of the 28S rRNA gene. The entire *Alexandrium tamarense* species complex was selected for probe development from our SSU rDNA data set and probes for the geographic clades were designed from LSU rDNA data set, using the ARB program. A general eukaryote-specific probe EUK1209R (Lim et al. 1993) was used as a positive control in dot blot and whole-cell hybridization experiments. All probes used in this study are listed in Table 2.

*DNA dot blot hybridization*

Unmodified oligonucleotides were supplied by MWG-Biotech (Ebersberg, Germany) and labelled with Digoxigenin (DIG) for non-radioactive DNA dot blot experiments using the 3' Oligonucleotide Tailing Kit (Roche, Mannheim, Germany) according to the manufacturers instructions. DNA extractions and PCR reactions for the dot blot experiments were done as described before (Simon et al. 2000). Approximately 100 ng of amplified PCR product per sample were denatured for 10 min at 95°C and kept on ice until spotted onto a positively charged nylon membrane (Roche, Mannheim, Germany) and fixed by 90s double-sided exposure of the membrane using a standard UV-transilluminator. Four hours of pre-hybridization followed by overnight hybridization were done in roller tubes in 10 mL hybridization buffer (5X Sodium-Sodium Citrate (SSC), 0.1 % (w/v) N-lauroylsarcosine, 0.02 % (w/v) Sodiumdodecylsulfate (SDS), 1% (w/v) Blocking reagent (Roche, Mannheim,

Germany), 0.1 mg $mL^{-1}$ Poly (A)) at a temperature of 54°C (probe ATME04 at 54.5°C). For hybridization, 0.1 pMol $mL^{-1}$ DIG-labelled probe was added to the hybridization buffer. Washing of filters consisted of two 5 min washes with 2X SSC/0.1% SDS at room temperature, and two 15 min washes with 1X SSC/0.1% SDS at hybridization temperature. Detection was performed with the DIG Luminescent Detection Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions, and membranes were exposed to X-ray film (Amersham, Freiburg, Germany) for 1 to 3 h.

*In situ hybridization of dinoflagellate cells*

Whole cells from lab cultures were hybridized with FITC-labelled (Fluoresceinisothiocyanate) probes (Interactiva, Ulm, Germany) using a modified protocol from Scholin et al. (1996, 1997) and examined by epifluorescence microscopy. Cells were carefully filtered onto 47 mm diameter 3.0 µm pore-size polycarbonate membranes (Isopore, Millipore, Bedford, MA, USA ) in a standard filtration unit (Millipore, Bedford, MA, USA) using not more than 100 mm Hg vacuum to prevent cell damage. Freshly prepared saline EtOH fixative (25 mL 100 % ethanol., 2 mL Milli-Q water, 3 mL 25X SET buffer (3.75 M NaCl, 25 mM EDTA, 0.5 M Tris/HCl, pH 7.8) was added to the filtration unit covering the cells which were fixed for 1 h at room temperature. The fixative was filtered through and the filter incubated with hybridization buffer (5X SET buffer, 0.1% IGEPAL-CA630, 30 ng $mL^{-1}$ poly A) for 5 min at room temperature. Afterwards, the rinsed filter was cut into pieces, with each piece placed onto a microscopic slide and hybridized separately with a different probe. Sixty µL hybridization buffer containing 20% formamide (0% formamide for probe EUK1209R) with FITC-labelled probes (5 ng $\mu L^{-1}$) were applied directly onto the filter pieces and incubated for 1 h at 50°C in a moisture chamber in the dark.. The filters were rinsed for 5 min in 100 µL of 1X SET buffer at hybridization temperature to remove excess unbound probe, before mounting in a mixture of CitiFluor (Citifluor Ltd., London, U.K.) as an anti-fade agent, with 4',6'-diamidino-2-phenylindole (DAPI; 1 µg $mL^{-1}$) as a counterstain. The cover slip was sealed with nail varnish and the slides analyzed using an Axioskop 20 epifluorescence microscope (Zeiss, Oberkochen, Germany) equipped with Zeiss filter sets 02 (DAPI) and 09 (FITC). Photographs were taken with 1600 ASA Fuji colour print film.

*AFLP: DNA digestion/ligation*

*Alexandrium* DNA (500ng) was digested over night at 37°C with 2 U *Eco*RI and 2 U *Mse*I (both New England BioLabs, Frankfurt am Main, Germany). Afterwards, the enzymes

were heat deactivated at 65°C for 15 min. One third of the restriction was added to a ligation mix consisting of 2.5 µL water, 1.7 µL ATP (10mM), 0.8 µL T4 ligase Buffer (10 x), 1µL *Eco*RI adapter (sequence see Table 3, 10 pmol $\mu L^{-1}$), 1µl *Mse*I adapter (sequence see Table 3, 50 pmol $\mu L^{-1}$), and 1µL T4 DNA ligase (Amersham Pharmacia Biotech, Freiburg, Germany). The mix was incubated at room temperature for 7 h and stopped by heating for 10 min at 65°C. The ligated DNA was diluted 1:5 in 0.1 x TE buffer and stored at – 20°C until preamplification.

*AFLP: Preamplification*

Preamplification was done with 10 µL of ligated DNA, 12.5 µL water, 3 µL 10 x *Taq* DNA polymerase buffer (Perkin Elmer-Applied Biosystems, Weiterstadt, Germany), 0.5 µL *Eco*RI + A primer (sequence see Table 3; 10 pmol $\mu L^{-1}$), 0.5 µL *Mse*I + C primer (sequence see Table 2; 10 pmol $\mu L^{-1}$), 3 µL 1 mM dNTPs (Amersham Pharmacia Biotech, Freiburg, Germany) and 0.5 µL *Taq* DNA polymerase (Perkin Elmer-Applied Biosystems, Weiterstadt, Germany). The PCR reaction was carried out in an Mastercycler Gradient (Eppendorf, Hamburg, Germany) with 22 cycles of 94°C for 30 s (denaturation), 56°C for 30 s (annealing) and 72°C for 1 min (extension). The PCR product was diluted 1:10 in 0.1 x TE buffer and stored at –20°C.

*AFLP: Amplification*

For the selective amplification step several *Eco*RI and *Mse*I primers with two or three additional bases (sequences see Table 3) were used. The reaction mix contained 5 µL of preamplified product, 9.9 µL of water, 2 µL 10 x *Taq* DNA polymerase buffer (Perkin Elmer-Applied Biosystems, Weiterstadt, Germany), 2 µL 1 mM dNTPs (Amersham Pharmacia Biotech, Freiburg, Germany), 0.6 µL *Mse*I + 3 primer (10 pMol $\mu L^{-1}$), 0.1 µL of IR800 labelled *Eco*RI + 3 primer (10 pMol $\mu L^{-1}$) and 0.4 µl *Taq* DNA polymerase (Perkin Elmer-Applied Biosystems, Weiterstadt, Germany). Amplification by touch-down PCR was performed with an initial denaturation of 94°C for 30 s and a first cycle of 94°C for 30 s (denaturation), 65°C for 30 s (annealing) and 72°C for 60 s (extension). During the next 12 cycles the annealing temperature was reduced by 0.8°C per cycle down to 56°C whereas the last 23 cycles were the same as described for preamplification.

*AFLP: Electrophoresis*

The selective amplification reaction was stopped by adding 15 µL loading buffer (95% formamide, 0.05% brom-phenol-blue, 0.05% xylene cyanol, 20 mM EDTA). After 5 min. denaturation at 80°C, 1 µL was loaded onto a 0.25 mm thick polyacrylamide gel, consisting of 18.2 mL water, 4 mL 10 x buffer (1390 mM Tris-base, 45 mM Boric acid, 25 mM disodium-EDTA), 16.8 g urea and 5.6 mL Long Ranger (Biozym, Hessisch Oldendorf, Germany). Electrophoresis was done on a LI-COR sequencer 4000L (MWG-Biotech, Ebersberg, Germany) in 1 x buffer at constant 50 W, 1700 V and 50°C for 4 hours and external reference marker (MWG-Biotech, Ebersberg, Germany) were used for later band size determination and normalization of gel lanes and bands, e.g., correction of “smile effect” of bands. Results were stored as TIFF files for further processing.

*AFLP: Gel processing*

Gels were processed and analyzed using the Software package BioNumerics (Applied Math, city, country). Bands were defined by using the "band auto search function" of the software set to 3% for "minimum profiling" and for “gray zone”. "Shoulder sensitivity" was set to 2 for finding band doublets and bands on shoulders. However, it was still necessary to correct the selected bands manually and errors by subjectively choosing bands could not be completely excluded. Band matching was done with an optimization of 0.04% and a position tolerance of ± 0.1% using the "tolerance & optimization analysis" option.

*AFLP: Data analysis*

For calculating a similarity matrix of the binary AFLP-data, the Dice similarity coefficient was used, taking in account the shared presence of bands and not the absence. The generated similarity matrix was converted to a dendogram with UPGMA (Unweighted Pair-Group Method with Arithmetic averages) cluster analysis. As a test of support for tree and cluster topology, 1000 bootstrap replicates (Felsenstein 1985) were performed with UPGMA analysis.

***Results***

*Design of specific rRNA probes for identification and characterization of A. tamarensespecies*

Molecular probes were developed for the entire *A. tamarense* species complex from the SSU rDNA sequences taken from John et al. (2002a, submitted)(ATAM01; Fig. 1 and

Table 2) and one probe each for the NA (ATNA02), WE (ATWE03), and ME clades (ATME04), which were all designed from LSU rDNA sequences taken from John et al. (2002a, submitted)(Fig. 2 and Table 2). The addition of *A. cohorticula* (GenBank: AF113935) to the data set has complicated the interpretation of the evolution of the species complex because the Tropical Asian clade is now sister to this taxon and pulled out of the species complex. A more complete discussion of this taxonomic problem can be found in John et al. (2002a, submitted). Our species complex level probe was developed prior to the addition of this taxon and it recognizes this taxon plus the species complex. All probes showed 100% specificity to their group of target organisms, and at least one mismatch in the probe region to all known non-target organisms. We re-designed the previously published probe NA1 (Miller and Scholin 1998) by shifting it five bases upstream of its target sequence, because the original probe was likely to produce a hairpin fold that may have prevented correct binding under low stringency conditions as in the original hybridization protocols. *In situ* hybridization tests showed that our probe gave slightly stronger signals than the Scholin NA1 (data not shown), but both are specific and can be used to detect the NA clade.

The probes were tested for specifity with DNA dot blots using PCR amplification products from 24 target and non-target species/strains and using the Eukaryote-specific probe EUK1209R (Lim et al. 1993) as a positive control. After optimization of hybridization conditions (temperature, probe concentration and washing conditions), all probes showed strong hybridization signals only with their respective target sequences (Fig. 3).

Two different strains of *Alexandrium tamarense* from the NA clade, one from the east coast, and one from the Orkney Islands, one strain from the WE clade, one from the ME Clade, and one strain of each *Alexandrium* species given in Table 1 were tested by whole-cell hybridization. The eukaryote-specific probe EUK1209R was used throughout to see if the *in situ* hybridization itself was successful. We were able to establish conditions under which all probes gave only signals with their specific targets (Fig. 4 and 5). Probe ATWE03 hybridized to their target cells displaying positive signals that differed from those of other probes. Instead of a rather uniform distribution of the signals inside the cells when binding to the ribosomes, the signals could only be seen near the center of the U-shaped nucleus (Fig. 5), presumably at the nucleolus. However, probe ATWE03 showed these kind of signals only with their target strains and hence can be considered specific.

A problem that can occur while doing *in situ* experiments is the masking of hybridization signals by strong autofluorescence of cells. Even with the chlorophyll having its emission maximum not in the wavelengths of FITC, it can be stronger than the probe itself.

The occurrence of autofluorescence is strongly species specific, e.g., *A. taylori* exhibited strong autofluorescence, whereas *A. tamarense* shows hardly any. Autofluorescence is also influenced by the physiological conditions of the cells. Therefore, lab cultures were used for the experiments that were harvested in exponential growth phase to obtain healthy cells. When this is not sufficient to get clear results or not possible, e.g. when analyzing field samples, either an elongated fixation time in which the ethanol bleaches the cells further is recommended or a two hour incubation of the filter after fixation in Dimethylformamide (DMF) at 4°C (data not shown).

As previously shown (Medlin et al. 1998) and demonstrated in Fig. 6, it was not possible to separate the isolates from North America and the Orkney Islands, Scotland, by analysis of the D1/D2 region of the LSU rDNA. Thus to resolve the relationship between the isolates from North America and the Orkney Islands, Scotland, we employed the finer scaled fingerprinting method of AFLP analysis.

*Establishment of the AFLP technique to study biodiversity in* Alexandrium tamarense

Before using AFLPs for studying *A. tamarense*, it was necessary to adapt the method to the organisms to obtain reproducible results. Some points were shown to be of special importance: AFLP is based upon restriction digestion and ligation of genomic DNA. Therefore, the quality of the extracted DNA in terms of purity and integrity was very important as this affects the resulting fingerprints. For the extraction of DNA two methods were tested, first a CTAB-based method was used (Doyle and Doyle 1990), including RNAse and Proteinase K treatment, but less DNA shearing and more reproducible results were obtained when the PAN plant DNA purification Kit was used (data not shown). The need for the use of one selective base extension on the preamplification primer to reduced the background noise according to (Mackill et al. 1996) was verified. During optimization selective primers with one to four selective bases were used and visualized using denaturating polyacryamide gel electrophoresis to specify the primer consistency that gave an appropriate number of bands to permit analysis. Primers consisting of three selective bases gave between 15 and 60 bands with fragment sizes between 50 and 400 bp, no significant difference was observed to that obtained when using four selective bases. When using less than three selective bases, the banding pattern was too complex for reliable analyses (data not shown). Reproducibility of the AFLP method was assessed by performing three successive DNA isolations and AFLP analysis with three different *A. tamarense* strains. Additionally, from those three strains five DNA concentrations (10, 50, 250, 500, and 1000ng) were analyzed,

showing that above 50 ng the reproducibility of the pattern within one gel was higher than 95% but below 50 ng 20% differences in the pattern reproducibility were observed (data not shown). Comparing samples between gels, minor variation in background and band intensity were observed, but banding patterns that exhibited ≥ 90% homology were always obtained. In this study six primer combinations were used to study the individual genetic diversity between and within strains of populations of *Alexandrium tamarense* species complex (Table 4) , whereas only three of the five pairs were used for computational analyses.

*AFLP data*

The Dice similarity coefficient between all pairwise comparisons of strains of the three AFLP-gels used in this study, ranged from approximately 0.0 to 0.95 with higher values meaning higher similarity or more shared bands between strains compared (shown for gel A3 and gel A5 in Table 5). The wide range of similarity values indicates a very high genetic diversity among all strains used in this study. The UPGMA dendogram (Fig. 7) calculated from the Dice coefficient matrix of gel A2, A3, and A5 shows four loose clusters built by the strains used in this study. Strains belonging to the NA clade and those belonging to WE one, were spread over the dendogram without forming a unique cluster representing one geographic population as shown for the 28S rDNA tree (Fig. 2). Only one or two bands can be seen as common to all the NA isolates in Fig. 7. In general the banding pattern is very heterogeneous with similarity values below 0.5 between the clades and low bootstrap values (Table 5 and Fig. 7). This is because most of the isolates were single strains from a single location within a much broader geographic clade. However, the multiple strains of the Orkney populations and the four strains representing the ME clade each clustered together with bootstrap values of 100% in all three gels. Within the cluster of the Orkney strains the Dice similarity coefficient between the strains ranged from 0.40 to 0.95 shown for gel A3 and A5 (Table 5), despite the isolates being taken from a single sampling site. Strains BAHME 200 and 184 are nearly identical with the most differences being seen in the longer fragments amplified. The bootstrap support for the clustering of the four strains within the Mediterranean clade range from 48 to 95% bootstrap supported (Fig. 7). The Dice similarity coefficient within that group in gel A3 and A5 ranged between 0.44 and 0.90 (Table 5).

***Discussion***

The phylogenetic trees for *Alexandrium* derived from the rRNA sequencing data have consistently never shown a separation of the A. *tamarense*/*catenella*/*fundyense* species complex into their morphotypes. Rather, the analysis of the LSU D1/D2 region has shown significantly different ribotypes representing different geographic clades (Scholin et al. 1994; Adachi et al. 1996a; Medlin et al. 1998; Higman et al. 2001, John et al. 2002a, submitted), along with some differences in branching order when new strains have been added and when other methods of analysis used. The consistency of the topology within the phylogenetic trees of *Alexandrium* spp. permitted the SSU and LSU rDNA sequences to be used to develop molecular probes. We were able to design clade specific probes for the *Alexandrium* species complex and for *A. tamarense* ribotypes present in European and North American waters and that those can be used to identify clearly their target strains in DNA dot blots and whole-cell hybridization. Of certain interest is the phenomenon of the ATWE03 probe, which only shows fluorescence near the center of the U-shaped nucleus (Fig. 5), presumably the location of the nucleolus as discussed in Adachi et al. (1996b). We think that this comes from binding of the probes only to pre-ribosomal structures in the nucleolus before they are processed into functional ribosomes. The probes were unable to bind to the mature ribosomes either because of confirmational changes in the rRNA molecule or because the probe binding site was covered by ribosomal proteins. More investigations will be done concerning this phenomenon, but nevertheless, the probe ATWE03 show these kind of signals only with their target strains and therefore can be considered specific.

The use of probes is of course not limited to lab cultures. John et al. (2002b, submitted) have shown that clade probes of the *A. tamarense* species complex can be applied to identify *Alexandrium* isolates and/or populations in field samples as well. This is interesting because toxicity has been correlated with specific ribotypes, viz., to the TA and the NA clades. Members of these toxic clades could be distinguish from non-toxic strains only if they belong to the *A. fundyense* or *A. catenella* morphotype, both of which are known to be exclusively toxic (Anderson et al. 1998; Scholin 1998). Because the morphological differences are so slight and they are able to produce progeny with variable morphologies, a morphological differentiation is elaborate, time consuming and mostly depends on taxonomic expertise, making detailed examination impractical for field studies. However, both the TA and the NA ribotypes contain toxic *A. tamarense* morphotype strains, which are visually indistinguishable from the non-toxic *A. tamarense* of the other geographic clades. Therefore,

these probes can be used in monitoring programs where isolates were examined whether they are toxic or non-toxic and additionally it could be clarified if a certain strain has been introduced by human activity, e.g., by ballast water or by shellfish stocks (Scholin et al. 1995; Rigby et al. 1999) or if they belong to the endemic population of an area. Toxic dinoflagellates are probably one of the best studied organisms used to assess the bioeconomic risk of ballast water introduction of nonindigenous marine pests (Hallegraeff 1998). Anderson et al. (2000) have estimated the annual economic impact of an HAB outbreak at an average cost of about $50 million per year. Therefore, it must be of great public interest to prevent introductions of toxic algae, via ballast water into new areas. Until a mandatory effective, safe, financially viable, and environmental friendly ballast water treatment can be established, probe technology may represent a potential alternative to help identify new introductions or to establish an early warning system for algal blooms. In coastal monitoring or in aquaculture the probes can be use to observe and monitor coastal zones where algal blooms normally occur and record if toxic strains are present or absent, without having to resort to costly sequence comparison studies, toxin analysis, or microscopic observations. Studies by John et al. (2002b, submitted) for *Alexandrium* and by Parsons et al. (1999) for *Pseudo-Ntizschia* have shown that the oligonucleotide probes have a great potential as tools for routine monitoring of HABs even when there are still some technical problems, like e.g., empty thecas, broken and unhealthy cells, resulting in fewer ribosomes and therefore in reduced fluorescence, which has to be solved. In monitoring programs, where toxin data is measured directly on board ship (Hummert et al. submitted., Medlin et al. 1998, John et al. 2002b, submitted) the FISH technology can be combined with flow cytometry to give near real time data about the species composition at the sampling stations (Lange et al. 1996; Simon et al. 1997, Brenner pers. comm.).

In order to understand the worldwide distribution of the *A. tamarense* species complex with its increase in intensity and frequency to form HABs (Hallegraeff 1995). An intensive observation of the species complex dynamics in monitoring programs using molecular probes, might forced our knowledge and help to predict bloom formation in future. However, beside that, we need a better understanding of the population and bloom dynamics itself. As mentioned in the introduction, rDNA sequences have their limits to elucidate the genetic diversity among certain geographic cladesespecially among isolates of a bloom, as demonstrated in Fig. 6. In this study we were able to show that AFLP can be reproducibly applied to investigate the genetic diversity of *Alexandrium tamarense*, and to our knowledge this is the first report of the use of this molecular marker in marine microalgae in general.

Although the AFLP method is relatively time consuming to establish in the laboratory, the results were highly reproducible after finding a DNA extracting method that lead to highly purified genomic DNA. The high reproducibility between 90-98% of AFLP even for intergel-correlation has been demonstrated in several studies, e.g., by Jones et al. (1997) who compared within a network of European laboratories the usability of RAPDs, AFLPs, and microsatellites. Other groups found the same reproducibility for sunflower (Hongtrakul et al. 1997), bacteria (Janssen et al. 1997; Hauben et al. 1999) or macroalgae (*Chondrus crispus*; Donaldson et al. 1998). Donaldson et al. (2000) explained that the high reproducibility might be based on incomplete replicates because they have used the same original DNA sample for their replicates, but Duim et al. (1999) and this study presented true replicates starting with different DNA extractions, and still a high reproducibility was obtained. Cases in which reproducibility, especially in band intensity, is reduced, are most likely because of variation in DNA quality obtained from different extractions (Donaldson et al. 2000). Marine algae are especially known to produce many secondary metabolites, such as phenols, and polysaccharides that can negatively influence the restriction and ligation efficiencies. But even including these few drawbacks, AFLPs have proven a reliable source of information for phylogenetic studies in microalgae.

AFLPs demonstrated the extremely high genetic diversity of the *Alexandrium tamarense* species complex even within one ribotype, when sequences of the ITS region and the hypervariable D1/D2 region of LSU rDNA gave no further resolution (Adachi et al. 1996a; Scholin et al. 1994; Medlin et al. 1998). The UPGMA cluster analysis and the bootstrap values for the members of the WE and the NA clade gave no clear resolution because in most cases only single strains from one location within a larger geographic clade were used, but when mulitiple strains isolated from the same area (e.g. the ME strains) or from one bloom (e.g., Orkney strains) were used then they grouped into clusters, that were well supported by bootstrap values and showed common bands unique for those groups. This indicates, that AFLPs can be used to investigate the diversity within mulitiple strains from a closely related geographic region and among strains of one bloom, but the genetic variation across the entire *A. tamarense*/*catenella*/*fundyense* species complex is too extensive to compare different clades by this technique, if only single representatives of each location are used. Donaldson (2000 and references within) have discussed up to which level of divergence a comparison between samples can be made. Although Kessler and Avise (1985) suggested that 25% divergence is the maximum, Upholt (1977) considered 15% as the level which must not be exceeded. In fact, most pairwise comparisons in this study exceeded these levels of

divergence, with some strains, such as UW42 had 100% polymorphic bands compared to some strains from the ME and NA clade (Table 5). This high percentage of polymorphic bands has also been observed by Donaldson et al. (2000) with 97% divergence, which led them to the suggestion that AFLPs were not useful for the high genetic variability inside *Chondrus crispus*. When other microalgal groups, e.g., the prymnesiophyte *Calcidiscus leptoporus* were analysed by AFLPs, the resulting banding pattern appear much more homogenous (A. Saez, pers. comm.), and we suggest that the life cycle and the population dynamics of *Alexandrium* could be the reason for such high genetic diversity. *Alexandrium* species are meroplanktonic; they spend only few weeks in the plankton where they undergo vegetative growth. The vegetative phase ends with gametogenesis and the formation of diploid zygotes, known as planozygotes, which remain motile for a couple of weeks (Wyatt and Jenkinson 1997; Anderson et al. 1998). The planozygotes mature and become cysts, which settle to the sediments, and can accumulate in seedbeds, where they may remain dormant for several years. Each year some fractions of the cysts from different year classes hatch and provide the new generation of vegetative cells (Dale 1977; Wyatt and Jenkinson 1997; Anderson et al. 1998). This results in heterogeneous and genetically diverse blooms even if a bloom is considered to be monospecific, which means that a bloom of *Alexandrium* species is not a clonal phenomenon. Barker et al. (1994) also inferred that a high frequency of sexual reproduction must be taking place in *Emiliania huxleyi* because of the compelx RAPD banding patterns found in the bloom isolates of *Emiliania huxleyi* found in the North Atlantic.

Another possible use of AFLP markers is to isolate the bands from a gel that are common for strains from a distinct geographic area or a bloom (Fig. 7) and use them to develop specific PCR markers to identify the strain origin on a basis of bloom membership or from which bay or port the strain comes from as shown in the study of McLenachan et al. (2000) for investigating the ecology and evolution of closely related taxa. However, this AFLP-derived PCR markers might help if all preventive efforts fail and a cyst introduction via human activity occurs, to identify the source of the introduced strains and to hold perpetrators accountable. (Bolch et al. 1999) used RAPD markers to identify the source of introduction of the toxic dinoflagellate species *Gymnodinium catenatum* to mainland Australian waters and found it most likely to be a translocation from Tasmania.

To conclude, molecular probes were designed for the *Alexandrium tamarense* species complex and its geographic clades. The probes have a high potential to facilitate the monitoring of the distribution and the bloom dynamics of the species complex, without taxonomic expertise. We have demonstrated that the molecular marker AFLP is sensitive,

reproducible, and efficient for the study of microalgae. For the future, AFLPs may provide deeper look in the genetic diversity of a particular ribotype, geographic area or a bloom and overcome the limits in resolution of rDNA sequences.

*Acknowledgements*

The authors thank Helga Mehl for her technical assistance. The work was supported by BMBF TEPS (Project No. 03F0161) and by the EU project AIMS (MAS3-CT97-0080).

*Reference List*

Adachi M, Sako Y and Ishida Y 1996a. Analysis of *Alexandrium* (Dinophyceae) species using sequences of the 5.8S ribosomal DNA and internal transcribed spacer regions. *J. Phycol.* 32:424-32.

Adachi M, Sako Y and Ishida Y 1996b. Identification of the toxic dinoflagellates Alexandrium catenella and A. tamarense (Dinophyceae) using DNA probes and whole-cell hybridization. *J. Phycol*. 32:1049-52.

Anderson, D.M. 1998. Physiology and bloom dynamics of toxic *Alexandrium* species, with emphasis on life cycle transitions. *In* Anderson, D.A., Cembella, A.D., & Hallegraeff, G.M., [Eds.] Springer, Berlin, pp. 29-48

Anderson, D.M., Hoagland, P., Kaoru, Y. and White, A.W. 2000. Estimated annual economic impacts from harmful algal blooms (HABs) in the United States. WHOI-T-00-00297.

Anderson, D.M. andWall, D. 1978. Potential importance of benthic cysts of *Gonyaulax tamarensis* and *G. excavata* in initiating toxic dinoflagellate blooms. *J. Phycol*. 13:224-34.

Balech, E. 1995. The Genus *Alexandrium* Halim (Dinoflagellata) Sherkin Island Marine Station, Sherkin Island Marine Station, Ireland.

Balech, E. andTangen, K. 1985. Morphology and taxonomy of toxic species in the tamarensis group (Dinophyceae): *Alexandrium excavatum* (Braarud) *comb. nov*. and *Alexandrium ostenfeldii* (Paulsen) *comb. nov.* Sarsia 70:333-43.

Barker, G.L.A., Green, J.C., Hayes, P.K. and Medlin, L.K. 1994. Preliminary results using the RAPD analysis to screen bloom populations of Emiliania huxleyi (Haptophyta). *Sarsia* 79:301-06.

Barrett, B.A. and Kidwell, K.K. 1998. AFLP-based genetic diversity assessment among wheat cultivars from the Pacific Northwest. *Crop Science* 38:1261-71.

Bolch, C.J.S., Blackburn, S.J., Hallegraeff, G.M. and Vaillancourt, R.E. 1999. Genetic variation among strains of the toxic dinoflagellate *Gymnodinium catenatum* (Dinophyceae). *J. Phycol*. 35:356-67.

Cembella, A.D. 1988. Electrophoretic characterization of esterase isozymes in

*Protogonyaulax tamarensis*. *J. Phycol*. 24:14.

Cembella, A.D., Sullivan, J.J., Boyer, G.L., Taylor, F.J.R. and Andersen, R.J. 1987. Variation in paralytic shellfish toxin composition within the *Protogonyaulax tamarensis/catenella* species complex; red tide dinoflagellates. *Biochem. Syst. Ecol* 15:171-86.

Cembella, A.D. and Taylor, F.J.R. 1986. Electrophoretic variability within the *Protogonyaulax tamarensis/catenella* species complex: Pyridine linked dehydrogenases. *Biochem. Syst. Ecol* 14:311-23.

Cembella, A.D., Taylor, F.J.R. and Therriault, J.C. 1988. Cladistic analysis of electrophoretic variants within the toxic dinoflagellate genus *Protogonyaulax* . *Bot. Mar*. 31:39-51.

Dale, B. 1977. Cysts of the toxic red-tide dinoflagellate *Gonyaulax excavata* (Braarud) Balech from Oslofjorden, Norway. *Sarsia.* 63:29-34.

Donaldson, S.L., Chopin, T. and Saunders, G.W. 1998. Amplified Fragment Length Polymorphism (AFLP) as a source of genetic markers for red algae. *J. Appl. Phycol*. 10:365-70.

Donaldson, S.L., Chopin, T. and Saunders, G.W. 2000. An assessment of the AFLP method for investigating population structure in the red alga *Chondrus crispus* Stackhouse (Gigartinales, Florideophyceae). *J. Appl. Phycol*. 12:25-35.

Doyle, J.J. and Doyle J.L. 1990. Isolation of plant DNA from fresh tissue. *Focus* 12:13-15.

Duim, B., Wassenaar, T.M., Rigter, A. and Wagenaar, J. 1999. High-Resolution Genotyping of *Campylobacter* Strains Isolated from Poultry and Humans with Amplified Fragment Length Polymorphism Fingerprinting. *Appl. Env. Microbiol*. 65:2369-75.

Eppley, R.W., Holmes, R.W. and Strickland, J.D.H. 1967. Sinking rates of the marine phytoplankton measured with a fluorochrometer. *J. Exp. Mar. Biol. Ecol*. 1:191-208.

Felip, A., Martinez-Rodriguez, G., Piferrer, F., Carrillo, M. and Zanuy, S. 2000. AFLP analysis confirms exclusive maternal genomic contribution of meiogynogenetic sea bass (*Dicentrarchus labrax* L.). *Marine Biotechnology* 2:301-06.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*. 39:783-91.

Guillou, L., Moon-Van der Staay, S.Y., Claustre, H., Partensky, F. and Vaulot, D. 1999. Diversity and abundance of bolidophyceae (Heterokonta) in two oceanic regions. *Appl. Env. Microbiol.* 65:4528-36.

Hallegraeff, G.M. 1993. A review of harmful algal blooms and their apparent global increase. *Phycologia* 32:79-99.

Hallegraeff, G. M. 1995. Harmfull algal blooms: A global overview. *In* Hallegraeff, G. M., Anderson, D. M., and Cembella, A. D. [Eds.] Manual on Harmful Marine Microalgae, UNESCO, IOC Manual and Guides No. 33, pp. 1-22

Hallegraeff, G.M. 1998. Transport of toxic dinoflagellates via ships' ballast water: bioeconomic risk assessment and efficacy of possible ballast water management strategies. *Mar. Ecol. Prog. Ser*. 168:297-309.

Hauben, L., Vauterin, L., Moore, E.R.B., Hoste, B. and Swings, J. 1999. Genomic diversity of the genus *Stenotrophomonas*. *Int. J. Syst. Bacteriol*. 49:1749-60.

Higman, W.H., Stone, D.M. and Lewis, J.M. 2001. Sequence comparisons of toxic and non-toxic *Alexandrium tamarense* (Dinophyceae) isolates from UK waters. *Phycologia* 40:256-62.

Hongtrakul, V., Huestis, G.M. and Knapp, S.J. 1997. Amplified fragment length polymorphisms as a tool for DNA fingerprinting sunflower germplasm – genetic diversity among oilseed inbred lines. *Theoretical & Applied Genetics* 95:400-07.

Hummert, C., Rühl, A., Reinhardt, K., Hess, P., Luckas, B. and Gerdts, G. (2002). First evidence of neuro-toxic spirolides in European waters. *Euro. J. Phycol*., submitted.

Janssen, P., Coopman, R., Huys, G., Swings, J., Bleeker, M., Vos, P., Zabeau, M. and Kersters, K. 1996. Evaluation of the DNA fingerprinting method AFLP as a new tool in bacterial taxonomy. *Microbiology* 142:1881-93.

Janssen, P., Maquelin, K., Coopman, R., Tjernberg, I., Bouvet, P., Kersters, K. and Dijkshoorn, L. 1997. Discrimination of Acinetobacter genomic species by AFLP fingerprinting. *Int. J. Syst. Bacteriol*. 47:1179-87.

John, U. and Medlin, L.K. 2002a. Discovery of a new ribotype in the *Alexandrium tamarense* species complex (Dinophyceae) and the application of a molecular clock to explain the biogeographic distribution of the genus. *Mol. Biol. Evol*. Submitted.

John, U., Cembella, A.D., Hummert, C., Elbrächter, M., Groben, R. and Medlin, L.K. 2002b. Discrimination of the toxigenic dinoflagellates *Alexandrium tamarense* and *Alexandrium ostenfeldii* in co-occurring natural populations from Scottish coastal waters. *Euro. J. Phycol*. submitted.

Jones, C.J., Edwards, K.J., Castaglione, S., Winfield, M.O., Sala, F., Vandewiel, C., Bredemeijer, G., Vosman, B., Matthes, M., Daly, A., Brettschneider, R., Bettini, P., Buiatti, M., Maestri, E., Malcevschi, A., Marmiroli, N., Aert, R., Volckaert, G., Rueda, J., Linacero, R., Vazquez, A. and Karp, A. 1997. Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European Laboratories. *Molecular Breeding* 3:381-90.

Keller, M.D., Selvin, R.C., Claus, W. and Guillard, R.R.L. 1987. Media for the culture of oceanic ultraphytoplankton. *J. Phycol*. 23:633-38.

Kessler, A.G. and Avise, J.C. 1985. A comparative description of mitochondrial differentiation in selected avian and other vertebrate genera. *Mol. Biol. Evol*. 2:109-25.

Lange, M., Guillou, L., Vaulot, D., Simon, N., Amann, R.I., Ludwig, W. and Medlin, L.K. 1996. Identification of the Class Prymnesiophyceae and the Genus Phaeocystis with Ribosomal RNA-targeted Nucleic Acid Probes detected by Flow Cytometry. *J. Phycol*. 32:858-68.

Lim, E.L., Amaral, L.A., Caron, D.A. and DeLong, E.F. 1993. Application of rRNA-based probes for observing marine nanoplanktonic protists. *Appl. Environ. Microbiol*. 59:1647-55.

Mackill, D.J., Zhang, Z., Redoña, E.D. and Colowit, P.M. 1996. Level of polymorphism and genetic mapping of AFLP markers in rice. *Genome* 39:969-77.

Masiga, D.K., Tait, A. and Turner, C.M.R. 2000. Amplified Restriction Fragment Length Polymorphism in Parasite Genetics. *Parasitology Today* 16:350-53.

McLenachan, P.A., Stöckler, K., Winkworth, R.C., McBreen, K., Zauner, S. and Lockhart, P.J. 2000. Markers derived from amplified fragment length polymorphism gels for plant ecology and evolution studies. *Mol. Ecol*. 9:1899-903.

Medlin, L., Lange, M., Wellbrock, U., Donner, G., Elbrächter, M., Hummert, C. and Luckas, B. 1998. Sequence comparison links toxic European isolates of *Alexandrium tamarense* from the Orkney Islands to toxic north American stocks. *Europ. J. Protistol*. 34:329-35.

Medlin, L.K., Elwood, H.J., Stickel, S. and Sogin, M.L. 1988. The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene* 71:491-499.

Miller, P.E. and Scholin, C.A. 1998. Identification and enumeration of cultured and wild *Pseudo-nitzschia* (Bacillariophyceae) using species-specific LSU rRNA-targeted fluorescent probes and filter-based whole cell hybridization. *J. Phycol*. 34:371-382.

Parsons, M.L., Scholin, C.A., Miller, P.E., Doucette, G.J., Powell, C.L., Fryxell, G.A., Dortch, Q. and Soniat, T.M. 1999. *Pseudo-nitzschia* species (Bacillariophyceae) in Louisiana coastal waters: Molecular probe field trials, genetic variability, and domoic acid analyses. *J. Phycol*. 35:1368-78.

Rigby, G.R., Hallegraeff, G.M., and Sutton, C. Novel ballast water heating technique offers cost-effective treatment to reduce the risk of global transport of harmful marine organisms. *Mar. Ecol. Prog. Ser*. 191:289-293.

Rynearson, T.A. and Armbrust, E.V. 2000. DNA fingerprinting reveals extensive genetic diversity in a field population of the centric diatom *Ditylum brightwellii*. *Limnol. Oceanogr*. 45:1329-40.

Sako, Y., Kim, C.H., Ninomiya, H., Adachi, M. and Ishida, Y. 1990. Isozyme and cross analysis of mating populations in the *Alexandrium catenella/tamarense* species complex. *In* Granéli, E., Sundström, B., Edler, L., and Anderson, D.M., [Eds.] Toxic Marine Phytoplankton. Elsevier, New York, pp. 320-23

Scholin, C., Miller, P., Buck, K., Chavez, F., Cangelosi, G., Haydock, P., Howard, J., and Harris, P. 1996. DNA probe-based detection of harmful algal species using *Pseudo-nitzschia* species as models. *In* Yasumoto, T., Oshima, Y., and Fukuyo, Y. [Eds.] Harmful and Toxic Algal Blooms, IOC/UNESCO, Paris, pp. 439-42

Scholin, C., Miller, P., Buck, K., Chavez, F., Harris, P., Haydock, P., Howard, J. and Cangelosi, G. 1997. Detection and quantification of *Pseudo-nitzschia australis* in cultured and natural populations using LSU rRNA-targeted probes. *Limnol. Oceanogr*. 42:1265-72.

Scholin, C.A. 1998. Morphological, genetic, and biogeographic relatioships of the toxic dinoflagellates *Alexandrium tamarense*, *A. catenella*, and *A. fundyense*. *In* Anderson, D.M., Cembella, A.D., and Hallegraeff, G.M., [Eds.] Physiological Ecology of Harmful Blooms. Springer, Berlin, pp. 13-27

Scholin, C.A. and Anderson, D.M. 1994. Identification of group- and strain-specific genetic markers for globally distributed *Alexandrium* (Dinophyceae). I. RFLP analysis of SSU rRNA genes. *J. Phycol*. 30:744-54.

Scholin, C.A. and Anderson, D.M. 1996. LSU rDNA-Based RFLP Assays for Discriminating Species and Strains of *Alexandrium* (Dinophyceae). *J. Phycol*. 32:1022-35.

Scholin, C.A., Hallegraeff, G.M. and Anderson, D.M. 1995. Molecular evolution of the *Alexandrium tamarense* species complex (Dinophyceae)-dispersal in the North American and West Pacific regions. *Phycologia* 34:472-85.

Scholin, C.A., Herzog, M., Sogin, M. and Anderson, D.M. 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.

Seki, S., Agresti, J.J., Gall, G.A.E., Taniguchi, N. and May, B. 1999. AFLP analysis of genetic diversity in three populations of ayu *Plecoglossus altivelis*. *Fisheries Science* 65:888-92.

Simon, N., Brenner, J., Edvardsen, B. and Medlin, L.K. 1997. The identification of *Chrysochromulina* and *Prymnesium* species (Haptophyta, Prymnesiophyceae) using fluorescent or chemoluminescent oligonucleotide probes: a means for improving studies on toxic algae. *Euro. J. Phycol*. 32:393-401.

Simon, N., Campbell, L., Ornolfsdottir, E., Groben, R., Guillou, L., Lange, M. and Medlin, L.K. 2000. Oligonucleotide probes for the identification of three algal groups by dot blot and fluorescent whole-cell hybridization. *J. Eukar. Microbiol*. 47:76-84.

Smayda, T.J. 1990. Novel and nuisance phytoplankton blooms in the sea: evidence for a global epidemic. *In* Graneli, E., Sundström, B., Edler, L., and Anderson, D.M., [Eds.] Toxic Marine Phytoplankton. Elsevier, New York, pp. 29-40

Taylor, F.J.R. 1984. Toxic dinoflagellates: taxonomic and biogeographic aspects with emphasis on *Protogonyaulax*. *In* Ragelis, E. P. [Eds.] Seafood Toxins, American Chemical Society, Washington, D.C., pp. 77-97

Taylor, F.J.R. 1987. General and marine ecosystems. *In* Taylor, F.J.R., [Eds.] The Biology of Dinoflagellates Blackwell, Oxford, pp. 399-502

Upholt, W.B. 1977. Estimation of DNA sequence divergence from comparison of restriction endonuclease digests. *Nucl. Acids Res*. 4:1257-65.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Vandelee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. and Zabeau, M. 1995. AFLP – a new technique for DNA fingerprinting. *Nucl. Acids Res*. 23:4407-14.

Walsh, D., Reeves, R.A., Saul, D.J., Gray, R.D., MacKenzie, L., Bergquist, P.R. and Bergquist, P.L. 1998. Heterogeneity of SSU and LSU rDNA sequences of *Alexandrium* species. *Biochem. Syst. Ecol*. 26:495-509.

Wyatt, T. and Jenkinson, I. R. 1997. Notes on *Alexandrium* population dynamics. *J. Plankton Res*. 19:551-75.

Table 1. Designation and geographical origin of strains used in this study.

| *Species* | **Strain** | **Origin** | **Collector** |
|---|---|---|---|
| *Alexandrium affine* | CCMP 112 | Rio de Vigo (Spain) | I. Bravo |
| *Alexandrium catenella* | BAHME 255 | Tarragona (Spain) | M. Delgado |
| | BAHME 222 | Tarragona (Spain) | M. Delgado |
| | BAHME 217 | Tarragona (Spain) | M. Delgado |
| *Alexandrium fundyense* | GT 7 | Bay of Fundy (USA); | A. White |
| | CCMP 1719 | Portsmouth (USA) | D. Kulis |
| *Alexandrium lusitanicum* | BAHME 91 | Laguna de Obidos (Portugal) | E. Silva e Sousa |
| *Alexandrium minutum* | Al5T | Gulf of Trieste (Italy) | A. Beran |
| *Alexandrium ostenfeldii* | BAHME 136 | Timaru (New Zealand) | N. Berkett |
| *Alexandrium pseudogonyaulax* | AP2T | Gulf of Trieste (Italy) | A. Beran |
| *Alexandrium tamarense* | GTTP01 | Perch Pond, Falmouth, MA (USA) | D. Kulis |
| | GT-7 | Bay of Fundy (USA) | A. White |
| | AL18b | St. Lawrance (Canada) | A. Cembella |
| | AT-9 | Ofunata Bay (Japan) | Kodama |
| | OF 84423.3 | Ofunata Bay (Japan) | Kodama |
| | BAHME 181 | Orkney Island (Scotland) | M. Elbrächter |
| | BAHME 182 | Orkney Island (Scotland) | M. Elbrächter |
| | BAHME 184 | Orkney Island (Scotland) | M. Elbrächter |
| | BAHME 200 | Orkney Island (Scotland) | M. Elbrächter |
| | SZN 01 | Gulf of Naples (Italy) | M. Montresor |
| | SZN 08 | Gulf of Naples (Italy) | M. Montresor |
| | SZN 019 | Gulf of Naples (Italy) | M. Montresor |
| | SZN 021 | Gulf of Naples (Italy) | M. Montresor |
| | UW42 | Belfast (Nord Ireland) | W. Higman |
| | 31/9 | Cork Harbour (Ireland) | W. Higman |
| | 31/4 | Cork Harbour (Ireland) | W. Higman |
| | CCMP 115 | Tamar estuary (U.K.) | I. Adams |
| *Alexandrium taylori* | Ay1T | Lagoon of Marano (Italy); | A. Beran |
| *Prorocentrum lima* | CCMP 1743 | Gulf of Maine (USA) | M. Faust |
| *Prorocentrum micans* | BAHME 04 | Helgoland (Germany) | G. Drebes |
| *Prorocentrum minimum* | BAHME 137 | Vigo (Spain) | I. Bravo |

Table 2. Probes used in this study.

| Probe name used in this study | Standardized probe name[b] | Specific for | Probe sequence [5'- 3'] |
|---|---|---|---|
| **Universal** | | | |
| EUK1209R [a] | S-K-Euk-1209-a-A-16 | Eukaryotes | GGGCATCACAGACCTG |
| **Species level** | | | |
| ATAM01 | S-S-A.tam-0775 (A. tamarense)-a-A-18 | *A. tamarense* species complex | TTCAAGGCCAAACACCTG |
| **Clade level** | | | |
| ATNA02 | L-St-At.NA-373 (A. tamarense)-a-A-18 | *A. tamarense* – North American / Orkney strains | AACACTCCCACCAAGCAA |
| ATWE03 | L-St-At.WE-565 (A. tamarense)-a-A-18 | *A. tamarense* – Western European strains | GCAACCTCAAACACATGG |
| ATME04 | L-St-At.ME-484 (A. tamarense)-a-A-18 | *A. tamarense* – Mediterranean strains | CCCCCCCACAAGAAACTT |

[a] Lim et al. 1993; [b] Alm et al. 1996

Table 3: Oligonucleotide sequences used in AFLP analysis. Adapter and primer designed according to Vos *et al.* (1995); AS: adapter sequence, RS: restriction site sequence, SB: selective bases

| **Adapters** | **Sequence (5' – 3')** |
|---|---|
| *EcoR*I | CTC GTA GAC TGC GTA CC |
| | CAT CTG ACG CAT GGT TAA |
| *Mse*I | GAC GAT GAG TCC TGA G |
| | TA CTC AGG ACT CAT |

| **Primer** | ------AS---------- ---RS--- ---SB |
|---|---|
| *EcoR*I +A | GACTGCGTACC AATTC A |
| *EcoR*I +AAC | GACTGCGTACC AATTC AAC |
| *EcoR*I +AAG | GACTGCGTACC AATTC AAG |
| *EcoR*I +ACC | GACTGCGTACC AATTC ACC |
| *Mse*I +C | GATGAGTCCTGAG TAA C |
| *Mse*I +CAC | GATGAGTCCTGAG TAA CAC |
| *Mse*I +CTA | GATGAGTCCTGAG TAA CTA |
| *Mse*I +CTT | GATGAGTCCTGAG TAA CTT |

Table 4. Primer used in AFLP analysis

| **Gel number** | ***EcoR*I-primer** | ***Mse*I-primer** | **Used** |
|---|---|---|---|
| A1 | +AAG | +CAC | no |
| A2 | +AAG | +CTT | yes |
| A3 | +AAG | +CTA | yes |
| A4 | +AAC | +CTT | no |
| A5 | +ACC | +CTT | yes |
| A6 | +ACC | +CTA | no |

Table 5. Similarity matrices indicating the proportion of bands share between strains for gel A3 and A5 above and below the diagonal, respectively (- symbolize 100% similarity) Abbreviations in the first column stands for the geographic clades to which the strain belongs; ME: Mediterranean clade, NA: North American clade, NA (Ork): North American clade isolated at the Orkney Islands (Scotland), WE: Western Europe.

| | | WE | WE | NA | WE | NA | NA | NA | NA(Ork) | NA(Ork) | NA(ork) | Na(Ork) | ME | ME | ME | ME | WE |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Geographic clade** | **Strain / Strain** | **CCMP 1771** | **31-4** | **CCMP 1719** | **31-9** | **NEPCC 407** | **GTPP01** | **GTL121** | **BAHME 200** | **BAHME 184** | **BAHME 182** | **BAHME 181** | **SZN19** | **SZN01** | **SNZ08** | **SZN21** | **UW 42** |
| **WE** | **CCMP 1771** | - | 0.31 | 0.20 | 0.19 | 0.19 | 0.20 | 0.06 | 0.14 | 0.14 | 0.18 | 0.14 | 0.13 | 0.07 | 0.10 | 0.14 | 0.10 |
| **WE** | **31-4** | 0.26 | - | 0.16 | 0.15 | 0.03 | 0.13 | 0.03 | 0.23 | 0.11 | 0.13 | 0.12 | 0.29 | 0.12 | 0.14 | 0.25 | 0.16 |
| **NA** | **CCMP 1719** | 0.15 | 0.25 | - | 0.06 | 0.11 | 0.14 | 0.11 | 0.13 | 0.04 | 0.10 | 0.05 | 0.16 | 0.09 | 0.07 | 0.11 | 0.00 |
| **WE** | **31-9** | 0.19 | 0.16 | 0.14 | - | 0.07 | 0.09 | 0.07 | 0.12 | 0.16 | 0.06 | 0.15 | 0.20 | 0.16 | 0.14 | 0.21 | 0.17 |
| **NA** | **NEPCC 407** | 0.18 | 0.15 | 0.10 | 0.49 | - | 0.28 | 0.49 | 0.11 | 0.11 | 0.10 | 0.08 | 0.14 | 0.20 | 0.22 | 0.14 | 0.03 |
| **NA** | **GTPP01** | 0.27 | 0.19 | 0.14 | 0.45 | 0.36 | - | 0.33 | 0.20 | 0.17 | 0.17 | 0.18 | 0.16 | 0.07 | 0.06 | 0.17 | 0.05 |
| **NA** | **GTL 121** | 0.23 | 0.23 | 0.11 | 0.28 | 0.23 | 0.23 | - | 0.14 | 0.08 | 0.05 | 0.14 | 0.13 | 0.17 | 0.18 | 0.10 | 0.04 |
| **NA (Ork)** | **BAHME 200** | 0.16 | 0.11 | 0.12 | 0.19 | 0.15 | 0.22 | 0.24 | - | 0.81 | 0.40 | 0.43 | 0.04 | 0.03 | 0.03 | 0.04 | 0.08 |
| **NA (Ork)** | **BAHME 184** | 0.16 | 0.11 | 0.09 | 0.19 | 0.14 | 0.19 | 0.23 | 0.95 | - | 0.48 | 0.49 | 0.04 | 0.03 | 0.06 | 0.04 | 0.08 |
| **NA (Ork)** | **BAHME 182** | 0.14 | 0.11 | 0.09 | 0.23 | 0.18 | 0.22 | 0.26 | 0.71 | 0.76 | - | 0.42 | 0.03 | 0.05 | 0.02 | 0.03 | 0.10 |
| **NA (Ork)** | **BAHME 181** | 0.23 | 0.14 | 0.14 | 0.21 | 0.17 | 0.21 | 0.22 | 0.47 | 0.49 | 0.53 | - | 0.05 | 0.02 | 0.06 | 0.05 | 0.10 |
| **ME** | **SZN 19** | 0.11 | 0.06 | 0.03 | 0.04 | 0.19 | 0.07 | 0.09 | 0.10 | 0.10 | 0.03 | 0.05 | - | 0.50 | 0.44 | 0.80 | 0.00 |
| **ME** | **SZN 01** | 0.11 | 0.05 | 0.03 | 0.04 | 0.19 | 0.10 | 0.12 | 0.07 | 0.06 | 0.07 | 0.05 | 0.90 | - | 0.64 | 0.48 | 0.00 |
| **ME** | **SZN 08** | 0.18 | 0.16 | 0.11 | 0.19 | 0.28 | 0.12 | 0.20 | 0.16 | 0.15 | 0.13 | 0.15 | 0.79 | 0.70 | - | 0.46 | 0.04 |
| **ME** | **SZN 21** | 0.17 | 0.08 | 0.03 | 0.04 | 0.19 | 0.10 | 0.06 | 0.07 | 0.07 | 0.07 | 0.08 | 0.70 | 0.75 | 0.65 | - | 0.00 |
| **WE** | **UW 42** | 0.10 | 0.05 | 0.00 | 0.04 | 0.07 | 0.00 | 0.08 | 0.06 | 0.09 | 0.09 | 0.08 | 0.06 | 0.06 | 0.06 | 0.06 | - |

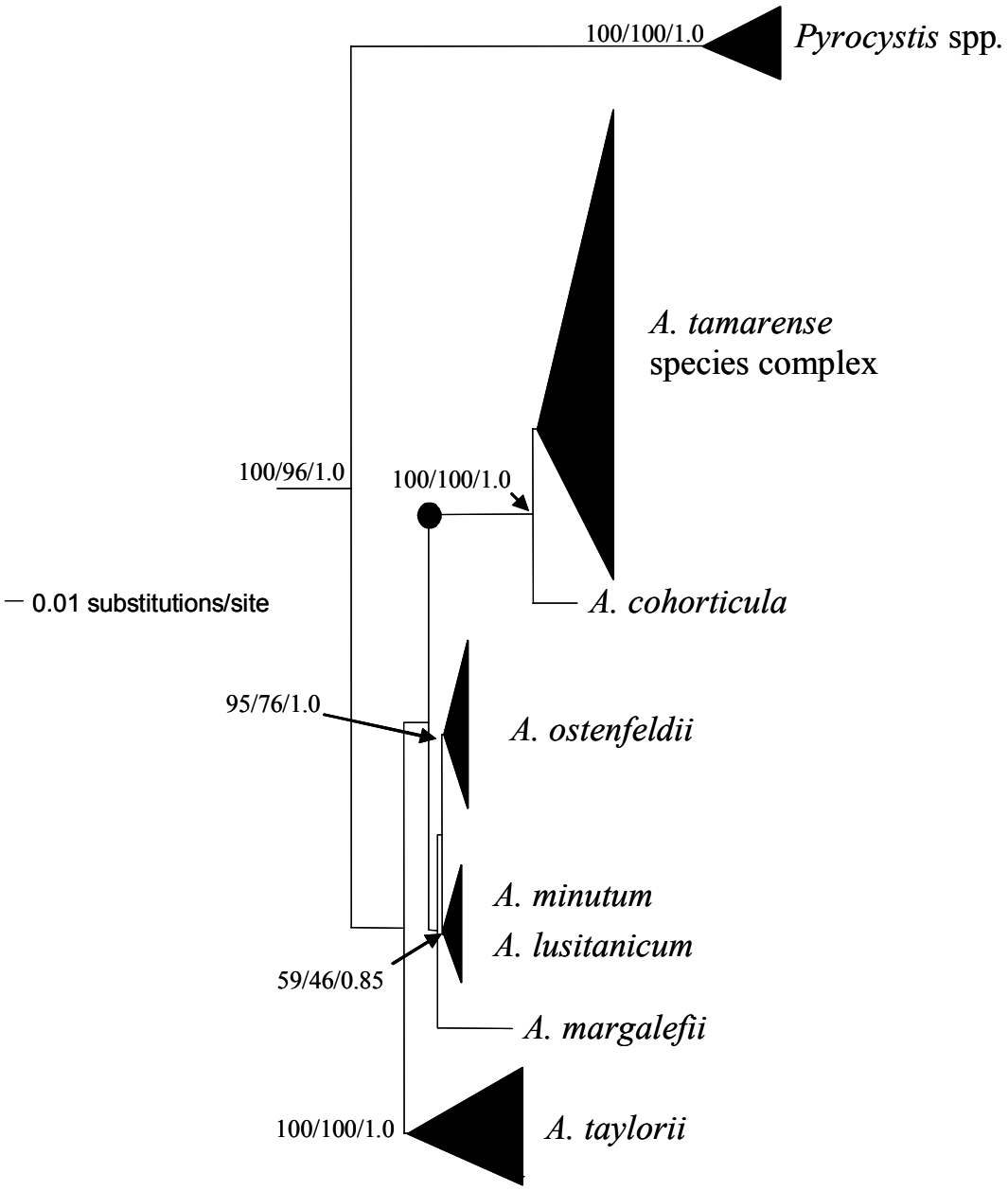



Fig. 1. Maximum likelihood phylogenetic tree of 18S LSU rRNA sequences from the genus *Alexandrium* modified from John et al. (2002a, submitted). Eleven outgroup species were pruned from the tree and the different branches of a certain taxa were condensed to a triangle for clarification. Circle symbolizes the node position for which the ATAM01 probe was designed. Bootstrap values (>50%) from a MP/NJ/BI analysis are placed close to each node or arrow show the correspondending node as described in detail in John et al. (2002a, submitted).

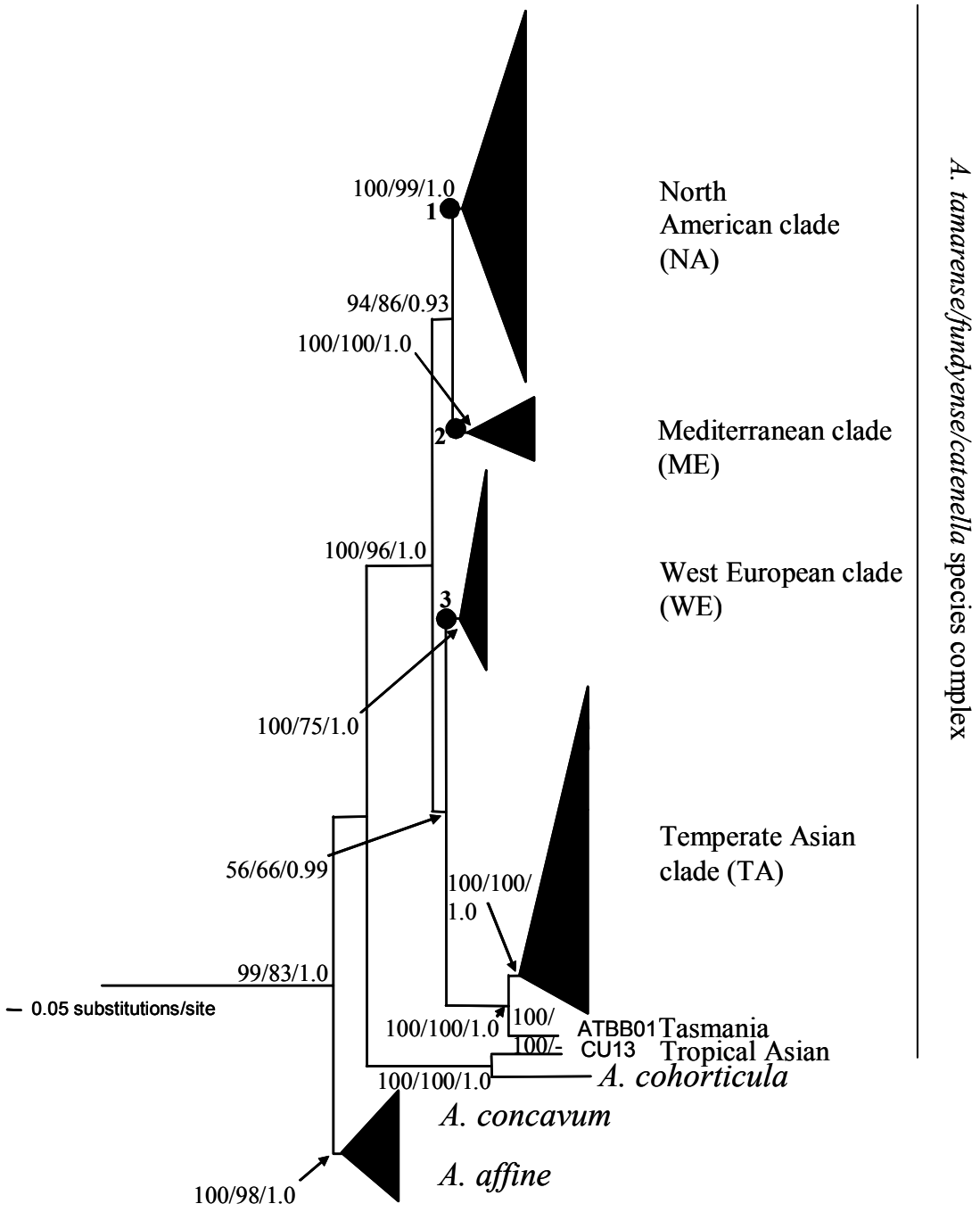



Fig. 2. Maximum likelihood phylogenetic tree of representatives of the genus *Alexandrium* based on their sequences of the D1/D2 region of the LSU rRNA modified from John et al. (2002a, submitted). Thirty-two outgroup species were pruned from the tree and the different branches of a certain taxa or geographic clade were conden to a triangle for clarification. Filled circle 1 shows the position of rRNA probe ATNA02., circle 2 that of ATME04, and circle 3 that of ATWE03, respectively. Bootstrap values (>50%) from a MP/NJ/BI analysis are placed close to each node or arrow show the correspondending node as described in detail in John et al. (2002a, submitted).

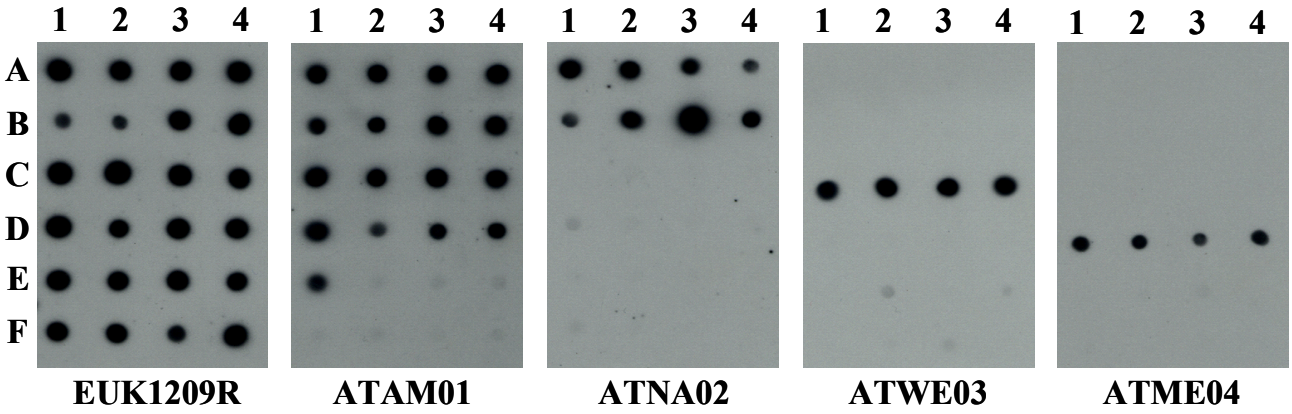



Fig. 3: Dot-blot hybridization of filter bound amplified SSU or LSU rRNA sequences with digoxigenin-labelled oligonucleotide probes (table 2). Rows A-D: *A. tamarense* species complex strains; row A: NA Orkney strains, A1: BAHME 181, A2: BAHME 182, A3: BAHME 184, A4: BAHME 200; row B: NA strains, B1: GT-7, B2: AL18b, B3: OF8423.3, B4: GTPP01; row C: WE strains, C1: CCMP 115, C2: 31/4, C3: 31/9, C4: UW42; row D: ME strains, D1: SZN01, D2: SZN08, D3: SZN19, D4: SZN21; E1: *A. catenella* (Asian clade); E2: *A.lusitanicum* (BAHME 91); E3: *A. minutum* (Al5T); E4: *A. pseudogonyaulax* (AP2T); F1: *A. ostenfeldii* (BAHME 136); F2: *A. taylori* (AY1T); F3: *A. affine* (CCMP 112); F4: *Prorocentrum minimum* (BAHME 137).

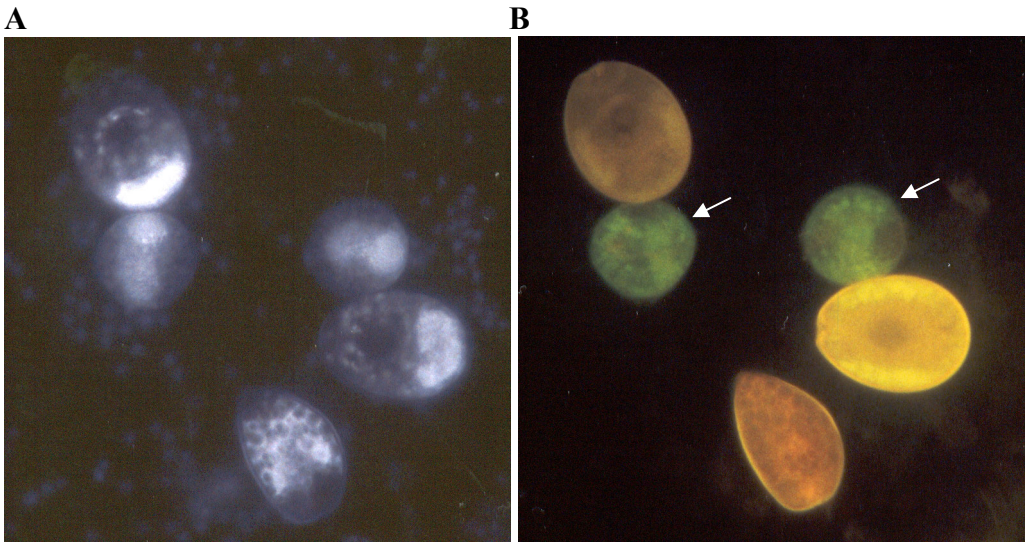



Fig. 4: Left: *In situ* hybridization of *A. tamarense* (SZN 01), *P. micans* (BAHME 04) and *P. lima* (CCMP 1743) using probe ATAM01. Arrows indicate the positive green signal of the *A. tamarense* cell. Right: DAPI counterstain of the cells.

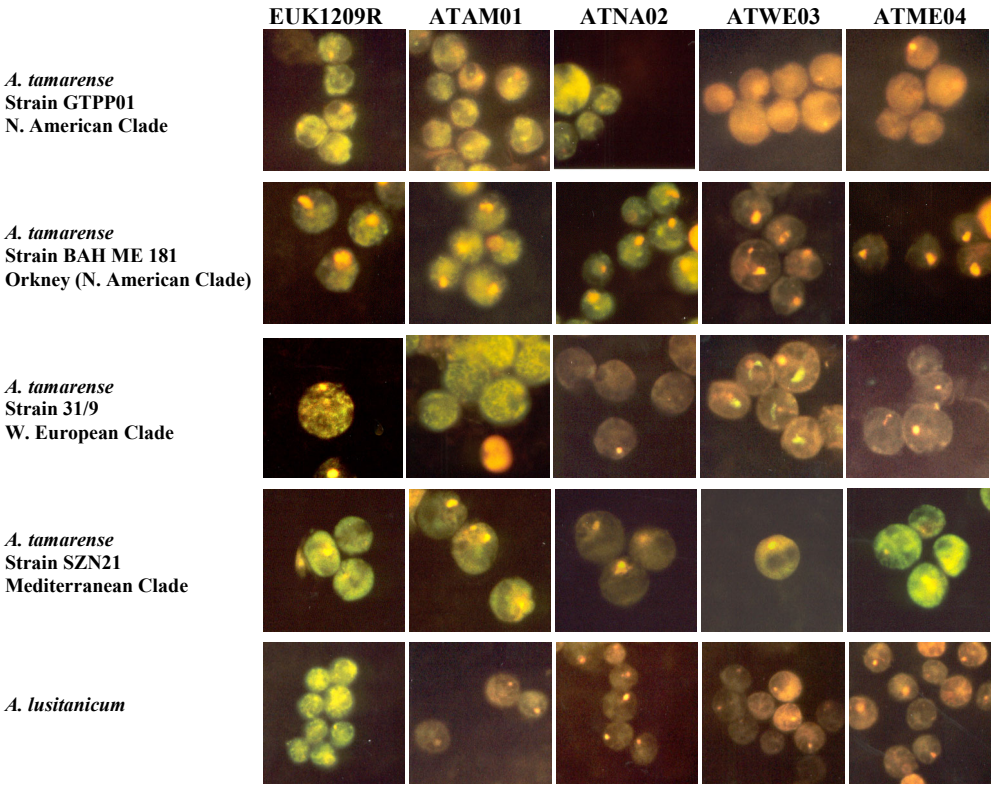



Fig. 5: Array of whole-cell hybridizations from five different *Alexandrium* strains representing of two strains from the NA clade, one from the WE clade, one the ME clade, and one *A. lusitanicum*. , and probed with the universal and clade specific probes listed in Table 2.

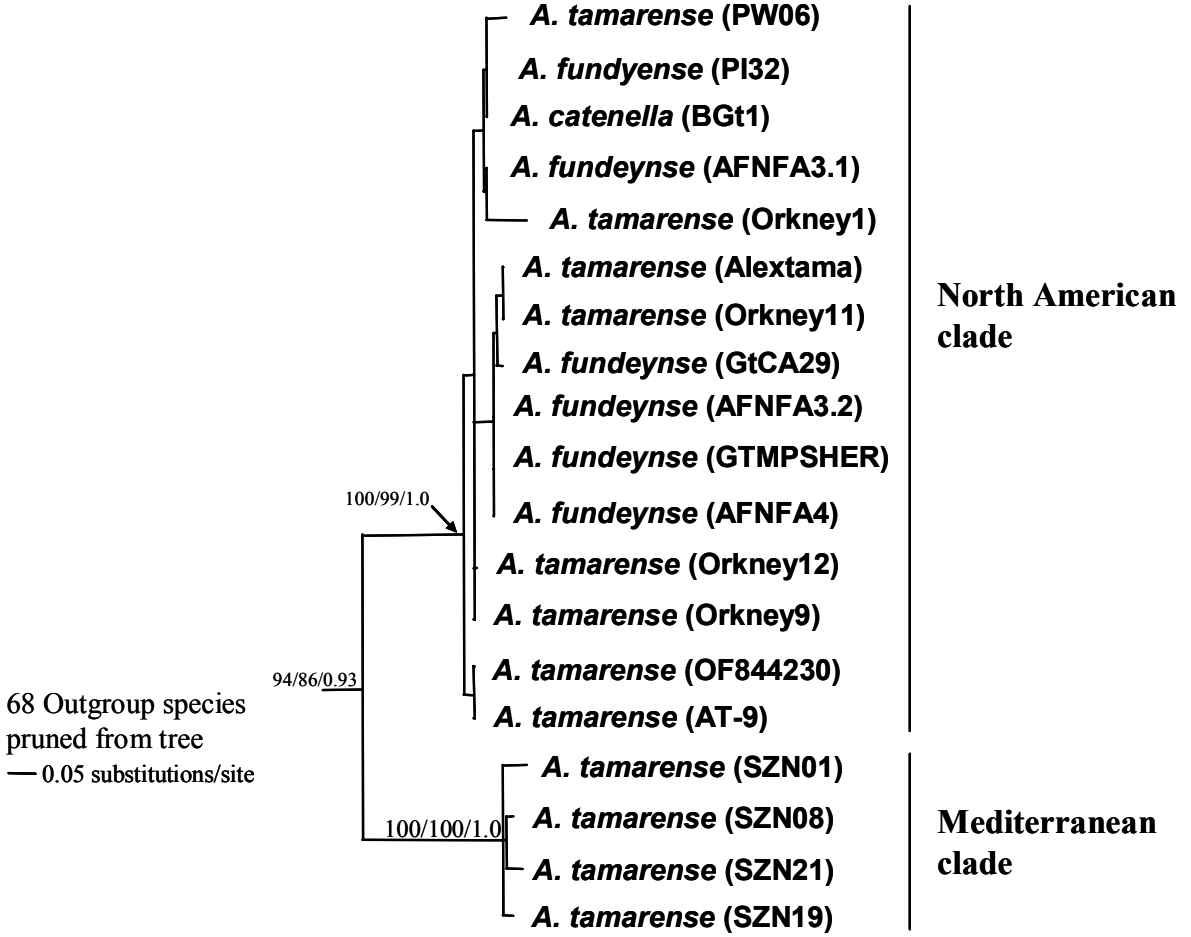



Fig. 6. Maximum likelihood phylogenetic tree of representatives of the genus *Alexandrium* based on their sequences of the D1/D2 region of the LSU rRNA modified from John et al. (2002a, submitted). Sixty eight outgroup species were pruned from the tree. Bootstrap values (>50%) from a MP/NJ/BI analysis are placed close to each node or arrow show the correspondending node asdescribed in detail in John et al. (2002a, submitted).

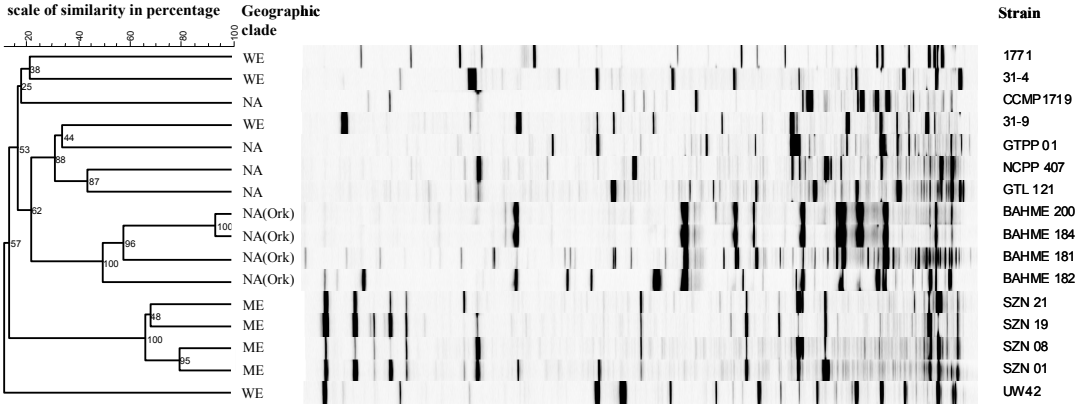



Fig. 7: AFLP analysis of fluorescently labelled fingerprints from different strains of the Alexandrium tamarense species complex. Strain names (Table 1) are listed at the right. AFLP fingerprints were generated from genomic DNA digested with *Eco*RI and *Mse*I. The specific primers used were ECORI+AAG and MSEICTT (Table 2 & 4). The dendrogram was constructed by using UPGMA. The scale indicates percentages of similarity, as determined with the Dice similarity coefficient (BioNumerics cluster analysis). Abbreviations in the last column stands for the geographic clades to which the strain belongs; ME: Mediterranean clade, NA: North American clade, NA (Ork): North American clade isolated at the Orkney islands (Scotland), WE: Western European clade.

## *2.9. Publication VII:* Discrimination of the toxigenic dinoflagellates Alexandrium tamarense and *Alexandrium ostenfeldii* in co-occurring natural populations from Scottish coastal waters

UWE JOHN[1], ALLAN CEMBELLA[2], CHRISTIAN HUMMERT[3], MALTE ELBRÄCHTER[4], RENÉ GROBEN[1] AND LINDA MEDLIN[1]

[1] Alfred-Wegener-Institut für Polar- und Meeresforschung, Am Handelshafen 12, D-27570 Bremerhaven, Germany

[2] Institute for Marine Biosciences, National Research Council, 1411 Oxford Street, Halifax, NS, Canada B3H 3Z1

[3] Laboratory Wiertz-Eggert-Jörissen GmbH (WEJ), Stenzelring 14b, D-21107 Hamburg, Germany

[4] Deutsches Zentrum für Biodiversität, FI-Senckenberg, Wattenmeerstation Sylt, Alfred-Wegener-Institut für Polar- und Meeresforschung, Hafenstrasse 43, D-25992 List/Sylt, Germany

Correspondence to: U. John. E-mail: **ujohn@awi-bremerhaven.de**

*Abstract*

Blooms of the toxic dinoflagellate *Alexandrium tamarense* (Lebour) Balech, a known producer of potent neurotoxins associated with paralytic shellfish poisoning (PSP), are common annual events along the Scottish east coast. The co-occurrence of a second *Alexandrium* species, *A. ostenfeldii* (Paulsen) Balech & Tangen is reported in this study from waters of the Scottish east coast. The latter species has been suspected to be an alternative source of PSP toxins in northern Europe. Recent identification of toxic macrocyclic imines known as spirolides in *A. ostenfeldii* indicates a potential new challenge for monitoring toxic *Alexandrium* species and their respective toxins in natural populations. In mixed phytoplankton assemblages, *Alexandrium* species are difficult to discriminate accurately by conventional light microscopy. Species-specific rRNA probes based upon 18S and 28S ribosomal DNA sequences were developed for *A. ostenfeldii* and tested by dot-blot and fluorescence *in situ* hybridisation (FISH) techniques. Hybridisation patterns of *A. ostenfeldii* probes for cultured *Alexandrium* isolates, and cells from field populations from the Scottish east coast, were compared with those of rDNA probes for *A. tamarense* and a universal dinoflagellate probe. *Alexandrium* cell numbers in field samples determined by whole-cell *in situ* hybridisation were much lower that those determined by optical microscopy with the Utermöhl method involving sedimentation chambers, but the results were highly correlated (e.g., $r^2$ = 0.94; $n$ = 6 for *A. tamarense*). Determination of spirolides and PSP toxins by instrumental analysis on board ship demonstrated the presence of both toxin groups in plankton assemblages collected from surface waters near the Orkney Islands, and confirmed the association of *A. ostenfeldii* with spirolides in northern Europe. These results show that rRNA probes for *A. tamarense* and *A. ostenfeldii* are useful albeit only semi-quantitative tools to detect and discriminate these species in field studies.

**Key Words**: *Alexandrium tamarense*, *Alexandrium ostenfeldii*, molecular probes, phycotoxins, toxic algae

*Introduction*

Harmful algal blooms are a common occurrence in northern European waters, causing a wide variety of environmental and public health problems, including massive fish mortalities, seafood poisoning in humans, and biofouling of beaches and fishing gear (see references in Granéli *et al.,* 1990). On a global basis, there is some evidence for an increase in the frequency, intensity and distribution of harmful events associated with algal blooms (Smayda, 1990; Hallegraeff, 1993). In any case, it is beyond dispute that the social and economic consequences have become more severe in the last several decades, concomitant with increased exploitation of non-traditional seafood products, fisheries stocks, and aquaculture species.

Among the several dozen reported toxigenic species of phytoplankton, those belonging to the marine dinoflagellate genus *Alexandrium* (Halim) (Balech, 1995) are perhaps the most thoroughly investigated for their toxic properties. *Alexandrium* species are frequently implicated as the cause of paralytic shellfish poisoning (PSP) in human consumers of contaminated seafood. The taxonomic history of *Alexandrium* is long and complex, with many issues remaining unresolved. *Alexandrium ostenfeldii* was first described (as *Goniodoma*) from Iceland and other locations in Scandinavia (Paulsen, 1904; 1949), but inadequacies in the plate tabulation warranted a redescription from Norwegian specimens (Balech & Tangen, 1985). The best-known species of the genus, *Alexandrium tamarense* (Lebour) Balech was first described as *Gonyaulax tamarensis* from the Tamar estuary near Plymouth, UK (Lebour, 1925). This species is now widely reported from temperate to sub-Arctic regions, and even tropical latitudes (Taylor, 1984). Braarud (1945) recognized three varieties of *G. tamarensis* (var. *tamarensis*; var. *globosa*; and var. *excavata*) from Norwegian waters. Later, these varieties were assigned specific status within the genus *Gonyaulax* Diesing. In his classic monograph on the morphology of *Alexandrium*, Balech (1995) considers "excavatum" to be a minor variant of *Alexandrium tamarense* and "globosa" as synonymous with *Alexandrium ostenfeldii* (Paulsen) Balech & Tangen.

Although *A. ostenfeldii* has been suspected as a possible source of PSP toxicity in Norwegian shellfish (Balech & Tangen 1985), this has not been definitively established because of a temporal overlap with the presence of *A. tamarense* blooms in locations such as Oslofjord. Certain cultured strains of *A. ostenfeldii* from Limfjord, Denmark were found to produce low levels of PSP toxins (Hansen *et al.,* 1992), and a few isolates from New Zealand were shown to be very toxic (Mackenzie *et al.*, 1996).

A group of novel marine toxins, macrocyclic imines known as spirolides, was isolated and characterised from shellfish (Hu *et al.,* 1995; 1996) and later from plankton (Cembella *et al.,* 1998; 1999) collected from the coastal waters of Nova Scotia, Canada. The causative organism of spirolide toxicity in shellfish was recently identified as *A. ostenfeldii* (Paulsen) Balech & Tangen (Cembella *et al.*, 2000), and certain isolates of this species can produce a wide diversity of spirolides in unialgal batch culture (Hu *et al.*, 2001). The association of spirolides with *A. ostenfeldii* is important, given that this species is broadly distributed, particularly in north temperate latitudes. In northern Europe, cultured isolates from Danish waters have also been found to produce these toxins (Cembella *et al.*, 2000; Cembella & Quilliam, unpubl. data).

Under casual microscopic observation, such as is routinely performed in monitoring programs for harmful algae, cells of *A. ostenfeldii* are difficult to discriminate reliably from those of *A. tamarense.* The vegetative cells of the former species are typically larger and more 'globose' than those of the latter (Balech, 1995; Cembella *et al.*, 2000), but there is considerable variation in gross morphology among cells of these species. Key diagnostic features, such as the size and shape of the ventral pore at the margin of the first apical (1') thecal plate, must be examined individually for each specimen – a tedious procedure. Thus even in the absence of other *Alexandrium* taxa, a species-specific probe for *A. ostenfeldii* would be a useful complement for conventional monitoring of phytoplankton.

Since populations of these *Alexandrium* species may coincide in nature (Cembella *et al.*, 1998; Levasseur *et al.*, 1998), whereas their toxic properties are very divergent, more reliable and rapid methods for species discrimination are desirable. *Alexandrium tamarense* is frequently dominant when the species co-occur and is better known. As a consequence, it is likely that the relative abundance of *A. ostenfeldii*, and hence the risk of spirolide toxicity, has been underestimated in field samples.

The application of taxon-specific nucleic acid probes is one emerging method for discriminating among phytoplankton species. At least in principle, such probes can be used for identifying, quantifying and mapping the distribution of various taxa in natural plankton populations, but these techniques have not yet been widely applied. Nucleic acid probes have been developed for a broad range of algae and certain hierarchical groups (e.g., Lange *et al.*, 1996; Scholin *et al.*, 1996; Miller & Scholin, 1998; Simon *et al.*, 2000), and in some cases they have been used to determine the abundance and diversity of Prymnesiophyceae (Moon-van der Staay *et al.*, 2000) and Bolidophyceae (Heterokonta) (Guillou *et al.*, 1999) in field samples. A few molecular probes have also been developed for toxic microalgal taxa,

including both diatoms and dinoflagellates (Scholin *et al.,* 1996; 1997; Miller & Scholin, 1998; Simon *et al.*, 1997). Species-specific rRNA probes for the potentially toxic pennate diatom *Pseudo-nitzschia australis* (Bacillariophyceae) have been used to discriminate this species from other co-occurring *Pseudo-nitzschia* species in culture and field samples, and to quantify cells of these respective taxa (Scholin *et al.,* 1996; Miller & Scholin, 1998). To date, only a single rRNA probe has been published for the genus *Alexandrium* (Miller & Scholin, 1998). This probe is specific for the North American clade of *A. tamarense*, but more rRNA probes for this group and other geographic clades of the species are under development (John *et al*., 2002 submitted).

The study reported here represents the first concerted attempt to apply molecular probes for the discrimination of these *Alexandrium* species in natural populations, combined with shipboard analysis of the respective toxin composition of these taxa. We present the results of the application of these taxon-specific probes to discriminate between cells *A. ostenfeldii* and *A. tamarense* in field plankton samples collected from the off the Scottish coast near the Orkney Islands, where annual blooms of toxic dinoflagellates are known to occur (Medlin *et al.*, 1998; Higman *et al.*, 2001).

*Material and Methods*

*Field sampling*

The cruise track of the research vessel *Heincke* in May 2000 extended over an area of 420 X 105 km, from the Orkney Islands to the Firth of Forth along the east coast of Scotland. In the present study, six sampling sites were selected along the coast between Aberdeen and Edinburgh (Fig.1). For cell identification and counts by optical microscopy, surface seawater samples were collected by pumping from a buoy, with the hose orifice fixed at 1 m depth. Taxonomic samples were preserved with formalin (2% final concentration). Parallel samples for nucleic acid probing were also collected via this pumping method. For toxin analysis, plankton samples (non-quantitative) were obtained with a plankton net (20 µm mesh size) from sub-surface water (approximately 1 to 3 m depth). Net planktonic material was concentrated by application of low vacuum onto 0.45-µm PTFE filters (50 mm diameter) and rinsed with 0.2 µm-filtered seawater.

*Plankton identification and counting*

Plankton identification and quantitation was performed by the inverted-microscope method (Utermöhl, 1958) with 25-ml sedimentation chambers. Critical identification of *Alexandrium* cells was carried out on a Leitz Fluovert FS inverted microscope equipped with epifluorescence optics, after the direct addition of the optical brightener calcofluor (0.002% final concentration) (Fritz & Triemer, 1985) to the formalin-fixed samples. Species of *Alexandrium* were discriminated by size, shape, and characteristic thecal features, including presence or absence of the ventral pore on the first apical (1') plate, form of the apical pore complex (APC), and the shape of the sixth precingular (6'') and posterior sulcal plates (Kofoid notation). *Alexandrium* cells were identified in two steps: by bright-field light microscopy, and then by epifluorescence after calcofluor staining of the thecal plates. As a check on the accuracy of the identifications and counts by the Utermöhl method, for a number of net tow samples, 100 *Alexandrium* cells randomly found in the observation field were carefully identified under epifluorescence microscopy, by manually rotating each cell until the ventral pore could be observed (320X magnification).

*Cultures and growth conditions*

For validation of selectivity and sensitivity, oligonucleotide probes were tested with unialgal cultured strains of various taxa from a range of geographical locations. A list of the cultured algal strains used in this study is presented in Table 1. Unialgal cultures were grown in 500-ml Erlenmeyer flasks in IMR½ growth medium (Eppley *et al.*, 1967), supplemented with 10 nM selenite (for *Alexandrium tamarense*, *A. catenella*, *A. fundyense*, A. *pseudogonyaulax*, *A. taylori*, *A. minutum* and *A. lusitanicum*), or K medium (Keller *et al.*, 1987) (for *A. affine*, *A. ostenfeldii*, and *Thalassiosira rotula*). All cultures were maintained at 15 $^{o}$C in a controlled growth chamber on 14:10h light:dark photocycle, at a photon flux density of 150 µmol $m^{-2}$ $s^{-1}$, except for *A. ostenfeldii* and *T. rotula* (90 µmol $m^{-2}$ $s^{-1}$).

*DNA preparation*

DNA extractions were made from 500 ml of culture in logarithmic growth phase, using a PAN Plant kit (PAN Biotech, Aldenach, Germany) according to the manufacturer's instructions, with minor modifications as follows. Cultures were filtered onto 47-mm, 3-µm pore-size polycarbonate filters (Isopore, Millipore, Bedford, MA, USA), then the cells were washed from the filter into 1.5-ml reaction tubes with 400 µl preheated (65 $^{o}$C) lysis buffer. Thirty µl of proteinase K (10 mg $ml^{-1}$) were added, followed by 90 minutes incubation at 65 $^{o}$C in a thermo-shaker. After cell lysis, 40 µl RNase A (10 mg $ml^{-1}$) were added and the extract was incubated at room temperature for 30 min. Extraction and cleaning of the genomic DNA was performed on a silica membrane supplied with the kit. DNA concentration was measured spectrophotometrically at 260 nm, and DNA quality was verified by agarose-gel electrophoresis.

*PCR amplification of rRNA genes and sequencing*

PCR amplification of the 18S rDNA gene and the D1/D2 region of the 28S rDNA gene was done in a thermo-cycler (MWG, Ebersberg, Germany) with the primer 1F and 1528R for 18S (Chesnick *et al.*, 1997), and Dir1F and D2CR for 28S (Scholin *et al.*, 1994), respectively. Conditions for PCR were as described in Chesnick *et al.* (1997) and Medlin *et al.* (1998)for the 18S and 28S rRNA genes, respectively. Up to three PCR products were pooled and cleaned with a PCR purification kit (Qiagen, Hilden, Germany) and sequenced with the Long Read kit (Biozym, Hessisch Oldendorf, Germany) and a LiCor 4000L

automatic sequencer (MWG, Ebersberg, Germany), with the same primers as in the PCR for the 28S rRNA and internal primers for the 18S rRNA gene (Elwood *et al.*, 1985). All sequencing primers were infrared-labelled. Sequences were compiled by DNASIS (Amersham, Freiburg, Germany). Sequence alignment was done with CLUSTAL software, and improved by eye for the 28S sequence and for the 18S sequence. The Neighbor-joining tree option in the ARB program (**http://www.mikro.biologie.tu-muenchen.de/pub/ARB/**) was used to identify the clade containing *A. ostenfeldii* from both the 18S and 28S rRNA tree for selected probe development.

*Design of oligonucleotide probes*

Ribosomal RNA oligonucleotide probes were designed with the ARB software package, according to Simon *et al.* (2000). Databases consisting of more than 450 published and unpublished algal 18S rRNA sequences and 150 28S rRNA sequences were consulted. Two functional probes were developed for *A. ostenfeldii*: AOST01 was targetted to the 28S rRNA, and AOST02 to the 18S rRNA of this species (Table 2). For comparison, a specific probe for the entire *Alexandrium tamarense*/*fundyense*/*catenella* species complex was selected from the 18S rRNA gene, and one probe was also selected from the 28S rRNA gene for the toxic North American clade of the *A. tamarense* group (John *et al*., 2002, submitted). The 28S rRNA probe for *A. tamarense* was shifted four bases from that published by Scholin *et al.* (1997) because of a potential hair-pin loop in the previously published sequence. A Dinophyceae-specific probe, DINO01 (Groben *et al.*, 2002, in prep.), was used as a positive control in dot blot and whole-cell hybridisation experiments.

*DNA dot-blot hybridisation*

Unmodified oligonucleotides were supplied by MWG-Biotech (Ebersberg, Germany) and labelled with digoxigenin (DIG) for non-radioactive DNA dot-blot experiments, using the 3' Oligonucleotide Tailing Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Approximately 100 ng of amplified PCR product per sample was denatured for 10 min at 95 °C, spotted onto a positively charged nylon membrane (Roche, Mannheim, Germany) and fixed by 90 s exposure of both sides of the membrane to standard UV illumination. Four hours of pre-hybridisation followed by overnight hybridisation were done in roller tubes in 10 ml hybridisation buffer [5X sodium-sodium citrate (SSC), 0.1 % (w/v) N-

lauroylsarcosine, 0.02 % (w/v) sodium dodecylsulfate (SDS), 1% (w/v) Blocking reagent (Roche, Mannheim, Germany), 0.1 mg $ml^{-1}$ Poly A] in a hybridisation oven with probe-dependent temperatures (Table 2). For hybridisation, 0.1 pmol $ml^{-1}$ DIG-labelled probe was added to the hybridisation buffer. Washing of filters consisted of two 5-min washes in 2X SSC/0.1% SDS or 1X SSC/0.1% SDS, according to the different probes (Table 2) at room temperature, and two 15-min washes at hybridisation temperature. Detection was performed with the DIG Luminescent Detection Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions and membranes were exposed to X-ray film (Amersham, Freiburg, Germany) for 1 to 3 h.

In situ *hybridisation of whole dinoflagellate cells*

Whole cells from laboratory cultures were hybridised with FITC-labelled (fluorescein-isothiocyanate) probes (Interactiva, Ulm, Germany) by a modified protocol from Scholin *et al.* (1996) and examined by epifluorescence microscopy. Cells were carefully filtered onto white 47-mm diameter (3.0 µm pore-size) polycarbonate membranes (Isopore, Millipore, Bedford, MA, USA) in a standard filtration unit (Millipore, Bedford, MA, USA) under <100 mm Hg vacuum to prevent cell damage. Freshly prepared saline EtOH fixative [25 ml 100% ethanol, 2 ml Milli-Q water, 3 ml 25X SET buffer (3.75 M NaCl, 25 mM EDTA, 0.5 M Tris/HCl, pH 7.8)] was added to the filtration unit and cells were fixed for 1 h at room temperature. The fixative was filtered through and the filter incubated with hybridisation buffer (5X SET buffer, 0.1% IGEPAL-CA630, 30 ng $ml^{-1}$ Poly A) for 5 min at room temperature. The rinsed filter was cut into pieces; each piece was put onto a microscopic slide and hybridised separately with a different probe. Sixty µl hybridisation buffer containing 10 to 20% formamide with FITC-labelled probes (5 ng $\mu l^{-1}$) were applied directly onto the filter pieces and incubated for 1 h at 50 to 55°C in a humid chamber in the dark (Table 2). The filters were rinsed for 5 min in 100 µl of 1X SET buffer under the same conditions to remove excess unbound probe, before mounting in a mixture of CitiFluor (Citifluor Ltd., London, UK) as an anti-fade agent, with 4',6'-diamidino-2-phenylindole (DAPI) (1 µg $ml^{-1}$) as a counterstain. The cover slip was sealed with clear nail varnish and the slides analysed with an Axioskop 20 epifluorescence microscope (Zeiss, Oberkochen, Germany) equipped with Zeiss filter sets 02 (DAPI) and 09 (FITC). Photographs were taken on 1600 ASA Fuji colour print film.

For the first tests of the rRNA probes, ~100 ng $\mu l^{-1}$ of 27 different PCR products from target and non-target species were pipetted onto a positively charged nylon membrane. The DINO01 probe was used as a positive control.

***Probe application to field samples***

One litre of seawater collected by pumping from a buoy (see *Field sampling* protocol) at 1 m depth from each of six stations was filtered onto a 47-mm diameter (3 μm pore-size) white polycarbonate filter (Isopore, Millipore, Bedford, MA, USA) on board ship. The cells were fixed for *in situ* hybridisation as described above. Filters were stored dry at 4 °C for analysis in the laboratory within two weeks. For analysis, half of a filter was cut into four equal-sized pieces and hybridised with different probes (Table 2). Samples were analysed as described above with a Zeiss Axioskop 20 epifluorescence microscope (Zeiss, Oberkochen, Germany). The entire filter slice was scanned at 400X magnification and all positive signals were counted in each quadrant.

*Extraction of algal toxins*

For spirolide analysis, filtered planktonic material collected by 20-μm plankton net tow was extracted for 1 min in a 2-ml Eppendorf vial containing 1.0 ml methanol:water (1:1 v:v) with a Sonopuls HM 70 ultrasonicator (Bandelin, Germany). Crude extracts were centrifuged for 10 min (2,980 x *g*) and the supernatant was passed through a 0.45 μm nylon filter (Rotilab, Carl Roth GmbH, Karlsruhe, Germany).

For PSP toxin determination, plankton samples were extracted on board ship for subsequent analysis. Plankton cells were extracted from filter slices by adding 1.0 ml of 0.03 N acetic acid and ultrasonicating for 10 min. The sonicate was centrifuged for 10 min (2,980 x *g*), and passed through a 0.45-μm nylon filter (Rotilab, Carl Roth GmbH, Karlsruhe, Germany; 10 mm diameter). All crude extracts were analysed on board, then stored at -28 $^{o}$C and re-analysed in the laboratory within three months after the research cruise.

*Liquid Chromatography/Mass Spectrometry determination of spirolides*

Plankton extracts were directly analysed on board ship by liquid chromatography with mass spectrometric detection (LC-MS) using a PE Series 200 quaternary pump and a PE Series 200 autosampler (Perkin-Elmer, Langen, Germany), according to minor modifications of the analytical method published in Cembella *et al.* (1999). Briefly, the separation of spirolides was carried out on a reversed-phase column (Luna 3 μm C18, 150 x 4.60 mm ID,

Phenomenex, Aschaffenburg, Germany) with an ammonium formate/formic acid buffer system and acetonitrile/methanol as the mobile phase (Hummert *et al*., 2002, submitted). Acetonitrile and methanol (Baker BV, Deventer, Netherlands) were HPLC-grade. Water was purified to HPLC-grade with a Millipore-Q RG ultra-pure water system (Millipore, Milford, USA). The quality of all other reagents was at least analytical grade (p.a.).

Spirolides were detected by an API 165 mass spectrometer equipped with an atmospheric pressure ionisation (API) source operating in turbo ion spray (TIS) mode (Applied Biosystems, Concord, Canada). Selected ion monitoring (SIM) mode was used for the determination of spirolides, according to the published mass/charge (*m/z*) values of known spirolides (Hu *et al.,* 1995; 1996; Cembella *et al.,* 1999; 2000), whereby *m/z* = 692.5, 694.5, 706.5, 708.5, 710.5, and 712.5 $[M+H]^+$ were monitored.

In addition, spirolides found in field samples were characterized by LC/MS/MS with an API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Concord, Canada) (Hummert *et al*., 2002, submitted). The spirolide content at each sampling site was calculated by integration of all chromatogram peaks that were clearly identified as spirolides: *m/z* 692.5 and 706.5, corresponding to spirolides A/desmethyl spirolide C and spirolide C, respectively. The only available quantitative standard for spirolides was a calibration solution containing spirolide D (2.0 μg $ml^{-1}$) obtained from the Institute for Marine Biosciences, NRC, Halifax, Canada. Spirolide concentrations were determined from a calibration curve prepared with spirolide D, assuming equal molar response factors for the other spirolides.

*Liquid chromatography/fluorescence determination of PSP toxins*

Paralytic shellfish poisoning (PSP) toxins in plankton samples were analysed by high-performance liquid chromatography (HPLC), with reference to a well-established method (Hummert *et al.*, 1997; Yu *et al.*, 1998) based on ion-pair chromatography with octane sulfonic acid, post-column oxidation with periodic acid, and fluorescence detection. Certified quantitative standards (NRC-PSP-1B) for saxitoxin (STX), neosaxitoxin (NEO), and gonyautoxins (GTX1, GTX2, GTX3 and GTX4), also containing un-quantified traces of decarbamoyl derivatives (dcGTX2 and dcGTX3), were purchased from the Institute for Marine Biosciences, National Research Council, Halifax, Canada. Calibration curves of different PSP toxins were used to calculate the respective toxin concentrations in sample extracts.

*Results*

*Morphology of A. ostenfeldii and A. tamarense*

The basic thecal plate tabulation for both *A. ostenfeldii* and *A. tamarense*, according to the modified Kofoid notation applied to *Alexandrium* (Balech, 1995), is expressed as: Po, 4', 6", 6c, 9-10s, 5''', and 2"". Detailed descriptions of these respective species from various locations are available in the literature (Balech, 1995; Balech & Tangen, 1985; Jensen & Moestrup, 1997; Moestrup & Hansen, 1988). In general, field specimens of these species from the east coast of Scotland are consistent with the description of material from other North Atlantic areas, including the coast of Spain (Balech, 1995), Norway (Balech & Tangen, 1985), and Nova Scotia, Canada (Cembella *et al.*, 2000).

*Alexandrium ostenfeldii* is morphologically similar to *A. tamarense* and the use of gross morphological characteristics to distinguish these species is often confounded, particularly after time in culture. For example, in field material from the waters near the Orkney Islands, *A. tamarense* was often found in chains of two cells (in culture up to four cells), but no chains of *A. ostenfeldii* were observed. In culture, a small fraction of *A. ostenfeldii* produced chains of two cells, particularly when growth was rapid. Vegetative cells of *A. ostenfeldii* are typically larger than those of *A. tamarense,* but the size ranges overlap. In field specimens from the Orkney Islands, most *A. ostenfeldii* cells were >45 µm, and only 10 to 15% of cells were <40 µm in trans-diameter. *Alexandrium tamarense* cells from this area were typically <35 µm, although 15 to 20% of cells were larger, to a maximum of 47 µm. In cultured isolates from the Orkney Islands, the mean diameter of *A. ostenfeldii* cells was <35 µm; this size reduction in culture was also described by Cembella *et al*. (2000) from cultured Nova Scotian populations. Representative specimens of these species from northern Europe stained with calcofluor and viewed under epifluorescence- (Fig. 2) and scanning electron- (Fig. 3) microscopy are shown photographically. For samples from the Scottish east coast, the percentage of *A. ostenfeldii* cells in relation to the total count of *Alexandrium* cells determined by careful observation of calcofluor-stained specimens under epifluorescence microscopy was similar to that determined by the Utermöhl technique.

As noted by Braarud (1945), for *A. ostenfeldii* (as *Gonyaulax tamarensis* var. *globosa*) and in the later redescription (Balech & Tangen, 1985) from Norway, cells tend to be "globose" and less angular than those of *A. tamarense.* In Orkney specimens of the former species, the sulcus is not markedly indented, and in contrast to *A. tamarense* cells, there are no clearly visible sulcal lists. In *A. tamarense*, the sulcal lists of most cells are visible even under low magnification (100X) in the inverted microscope. In comparison to *A. tamarense*, cells of

*A. ostenfeldii* from field samples have a rather delicate theca, with ill-defined plate sutures, and a shallow cingulum. *Alexandrium tamarense* from the Orkney Islands does have the deeply indented sulcal region and angular features characteristic of most specimens of *Alexandrium* cf. *excavatum sensu* Balech (1995) from the Estuary and Gulf of St. Lawrence in eastern Canada (Destombe & Cembella, 1990).

These species may be discriminated by careful observations of the details of particular thecal plates. Both species exhibit a ventral pore on the margin of the first apical (1') plate, but the pore is large and kidney-shaped in *A. ostenfeldii* and smaller and circular in *A. tamarense*. In field material from the east coast of Scotland, these ventral pores are easily distinguished at high magnification (>250X) of calcofluor-stained cells under epifluorescence microscopy. The ventral pore may be occluded by membranaceous material, particularly in cultured cells, but the margin can usually be visualized after fluorescence staining (Fig. 2). Fixation and preparation for SEM can result in occlusion to the extent that only the faint outline of the ventral pore (indicated as V.p.) remains visible (Fig. 3).

In typical specimens of *A. ostenfeldii* from the Orkney Islands, the sixth precingular (6") plate is much wider than long, whereas in *A. tamarense* it is more or less equal. In *A. minutum*, which was also present in very low numbers in several samples, the 6" plate is narrow. In *A. ostenfeldii*, the 2"" plate is extremely large and regularly shaped - more or less rectangular with only small deviations at the sutures where it joins the posterior sulcal (S.p.) plate and the 1"" plate, making it slightly pentagonal. In *A. tamarense*, the 2"" plate is more asymmetrical.

*Dot-blots*

Both *Alexandrium ostenfeldii* specific probes (AOST1 and AOST2) only gave clearly positive hybridisation signals with the target amplicons of the four *A. ostenfeldii* cultured strains in the first line of the dot-blots (Fig. 4). However, very weak and unspecific signals were detected for the AOST1 probe in PCR products from other dinoflagellates used as negative controls only after 90 min exposure time during autoradiography. All attempts to increase the stringency, such as lowering probe concentration, raising hybridisation temperature, and decreasing salt concentration of the wash buffers, did not remove these weak signals when the membranes were subjected to prolonged exposure. However, in all

cases, the weakly negative signals could be clearly distinguished from strong positive signals of the target samples.

*Fluorescence* in situ *hybridisation (FISH)*

Whole-cell hybridisation experiments with FITC-labelled rRNA probes were carried out with two cultured strains of *A. ostenfeldii* (AOSH1, K0287) and *A. tamarense* (GTTP01, BAHME181), and one strain each of *A. lusitanicum* (BAHME91), *A. taylori* (AY1T), *A. pseudogonyaulax* (AP2T), and *A. affine* (CCMP112) as controls. These strains were used to find the most stringent hybridisation conditions under which the probes were specific for the target cells. All *Alexandrium* strains exhibited a clearly positive (green) signal after hybridisation with the dinoflagellate-specific probe (DINO01) (Fig.5; A1-D2). In contrast, the probes AOST1 and AOST2 gave positive signals only when *A. ostenfeldii* cells were present. All controls were clearly negative and showed only weak yellowish colour due to residual pigment autofluorescence.

For whole-cell hybridisations from field material, half of the filter was divided into four equal slices and probed as follows: Quadrant 1: Dinophyceae-specific probe (DINO01); Quadrant 2: *Alexandrium tamarense* (specific for entire species complex) (ATAM01); Quadrant 3: *Alexandrium tamarense* (specific for the North American clade)(ATNA02); and Quadrant 4: *Alexandrium ostenfeldii* (AOST01). The DINO01 probe detected dinoflagellates in all assayed samples (Fig. 6). A specific probe for *A. ostenfeldii* (AOST01) gave positive signals for all samples within which spirolides were found (Fig. 7), whereas the probes for *A. tamarense* (ATAM01 and ATNA02) yielded positive results for all samples containing PSP toxins (Fig. 8).

The total dinoflagellate concentrations in the samples determined from counts performed by the *in situ* hybridisation technique (DINO01 probe) and Utermöhl techniques differed substantially, with consistently higher concentrations found by the latter method. Furthermore, the cell concentrations of *Alexandrium* spp. determined by the Utermöhl method were always much higher than those detected by *in situ* hybridisation, by up to an order of magnitude. At all six sample stations assayed with the FISH probes, more *A. tamarense* cells were detected than those of *A. ostenfeldii*, with both the *in situ* hybridisation and Utermöhl counting methods (Fig.7, 8). For *A. tamarense*, the same quantitative trend was apparent with both counting methods at all stations - highest cell concentrations were found at Station 7 and lowest concentrations at Station 10. Counts performed by these alternative techniques were highly correlated (Pearson correlation coefficient; $r^2 = 0.94$; $n = 6$). The same

covariance trend ($r^2$ = 0.67; $n$ = 6) was observed for *A. ostenfeldii*, with two obvious exceptions: at Stations 10 and 33, no *A. ostenfeldii* cells were detected in the Utermöhl counting chamber (25 ml), but this species was detected by *in situ* hybridisation.

*Analysis of spirolides and PSP toxins*

Spirolides were detected at all sampling sites, with the highest concentration of total spirolides found at Station 14 (155 ng $ml^{-1}$ net-plankton extract). Only three main spirolide compounds (at *m/z* 692.5 and *m/z* 706.5) were present in sufficient amounts for identification and quantitation. Ion *m/z* 692.5 $[M+H]^+$ refers to two different spirolides, designated as spirolide A and des-methyl C, both with MW 691.5. Ion *m/z* 692.5 was not detected at Station 29. Ion *m/z* 706.5 $[M+H]^+$ refers to spirolide C, which was the major derivative at all sampling sites. Two unconfirmed "spirolide-like" compounds with MW 691.5 were present only in trace amounts. There was an obvious discrepancy between detected spirolide concentration and the abundance of *A. ostenfeldii* cells counted by the Utermöhl technique ($r^2$ = 0.01; $n$ = 6). For example, at Station 10 and 33, no *A. ostenfeldii* cells were counted (Fig. 7), but 59 and 7 ng spirolides $ml^{-1}$ net-plankton extract, respectively, were detected at those stations. The trend in *A. ostenfeldii* cell abundance determined by the FISH probe tracked the spirolide concentration better than did the Utermöhl method (Fig. 7) ($r^2$= 0.37; $n$ = 6).

The total PSP toxin content in field samples determined by the HPLC technique generally followed a trend similar to that of the cell abundance of the putative toxigenic species, although correlations were not high for either counting method (Fig. 8). For example, in comparing PSP toxin concentration versus Utermöhl counts, the correlation ($r^2$ = 0.56; $n$ = 6) was similar to that for the FISH probe counts ($r^2$ = 0.43 ; $n$ = 6). The highest concentrations of total PSP toxins were measured at Station 7 and Station 14 (295 ng $ml^{-1}$ and 1100 ng $ml^{-1}$ of plankton extract, respectively); however, the concentration of *A. tamarense* cells was much higher at Station 7 than at Station 14 (10,400 versus 7,000 cells $l^{-1}$, respectively). An additional potential source of PSP toxins, *A. minutum* cells, was also present but in low abundance (10 cells $l^{-1}$ at Station 7 and 400 cells $l^{-1}$ at Station 14).

*Discussion*

The current study based upon samples collected during the RV *Heincke* cruise along the east coast of Scotland represents the first large-scale integrated field observations of the co-distribution of *Alexandrium ostenfeldii* and *A. tamarense*, and their respective toxins, spirolides and PSP toxins. These observations are significant, particularly given the long historical reports of the occurrence of these species in northern European waters (Paulsen, 1904; 1949; Lebour, 1925; Braarud, 1945; Balech & Tangen, 1985), and the recent discovery of spirolides in cultured isolates from Denmark (Cembella *et al*., 2000). The distribution of PSP toxicity on the Scottish east coast, circumstantially linked to blooms of *A. tamarense* via a density-driven circulation model (Brown *et al*., 2001), has been associated with occasional outbreaks of human illnesses in the region.

Prior research raised suspicions that *A. ostenfeldii* might pose some risk of causing shellfish toxicity in northern Europe, owing to the presence of PSP toxins (Balech & Tangen, 1985). More recent findings of low PSP toxicity of cultured isolates of *A. ostenfeldii* from the region (Hansen *et al*., 1992; A. Cembella, unpubl. data) have tended to reduce this concern. Nevertheless, an early report linking the presence of *Pyrodinium phoneus* (Woloszynska & Conrad, 1939) (probably a description of *A. ostenfeldii*) with symptoms of human intoxication resembling those of PSP, after consumption of mussels from canals in Belgium, suggests that this risk should not be ignored.

Spirolides are novel toxins with unknown toxicological effects on marine ecosystems and human consumers of shellfish. The use of direct LC/MS analysis on board ship has proven to be an effective research tool for surveying the distribution of spirolides in the water column. We assume that the reason why spirolides have not been detected before in European waters is because there have been no previous efforts to analyse plankton or shellfish samples for the presence of these compounds. Analytical standards are not readily available and detection relies on sophisticated analysis by LC/MS, a technology available in only a few research laboratories. Thus the occurrence of spirolides in the plankton, now known to be associated with *A. ostenfeldii*, is most likely not a new phenomenon, but only a newly discovered one.

Emerging evidence of the high potency of spirolides (Richard *et al*., 2002), even relative to other phycotoxins, and the accumulation of spirolides in bivalve shellfish in Atlantic Canada (Hu *et al*., 1995), underscores the importance of monitoring for these compounds and the causative organism(s) in the water column, especially in shellfish harvesting areas. In most toxic

phytoplankton monitoring programs, *A. ostenfeldii* has been of marginal interest due to its typically weak ability to produce PSP toxins, at least in north temperate waters. During a previous cruise of the RV *Heincke* in 1997, substantial concentrations of *A. ostenfeldii* cells were observed along the east coast of Scotland (M. Elbrächter, unpubl. obs.). Now that the spirolide-producing capability of this species has been clearly demonstrated along both the European and North American Atlantic coasts, the requirements for monitoring *A. ostenfeldii* have fundamentally changed.

To date, there are only a few *A. ostenfeldii* strains in culture collections, perhaps as a reflection of the difficulty in the successful isolation and maintenance of strains in clonal culture (Cembella *et al*., 2000). Few data are available on the occurrence of natural bloom populations in the field. As a consequence, the biogeographical distribution of *A. ostenfeldii* and its spirolide-producing capabilities remain cryptic.

In contrast to *A. ostenfeldii*, shellfish toxicity derived from *A. tamarense* populations in European waters has been a well-known phenomenon for many decades (Ayres, 1975). Yet there have been few concerted studies on the biogeographical distribution of blooms of this species in relation to the occurrence of PSP toxins in the water column. In the current study, application of HPLC-FD to field populations yields convincing links between the presence of PSP toxins and toxic dinoflagellate cells, while providing information on toxin composition.

In general, the autecological requirements of *A. tamarense* and *A. ostenfeldii* are somewhat different with respect to temperature and light. *Alexandrium ostenfeldii* clones from the northwest Atlantic tend to thrive in colder waters accompanied by lower light intensities than do members of the *A. tamarense* species complex (Cembella *et al*., 2000). In Atlantic Canada, *A. ostenfeldii* occurs frequently as a subdominant species within phytoplankton assemblages together with other toxigenic *Alexandrium* species (Cembella *et al*., 1998). While isolates from Danish coastal waters appear to grow well in culture at elevated temperatures (Jensen & Moestrup, 1997), *A. ostenfeldii* tends to bloom earlier than does *A. tamarense* in northern Europe, although there may be some overlap (Balech & Tangen, 1985). In this study, the co-occurrence of these two species has now been substantiated for the Orkney Islands, as well.

As noted previously, co-occurrence of *Alexandrium* species in northern Europe (Braarud, 1945; Balech & Tangen, 1985), now known to exhibit different cellular toxicity and toxin spectra, complicates the interpretation of toxic plankton monitoring data based on cell counting and identification by conventional optical microscopy. According to Cembella *et al.* (2000), based largely upon experience in Atlantic Canada, *A. ostenfeldii* is under-reported,

particularly in routine phytoplankton monitoring programs. This is due to morphological similarities to other toxigenic species such as *A. tamarense*. Thus, the successful application of FITC-labelled rRNA probes that are differentially specific for *A. ostenfeldii* versus *A. tamarense* cells is a major advance. The validity of this molecular probe approach, by comparison with thecal staining and epifluorescence microscopy, and the use of SEM – both techniques that are complex and often impractical to apply for routine analysis – has now been demonstrated with field populations of *Alexandrium* from European waters. Use of molecular probes as taxon-specific tools for the discrimination of *Alexandrium* species has the additional advantage that it does not require sophisticated taxonomic expertise for species recognition. For all *Alexandrium* species (but particularly *A. ostenfeldii*), the thecal plates, the primary diagnostic criteria, are rather fragile and readily shed in fixed samples, and with that, most of the distinguishing cell features are lost.

The co-occurrence of both toxic and non-toxic strains of *A. tamarense* and *A. ostenfeldii* is especially problematic for toxin risk assessment in areas where no toxin monitoring is done. The fact that these species may be capable of synthesising chemically unrelated families of marine phycotoxins poses an additional complexity for effective plankton monitoring based upon counts of total *Alexandrium* cells. For example, *A. ostenfeldii* alone could cause spirolide toxicity in shellfish during periods when toxigenic blooms of *A. tamarense* are absent, but this lipophilic toxicity would remain undetected by the routine mouse bioassay for PSP toxins, which involves an aqueous acid extraction. Furthermore, in mixed populations with *A. tamarense*, where *A. ostenfeldii* cell numbers are substantial, use of total *Alexandrium* cell concentrations for toxin risk assessment and early warning of toxicity may be seriously biased. In North America and Europe, where populations of members of the *Alexandrium tamarense* species complex are usually assumed to produce only PSP toxins, a monitoring program based exclusively on enumeration of cells of *Alexandrium* spp. and/or determination of PSP toxins could miss recurring threats of spirolide toxicity resulting from blooms of *A. ostenfeldii.*

In Europe, the constraints on designing effective taxon-specific probes for species discrimination within the genus *Alexandrium* may be particularly acute. Populations belonging to different clades of the *A. tamarense* species complex are found in European waters (Medlin *et al*., 1998; John *et al*., submitted). In addition to the two toxic *Alexandrium* clades described from North American and temperate Asian regions (Medlin *et al*., 1998) populations belonging to the non-toxic Western European and Mediterranean clades (John *et al*., 2002, submitted) have been identified in Europe. Given this complexity, it was critical to

design alternative rDNA probes with different hybridisation specificities for application to European field populations of *A. tamarense*. Similarly, in the absence of evidence regarding possible different clades of *A. ostenfeldii* in Europe, it was prudent to design and apply two rRNA probes, one specific for 18S rRNA and the other for the D1/D2 region of the 28S rRNA, to northern European populations.

The DNA dot-blot test is a powerful tool for probe development, offering the opportunity to test probe specificity over a broad range of control strains. In addition, dot blots are a sensitive technique well suited for detection of target species in mixed phytoplankton assemblages. Dot-blots can be used in field studies to analyse large sample series simultaneously, especially when PCR products of the target gene are used, as was recently demonstrated for bacteria (Raskin *et al.*,1994). All these rRNA probes designed for *Alexandrium* work at the DNA level with the dot-blot technique. Polymerase chain reaction (PCR) products were used in this study to avoid false positive hybridisation signals with short oligonucleotides (~20 bases) as parts of the genomic DNA.

The fluorescence *in situ* hybridisation (FISH) arrays (Fig. 5, 6) clearly show that the *A. ostenfeldii* probes AOST01 and AOST02 are effective as species-specific probes in whole-cell hybridisation experiments. Although both probes proved to be specific in these experiments, probe AOST01 is recommended for FISH, because AOST02 provides only a single base mismatch to the *A. minutum/lusitanicum* species complex. A single nucleotide is sufficient to distinguish target from non-target species under stringent laboratory conditions, but discrimination might be more difficult in field studies where the rRNA population is reduced. Since two probes are available, the application of the less problematic AOST01 probe is suggested for field studies.

The FITC-labelled probes showed that in addition to their usefulness in discriminating among *Alexandrium* species in culture, they have the capability to distinguish among taxa in complex field plankton assemblages. The application of molecular probes to field plankton studies has been shown before for diatoms (Miller & Scholin, 1998). In the study reported here, the cell concentrations determined by FISH for each *Alexandrium* species were positively correlated with those enumerated by the classic Utermöhl technique, although the total cell counts by the latter method were up an order of magnitude higher. In exceptional cases, such as at Station 10 and 33, *A. ostenfeldii* cells were detected by the FISH technique with the AOST1 probe, whereas no cells were counted by the Utermöhl method. These quantitative discrepancies cannot be fully explained, but there are unlikely to be related to probe failure, since adequate controls were used. Fluorescence labelling of *Alexandrium* cells

from unialgal cultures with the appropriate probe generally achieved almost complete hybridisation (>95% of viable cells labelled). It is conceivable that cells from field samples could be in a diverse physiological states, whereby reduced metabolic activity in some cells might result in fewer ribosomes and hence in weaker or fewer hybridisation signals (Anderson *et al*., 1999). Nevertheless, the high percentage of DAPI-positive and well-pigmented *Alexandrium* cells (indicating intact nuclei and apparently "healthy" cells) suggests that this was not a major factor. A more likely scenario is that even among healthy cells, the recognition of the minimum degree of fluorescence labelling required to be considered "positive" is highly subjective to the human eye – a conservative approach could lead to serious underestimates of the numbers of the targetted taxon.

There are other possible methodological explanations for the difference in counts registered by the FISH technique versus the Utermöhl method. Particulate material, such as organic and inorganic debris, can bind probe constituents, and this non-specifically bound probe is no longer available for labelling target cells (Miller & Scholin, 1998). Excess debris may also hide cells that are then overlooked, but the efforts made to avoid overloading the filters would minimize this problem. Gross inaccuracies in the respective species counts by optical microscopy are unlikely, given the experience of the taxonomist responsible for this aspect. Furthermore, since Utermöhl counts of *A. ostenfeldii* were always much lower (about ten-fold) than those of *A. tamarense* at each station, simple misidentification of *A. ostenfeldii* cells as *A. tamarense* would not greatly affect the total count of the latter species. The apparent discrepancy between counting methods is reasonably attributable to sampling inconsistencies and differences in the volumes of water sampled. For example, the Utermöhl counts were based upon a settled 25-ml sample, whereas the cells filtered for FISH probing represented a one-litre volume of seawater. Uneven distribution of the cells on the filter is also a potential source of sampling error; this effect is compounded by the fact that only a portion of the filter was scanned for labelled cells. Observed variability among cell counts with FISH probes at several sample dilutions led Miller & Scholin (1998) to propose counting at least three entire filters. In any case, it is clear that further refinements must be made to the application of the FISH probes for the technique to be considered fully quantitative.

The biogeographical relationship between the distribution of *A. ostenfeldii* and *A. tamarense* and their respective toxins is also noteworthy. For example, by LC/MS, spirolides were detected at two stations where *A. ostenfeldii* was not recorded by the Utermöhl method but was detected by the FISH probe. To date, *A. ostenfeldii* has been considered to be the unique spirolide producer (Cembella *et al.*, 2000); thus there is circumstantial evidence that

Utermöhl counts performed on small volumes may not detect rare cells. Of course, the possibility that other protistan taxa or bacteria may also produce spirolides, or that spirolides might be bound in zooplankton fecal pellets and other detritus cannot be completely excluded. Nevertheless, these results illustrate an advantage of the FISH technique, especially when the cell concentration of a target species in field samples is low, because even events as rare as a single cell generate a clear fluorescence signal. The probability of encountering a rare event is further increased if an instrument, such as a solid phase cytometer (Chemunex), is used to scan the filter and to record all positive signals.

The relatively weak correlations between toxin concentrations and *Alexandrium* cell counts, in contrast to the results of Cembella *et al*. (2001), are best explained as artifacts of the procedures used to sample the surface waters for these different parameters (i.e., net tows versus pumping from discrete depths). Again, in addition to intact recognisable *Alexandrium* cells, there may have been some contribution to the toxin pool from other sources, including bacteria, detritus, and grazers upon *Alexandrium*. Cells of *A. minutum* and *A. ostenfeldii* were found at these stations, but they were not specifically isolated and tested for PSP toxicity. In any case, potentially toxic *A. minutum* cells were observed only at a few sampling sites as minor components (maximum 400 cells $l^{-1}$ at Station 14) and were not quantitatively important enough to have substantially biased the relationship between *A. tamarense* cell abundance and PSP toxin concentration. Interestingly, the highest PSP toxin concentration was found at Station 14, whereas the concentration of *A. tamarense* cells was highest at Station 7. Wide variability in PSP toxin content among field and cultured populations of cells of the *A. tamarense/fundyense/catenella* species complex has been attributed to both genetic differences and variability in localised environmental factors (Cembella, 1998). These results clearly show that rRNA probes are a powerful tool for taxonomic discrimination in field studies. Such probes can be used for monitoring species composition even in the absence of advanced taxonomic expertise. In combination with DNA dot-blots, rRNA probes permit a taxonomically precise identification of microalgal composition, with selectivity adjustable according to probe design. The application of hierarchical probes, as carried out during this study, allows computation of proportions of each algal group even if the complete taxonomic spectrum is unknown. By using rRNA probes with *in situ* hybridisation, large numbers of field samples can be simultaneously analysed. If coupled with flow cytometry, *in situ* hybridisation offers the potential to measure microalgal diversity on a larger scale, even in real-time on board research vessels. In addition, algal cells can be sorted automatically and can then be used for further studies or verification, for example, by gene sequencing.

In the study reported here, the validity of rRNA probes for qualitative and semi-quantitative discrimination of *A. tamarense* and *A. ostenfeldii* cells in field samples from northern Europe was demonstrated for the first time. Further work will be directed towards development of more accurate and precise quantitative application of such probes to field populations.

*Acknowledgements*

The authors thank M. Quilliam, Institute for Marine Biosciences, NRC, Halifax, Canada for generous provision of the spirolide calibration solution and for assistance with the application of the MS/MS method. N. Lewis (IMB, NRC) provided calcofluor-stained specimens and SEM micrographs of *Alexandrium* cells. Michael Schweikert (Friedrich-Schiller Universität) contributed technical assistance on board ship during the cruises. The work was supported by BMBF TEPS (Project No. 03F0161). This publication is NRC (Canada) No. 42357.

*References*

ALM, E.W., OERTHER, D.B., LARSEN, N., STAHL, D.A. & RASKI, L. (1996). The oligonucleotide database project. *Appl. Environ. Microbiol*., **62**: 3557-3559.

ANDERSON, D.M., KULIS, D.M.; KEAFER, B.A. & BERDALET, E. (1999). Detection of the toxic dinoflagellate *Alexandrium fundyense* (Dinophyceae) with oligonucleotide and antibody probes: Variability in labeling intensity with physiological condition. *J. Phycol*., **35**: 870-883

AYRES, P.A. (1975): Mussel poisoning in Britain with special reference to paralytic shellfish poisoning. Review cases reported 1814-1968. *Environ. Health* **83**: 261-265.

BALECH, E. (1995). The Genus *Alexandrium* Halim (Dinoflagellata) Sherkin Island Marine Station, Ireland. 151 pp.

BALECH, E. & TANGEN, K. (1985). Morphology and taxonomy of toxic species in the tamarensis group (Dinophyceae): *Alexandrium excavatum* (Braarud) *comb. nov*. and *Alexandrium ostenfeldii* (Paulsen) *comb. nov. Sarsia*, **70**: 333-343.

BRAARUD, T. (1945). Morphological observations on marine dinoflagellate cultures (*Porella perforata, Gonyaulax tamarensis, Protoceratium reticulatum*). *Avh. Norske Vidensk. - Akad. Oslo, Mat. - Naturv. Kl.* 1944, **11**: 1-18.

BROWN, J., FERNAND, L., HORSBURGH, A.E. & READ, J.W. (2001). Paralytic shellfish poisoning on the east coast of the UK in relation to seasonal density-driven circulation. *J Plank. Res.,* **23**: 105-116.

CEMBELLA, A.D. (1998)**.** Ecophysiology and Metabolism of Paralytic Shellfish Toxins in Marine Microalgae. In *Physiological Ecology of Harmful Algal Blooms* (Anderson, D.M., Cembella, A.D. & Hallegraeff, G.M., editors), NATO-Advanced Study Institute Series, V. 41, 381-404. Springer-Verlag, Heidelberg.

CEMBELLA, A.D., BAUDER, A.G., LEWIS, N.I. & Quilliam, M.A. (2001). Association of the gonyaulacoid dinoflagellate *Alexandrium ostenfeldii* with spirolide toxins in size-fractionated plankton. *J. Plankton Res*., **23**: 413-419.

CEMBELLA, A.D., LEWIS, N.I., & QUILLIAM, M.A. (1999). Spirolide composition of micro-extracted pooled cells isolated from natural plankton assemblages and from cultures of the dinoflagellate *Alexandrium ostenfeldii. Nat. Tox.*, **7**: 197-206.

CEMBELLA, A.D., LEWIS, N.I., & QUILLIAM, M.A. (2000). The marine dinoflagellate *Alexandrium ostenfeldii* (Dinophyceae) as the causative organism of spirolide shellfish toxins. *Phycologia*, **39**: 67-74.

CEMBELLA, A.D., QUILLIAM, M.A., LEWIS, N.I., BAUDER, A.G. & WRIGHT, J. L.C. (1998). Identifying the planktonic origin and distribution of spirolides in coastal Nova Scotian waters. In *Harmful Algae* (Reguera, B., Blanco, J., Fernández, M.L. & Wyatt, T., editors), 481-484. Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO,

Santiago de Compostela, Spain.

CHESNICK, J.M., KOOISTRA, W.H.C.F., WELLBROCK, U. & MEDLIN, L.K. (1997). Ribosomal RNA analysis indicates a benthic pennate diatom ancestry for the endosymbionts of the dinoflagellates *Peridinium foliaceum* and *Peridinium balticum* (Pyrrhophyta). *J. Euk. Micro.*, **44**: 314-320.

DESTOMBE, C. & CEMBELLA, A. (1990). Mating-type determination, gametic recognition and reproductive success in *Alexandrium excavatum* (Gonyaulacales, Dinophyta), a toxic red-tide dinoflagellate. *Phycologia*, **29**: 316-325.

ELWOOD, H.J., OLSEN, G.J. & SOGIN, M.L. (1985). The small subunit ribosomal gene sequences from the hypotrichous ciliates *Oxytrichia nova* and *Stylonychia pustulata*. *Mol. Biol. Evol*., **2**: 399-410.

EPPLEY, R.W., HOLMES, R.W. & STRICKLAND, J.D.H. (1967). Sinking rates of the marine phytoplankton measured with a fluorochrometer. *J. exp. mar. Biol. Ecol*. **1**: 191-208.

FRITZ, L. & TRIEMER, R.E. (1985). A rapid simple technique utilizing Calcofluor white M2R for the visualization of dinoflagellate thecal plates. *J. Phycol.,* **21**: 662-664.

GRANÉLI, E., SUNDSTRÖM, B., EDLER, L. & ANDERSON, D. M. (editors) (1990). *Toxic Marine Phytoplankton*. Elsevier, New York, 554 pp.

GUILLOU, L., MOON-VAN DER STAAY, S.Y., CLAUSTRE, H., PARTENSKY, F., & VAULOT, D. (1999). Diversity and abundance of bolidophyceae (Heterokonta) in two oceanic regions. *Appl. & Environ. Microbiol.,* **65**: 4528-4536.

HALLEGRAEFF, G.M. (1993). A review of harmful algal blooms and their apparent global increase. *Phycologia*, **32**: 79-99.

HANSEN, P.J., CEMBELLA, A.D., & MOESTRUP, Ø. (1992). The marine dinoflagellate *Alexandrium ostenfeldii*: Paralytic shellfish toxin concentration, composition, and toxicity to a tintinnid ciliate. *J. Phycol.,* **28**: 597-603.

HIGMAN, W.H., STONE, D.M. & LEWIS, J.M. (2001). Sequence comparisons of toxic and non-toxic *Alexandrium tamarense* (Dinophyceae) isolates from UK waters. *Phycologia*, **40**: 256-262.

HU, T., BURTON, I.W., CEMBELLA, A.D., CURTIS, J.M., QUILLIAM, M.A., WALTER, J.A. & WRIGHT, J.L.C. (2001). Characterization of spirolides A, C, and 13-desmethyl C, new marine toxins isolated from toxic plankton and contaminated shellfish. *J. Nat. Prod*., **64**: 308-312.

HU, T., CURTIS, J.M., OSHIMA, Y., QUILLIAM, M.A., WALTER, J.A., WATSON-WRIGHT, W.M. & WRIGHT, J.L.C. (1995). Spirolides B and D, two novel macrocycles isolated from the digestive glands of shellfish. *J. Chem. Soc., Chem. Commun*., 2159-2161.

HU, T., CURTIS, J.M., WALTER, J.A. & WRIGHT, J.L.C. (1996). Characterization of biologically inactive spirolides E and F: identification of the spirolide pharmacophore. *Tetrahed. Lett*., **37**: 7671-7674.

HUMMERT, C., RITSCHER, M., REINHARDT, K. & LUCKAS, B. (1997). Analysis of the characteristic PSP profiles of *Pyrodinium bahamense* and several strains of *Alexandrium* by HPLC based on ion-pair separation, post-column oxidation, and fluorescence detection. *Chromatographia*, **45**: 312-316.

HUMMERT, C., RÜHL, A., REINHARDT, K., HESS, P., LUCKAS, B. & GERDTS, G. (2002). First evidence of neuro-toxic spirolides in European waters. *Eur. J. Phycol*., submitted.

JENSEN, M.O. & MOESTRUP, Ø. (1997). Autecology of the toxic dinoflagellate *Alexandrium ostenfeldii*: Life history and growth at different temperatures and salinities. *Eur. J. Phycol.,* **32**: 9-18.

JOHN, U., MEDLIN, L.K. & GROBEN, R. (2002). Development of specific molecular probes and the application of Amplified Fragment Length Polymorphisms (AFLP) to analyse the *Alexandrium tamarense* species complex. *J. Phycol*. submitted.

KELLER, M.D., SELVIN, R.C., CLAUS, W. & GUILLARD, R.R.L. (1987). Media for the culture of oceanic ultraphytoplankton. *J. Phycol*., **23**: 633-638.

LANGE, M., GUILLOU, L., VAULOT, D., SIMON, N., AMANN, R..I., LUDWIG, W. & MEDLIN, L.K. (1996). Identification of the class Prymnesiophyceae and the genus *Phaeocystis* with ribosomal RNA-targeted nucleic acid probes detected by flow cytometry. *J. Phycol.,* **32**: 858-868.

LEBOUR, M.V. (1925). *The Dinoflagellates Of Northern Seas*. Mar. Biol. Ass. U.K., Plymouth, England, 250 p.

LEVASSEUR, M., BERARD-THERRIAULT, L., BONNEAU, E. & ROY, S. (1998). Distribution of the toxic dinoflagellate *Alexandrium ostenfeldii* in the Gulf of St. Lawrence. In *Harmful Algae* (Reguera, B., Blanco, J., Fernández, M.L. & Wyatt, T., editors), 54-57. Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO, Santiago de Compostela, Spain.

MACKENZIE, L., WHITE, D., OSHIMA, Y. & KAPA, J. (1996). The resting cyst and toxicity of *Alexandrium ostenfeldii* (Dinophyceae) in New Zealand. *Phycologia*, **35**: 148-155.

MEDLIN, L., LANGE, M., WELLBROCK, U., DONNER, G., ELBRÄCHTER, M., HUMMERT, C. & LUCKAS, B. (1998). Sequence comparison links toxic European isolates of *Alexandrium tamarense* from the Orkney Islands to toxic North American stocks. *Eur. J. Protistol*., **34**: 329-335.

MILLER, P.E. & SCHOLIN, C.A. (1998). Identification and enumeration of cultured and wild *Pseudo-nitzschia* (Bacillariophyceae) using species-specific LSU rRNA-targeted fluorescent probes and filter-based whole cell hybridisation. *J. Phycol.,* **34**: 371-382.

MOESTRUP, Ø. & HANSEN, P.J. (1988). On the occurrence of the potentially toxic dinoflagellates *Alexandrium tamarense* (= *Gonyaulax excavata*) and *A. ostenfeldii* in Danish and Faroese waters. *Ophelia,* **28**: 195-213.

MOON-VAN DER STAAY, S.Y., VAN DER STAAY, G.W.M.., GUILLOU, L., VAULOT, D., CLAUSTRE, H. & MEDLIN, L.K. (2000). Abundance and diversity of prymnesiophytes in the picoplankton community from the equatorial Pacific Ocean inferred from 18S rDNA sequences. *Limnol. Oceanogr.,* **45**: 98-109.

PAULSEN, O. (1904). Plankton-investigations in the waters round Iceland in 1903. *Meddelelser fra Kommision for Havundersoegelser Koebenhaven Serie Plankton*, 1-40.

PAULSEN, O. (1949). Observations on dinoflagellates. *K. Danske Vidensk. Selsk. Biol*., **6**: 1-67.

RASKIN, L., POULSEN, L.K., NOGUERA, D.R., RITTMANN, B.E. & STAHL, D.A. (1994). Quantification of methanogenic groups in anaerobic biological reactors by oligonucleotide probe hybridisation. *Appl. Environ. Microbiol.,* **60**: 1241-1248.

RICHARD, D., ARSENAULT, E., CEMBELLA, A.D. & QUILLIAM, M.A. (2002). Investigations into the toxicology and pharmacology of spirolides, a novel group of shellfish toxins. In *Proceedings of the IXth International Conference on Harmful Microalgae* (Hallegraeff, G.M., Blackburn, S.I., Bolch, C.J. & Lewis, R.J., editors), 383-386. Intergovernmental Oceanographic Commission of UNESCO, Paris.

SCHOLIN, C.A., BUCK, K.R., BRITSCHGI, T., CANGELOSI, G. & CHAVEZ, F.P. (1996). Identification of *Pseudo-nitzschia australis* (Bacillariophyceae) using rRNA-targeted probes in whole cell and sandwich hybridisation formats. *Phycologia,* **35**: 190-197.

SCHOLIN, C.A., HERZOG, M., SOGIN, M. & ANDERSON, D.M. (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.

SCHOLIN, C., MILLER, P., BUCK, K., CHAVEZ, F., HARRIS, P., HAYDOCK, P., HOWARD, J. & CANGELOSI, G. (1997). Detection and quantification of *Pseudo-nitzschia australis* in cultured and natural populations using LSU rRNA-targeted probes. *Limnol. Oceanogr.,* **42**: 1265-1272.

SIMON, N., BRENNER, J., EDVARDSEN, B. & MEDLIN, L.K. (1997). The identification of *Chrysochromulina* and *Prymnesium* species (Haptophyta, Prymnesiophyceae) using fluorescent or chemiluminescent oligonucleotide probes: a means for improving studies on toxic algae. *Eur. J. Phycol.,* **32**: 393-401.

SIMON, N., CAMPBELL, L., ORNOLFSDOTTIR, E., GROBEN, R., GUILLOU, L., LANGE, M. & MEDLIN, L.K. (2000). Oligonucleotide probes for the identification of three algal groups by dot blot and fluorescent whole-cell hybridisation. *J. Euk. Microbiol.,* **47**: 76-84.

SMAYDA, T.J. (1990). Novel and nuisance phytoplankton blooms in the sea: evidence for a global epidemic. In *Toxic Marine Phytoplankton* (Granéli, E., Sundström, B., Edler, L. & Anderson, D.M., editors), pp. 29-40. Elsevier, New York.

TAYLOR, F.J.R. (1984). Toxic dinoflagellates: taxonomic and biogeographic aspects with emphasis on *Protogonyaulax*. In *Seafood Toxins* (Ragelis, E.P., editor), 77-97. American

Chemical Society, Washington, D.C.

UTERMÖHL (1958). Zur Vervollkommnung der quantitativen Phytoplanktonmethodik. *Mitt. Int. Verh. Theor. Angew. Limnol*., **9**: 1-38.

WOLOSZYNSKA, J. & CONRAD, W. (1939). *Pyrodinium phoneus n. sp*., agent de la toxicité des moules du canal maritime de Bruges à Zeebruge. *Bull. Mus. Roy. Hist. nat. Belg.,* **15**: 1-5.

YU, R.C., HUMMERT, C., LUCKAS, B., QIAN, P.Y., LI, J. & ZHOU, M.J. (1998). A modified HPLC method for analysis of PSP toxins in algae and shellfish from China. *Chromatographia,* **48**: 671-676.

Table 1. Designation and geographical origin of strains used in this study.

| Species | Strain | Origin | Collector |
|---|---|---|---|
| *Alexandrium affine* | CCMP 112 | Ria de Vigo (Spain) | I. Bravo |
| *Alexandrium catenella* | BAH ME 255 | Tarragona (Spain) | M. Delgado |
| | BAH ME 217 | Tarragona (Spain) | M. Delgado |
| *Alexandrium fundyense* | GT 7 | Bay of Fundy, New Brunswick, (Canada) | A. White |
| | CCMP 1719 | Portsmouth, MA (USA) | D. Kulis |
| *Alexandrium lusitanicum* | BAH ME 91 | Laguna Obidos (Portugal) | E. Silva e Sousa |
| *Alexandrium minutum* | Al3T | Gulf of Trieste (Italy) | A. Beran |
| | Al5T | Gulf of Trieste (Italy) | A. Beran |
| *Alexandrium ostenfeldii* | BAH ME 136 | Timaru (New Zealand) | N. Berkett |
| | AOSH1 | Ship Harbour, Nova Scotia (Canada) | N. Lewis |
| | K0324 | Limfjord (Denmark) | P.J. Hansen |
| | K0287 | Limfjord (Denmark) | P.J. Hansen |
| *Alexandrium pseudogonyaulax* | AP2T | Gulf of Trieste (Italy) | A. Beran |
| *Alexandrium tamarense* | GTTP01 | Perch Pond, Falmouth, MA (USA) | D. Kulis |
| | NEPCC 407 | English Bay, Vancouver, British Columbia (Canada) | A. Cembella |
| | AL18b | St. Lawrence estuary, Quebec (Canada) | A. Cembella |
| | OF 84423.3 | Ofunato Bay (Japan) | M. Kodama |
| | BAH ME 181 | Orkney Islands (Scotland) | M. Elbrächter |
| | BAH ME 182 | Orkney Islands (Scotland) | M. Elbrächter |
| | SZN 019 | Gulf of Naples (Italy) | M. Montresor |
| | SZN 021 | Gulf of Naples (Italy) | M. Montresor |
| | 31/4 | Cork Harbour (Ireland) | W. Higman |
| | CCMP 115 | Tamar estuary (UK) | I. Adams |
| *Alexandrium taylori* | Ay1T | Lagoon of Marano (Italy) | A. Beran |
| | Ay2T | Lagoon of Marano (Italy) | A. Beran |
| *Thalassosira rotula* | CCAP 1085/4 | Fishgard (UK) | L.K. Medlin |

Table 2. Summary data of oligonucleotide probes used in this study.

| | Standardized probe name[1] | Specific For | Probe sequence [5'- 3'] | *In situ* conditions | Dot blot conditions |
|---|---|---|---|---|---|
| DINO1[2] | S-C-DINO-1404 *(A. tamarense)* -a-A-20 | Dinoflagellates (18S rRNA) | CCTCAAACTTCCTTGCITTA | 20% Formamide | 55 °C, 2xSSC, 0.1 % SDS |
| ATAM01 | S-S-A.tam-0775 *(A. tamarense)* -a-A-18 | *A.tamarense* (18S rRNA) species complex | TTCAAGGCCAAACACCTG | 20% Formamide | 56 °C, 1xSSC, 0.1 % SDS |
| ATNA02 | L-St-At.NA-373 *(A. tamarense)* -a-A-18 | *A.tamarense* (28S rRNA)– North American/ Orkney strains | AACACTCCCACCAAGCAA | 15% Formamide | 56 °C, 1xSSC, 0.1 % SDS |
| AOST01 | L-S-A. ost-484 *(A. ostenfeldii)* -a-A-18 | *A.ostenfeldii* (28S rRNA) | ATTCCAATGCCCACAGGC | 20% Formamide | 55 °C, 1xSSC, 0.1 % SDS |
| AOST02 | L-S-A. ost-0232 *(A. ostenfeldii)* -a-A-18 | *A.ostenfeldii* (18S rDNA) | CACCAAGGTTCCAAGCAG | 20% Formamide | 55 °C, 1xSSC, 0.1 % SDS |

[1] Alm et al. 1996, [2]Groben *et al*. 2002, in prep.

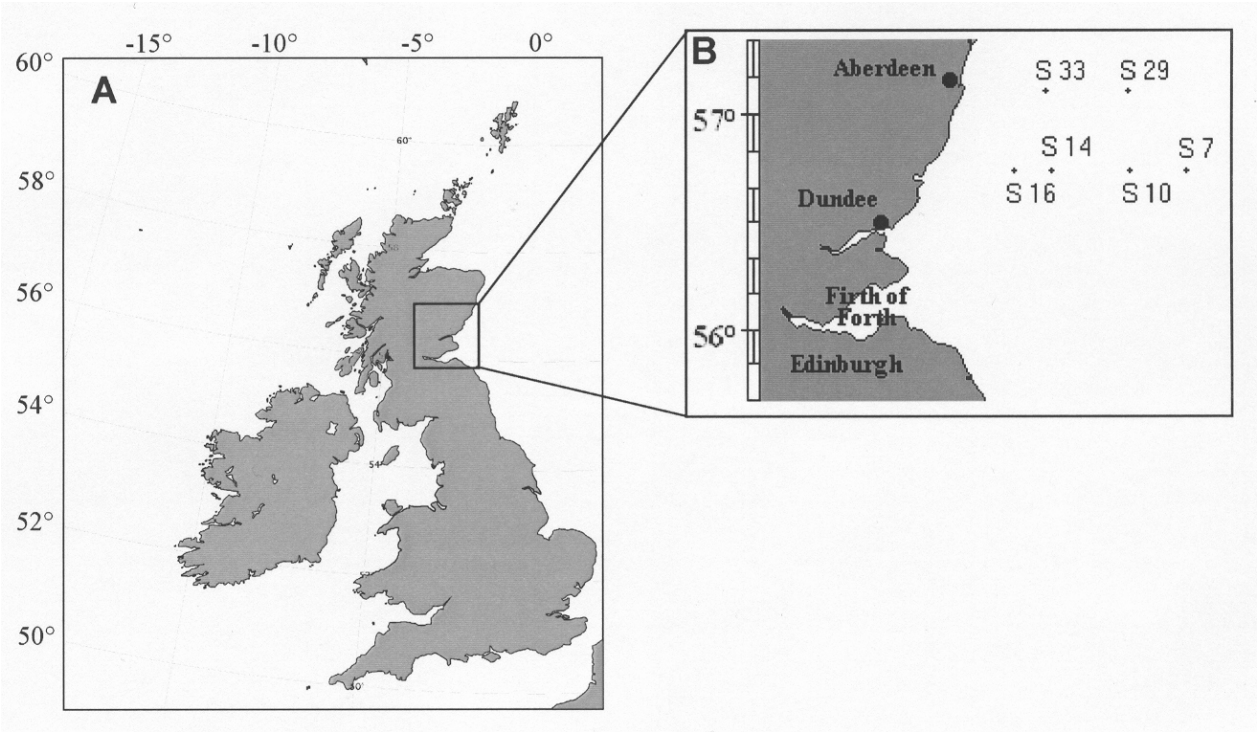



**Fig. 1:** Map showing the British Isles (**A**) and expanded view of the Scottish east coast (**B**) surveyed during the research cruise of the *RV Heinke* in May 2000. Sample stations (S) considered in this study are numbered, respectively.

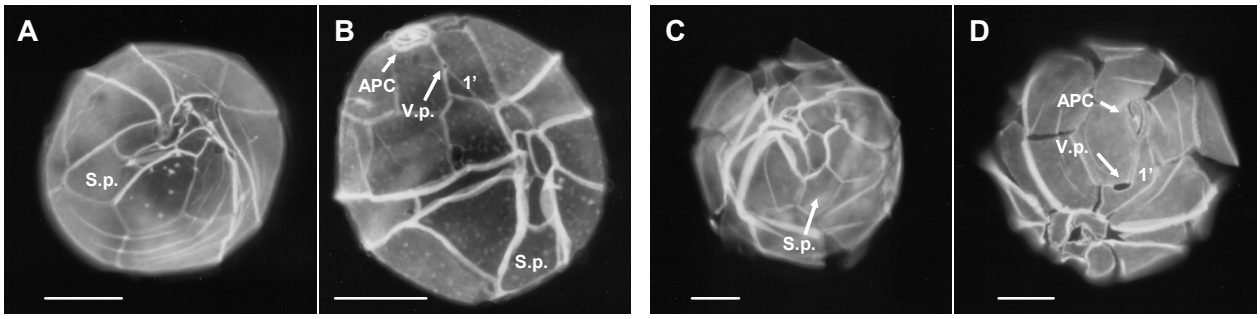



**Fig. 2:** Epifluorescence micrographs of calcofluor-stained thecal plates of *Alexandrium tamarense* (A,B) and *A. ostenfeldii* (C,D). APC, apical pore complex; V.p., ventral pore; 1', first apical plate; S.p., posterior sulcal plate. Scale bar = 10 μm.

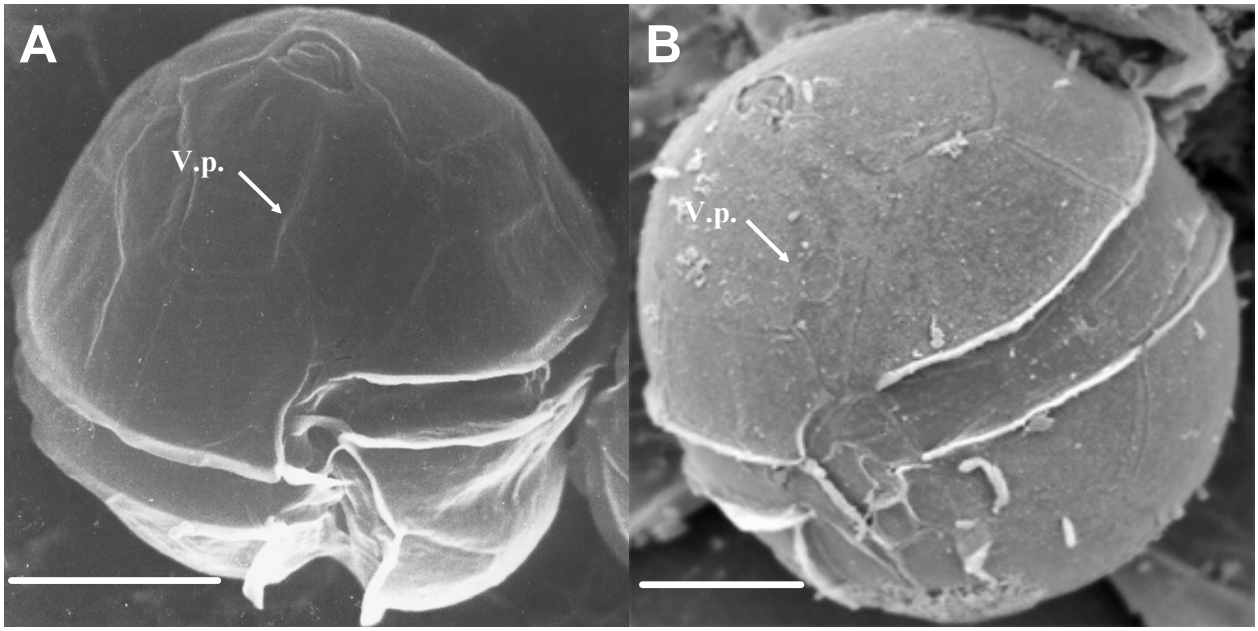



**Fig.3:** Scanning electron micrographs of entire vegetative cells of *Alexandrium tamarense* (A) and *A. ostenfeldii* (B) from northern European waters; V.p., ventral pore. Note that the V.p. is partially occluded by membranaceous material in these specimens. Scale bar = 10 µm.

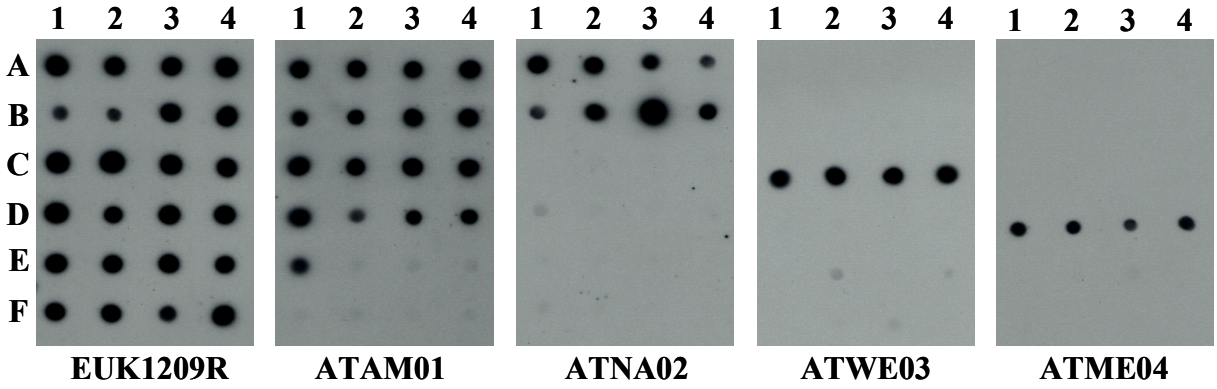



**Fig. 4**: Dot-blot hybridization of filter bound amplified SSU or LSU rRNA sequences with digoxigenin-labelled oligonucleotide probes (table 2). Rows A-D: *A. tamarense* species complex strains; row A: NA Orkney strains, A1: BAHME 181, A2: BAHME 182, A3: BAHME 184, A4: BAHME 200; row B: NA strains, B1: GT-7, B2: AL18b, B3: OF8423.3, B4: GTPP01; row C: WE strains, C1: CCMP 115, C2: 31/4, C3: 31/9, C4: UW42; row D: ME strains, D1: SZN01, D2: SZN08, D3: SZN19, D4: SZN21; E1: *A. catenella* (Asian clade); E2: *A.lusitanicum* (BAHME 91); E3: *A. minutum* (Al5T); E4: *A. pseudogonyaulax* (AP2T); F1: *A. ostenfeldii* (BAHME 136); F2: *A. taylori* (AY1T); F3: *A. affine* (CCMP 112); F4: *Prorocentrum minimum*

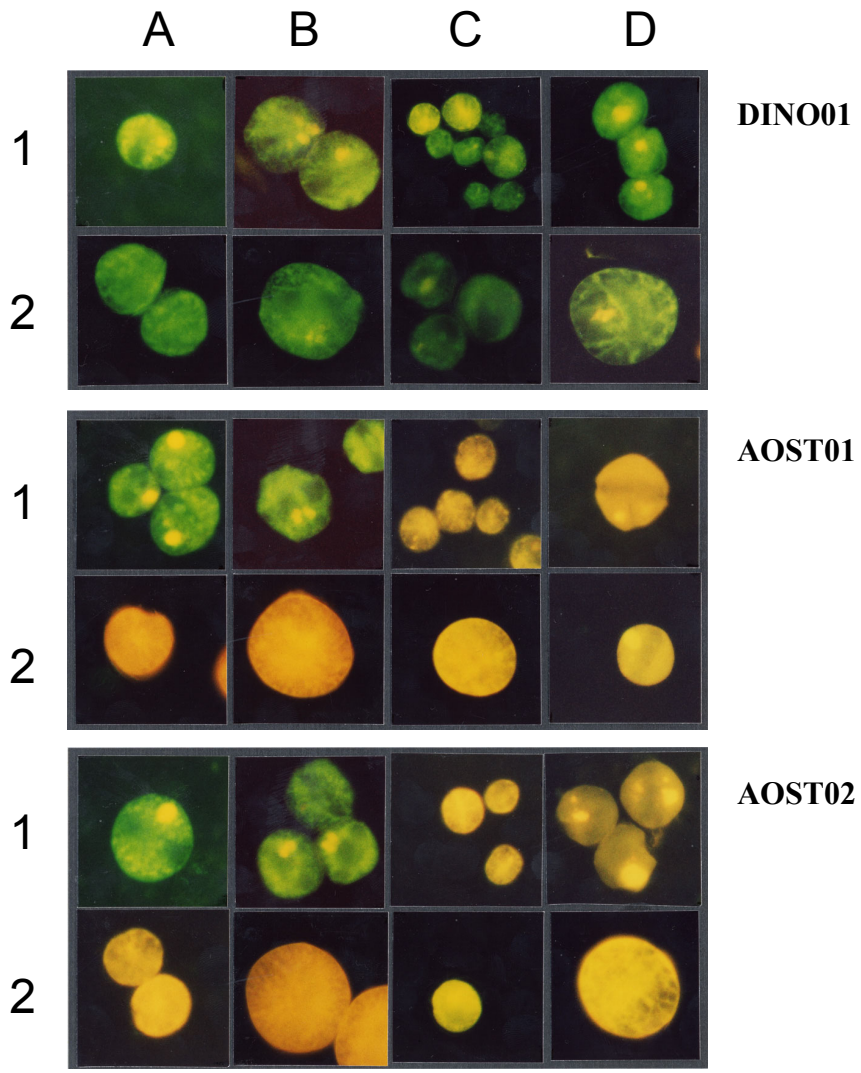



**Fig. 5:** Array of whole-cell hybridisations from eight *Alexandrium* strains representing six species. Dinoflagellates were hybridised with three different fluorescein-labelled rRNA probes: DINO01, AOST01, and AOST02. A1: *A. ostenfeldii* (AOSH1), B1: *A. ostenfeldii* (K0287), C1: *A. lusitanicum* (BAHME 91), D1: *A. tamarense* (BAH ME181), A2: *A. tamarense* (GTPP01), B2: *A. taylori* (Ay2T), C2: *A. pseudogonyaulax* (AP2T), D2: *A. affine* (CCMP 112).

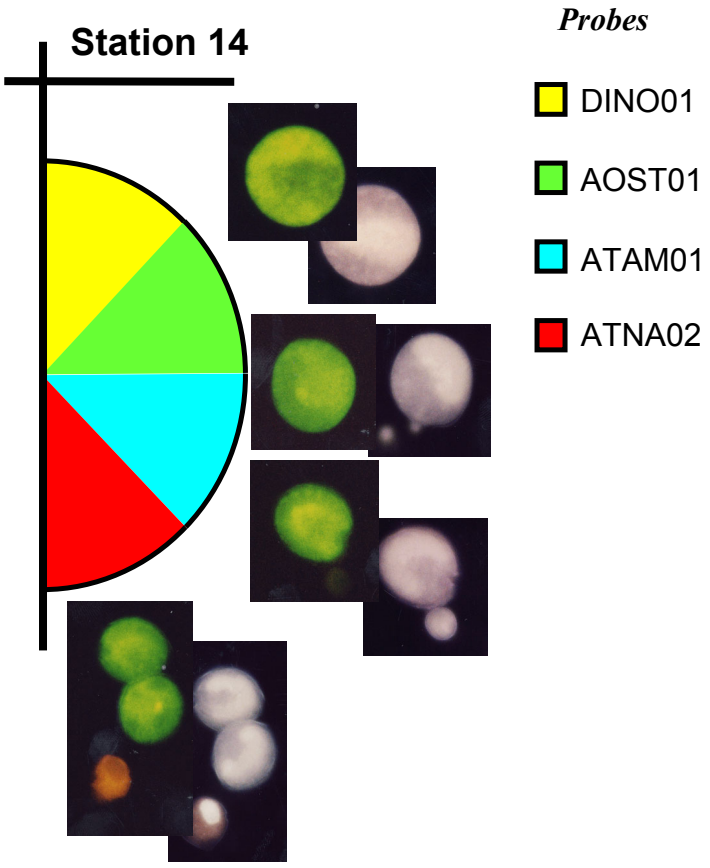



**Fig. 6**: An example of whole-cell hybridisation of plankton samples from Station 14. The semicircle represents half of the polycarbonate filter that was labelled. Quadrants labelled with different fluorescence-labelled rRNA probes are indicated in different colours as follows: DINO01 (yellow), AOST01 (green), ATAM01 (blue), ATNA02 (red). Positive hybridisation (greenish signal) is shown for all probes adjacent to the same specimens counterstained with DAPI.

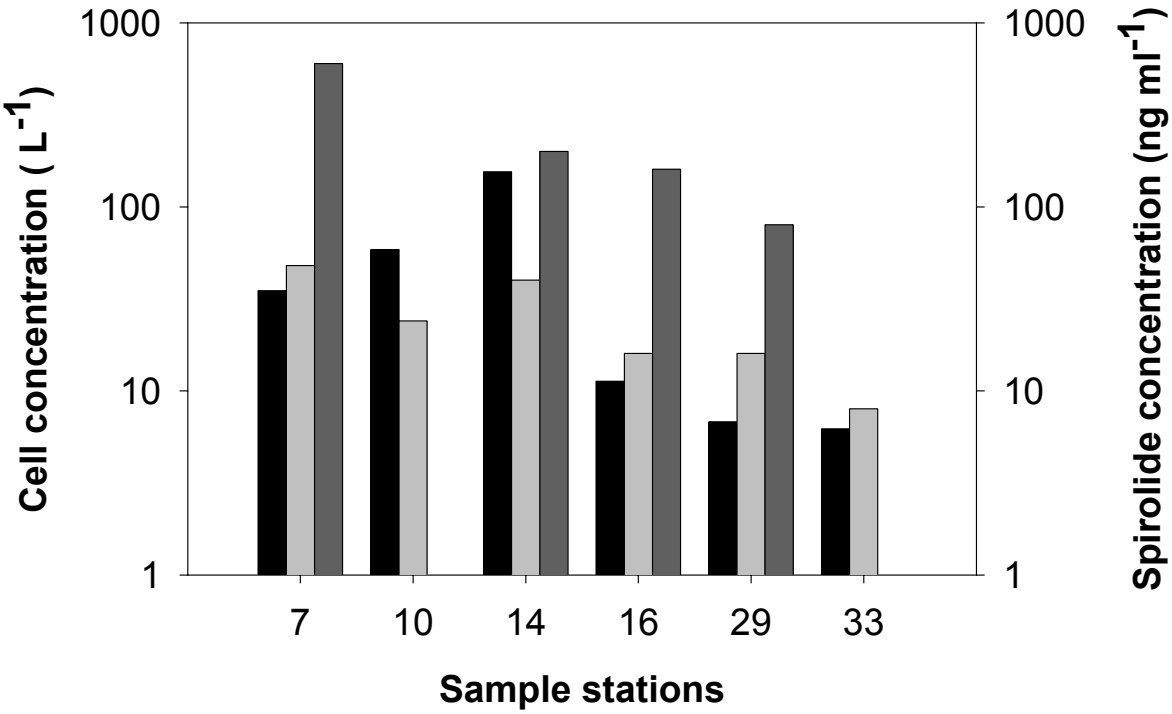



**Fig. 7**: Variation in concentration of *A. ostenfeldii* cells, counted as fixed cells in a Utermöhl chamber (dark grey bars) versus using FITC-labelled rRNA probes for *in situ* hybridisation (light grey bars) at six sampling stations. Total spirolide concentration (ng $ml^{-1}$) (black bars) at the respective sampling stations is presented for comparison.

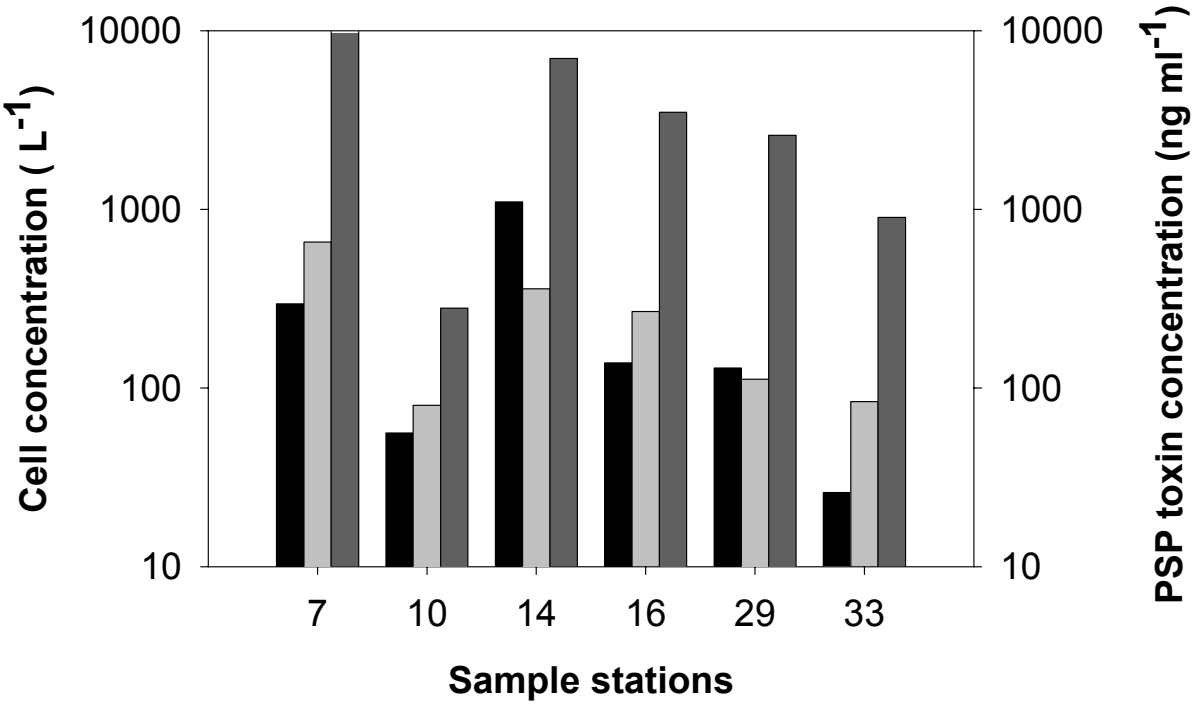



**Fig. 8**: Variation in concentration of *A. tamarense* cells, counted as fixed cells in a Utermöhl chamber (dark grey bars) versus using FITC-labelled rRNA probes for *in situ* hybridisation (light grey bars) at six sampling stations. Total PSP toxin concentration (ng $ml^{-1}$) (black bars) at the respective sampling stations is presented for comparison.

# 3. *Synthesis*

### *3.1 Phycotoxin and allelochemicals and their effect on grazing*

Harmful species are predominantly related to phycotoxins, which can be vectored through the food web, accompanied by broad-based trophodynamic effects and which can affect humans directly through several types of poisoning, e.g. PSP, DSP, Ciguatera etc. In contrast, allelochemical substances are usually directly targeted and therefore affect humans often indirectly with disturbed ecosystems or with losses in aquaculture. One part of this thesis was to understand the factors that may influence the formation of HABs. The results of **publications I** and **II** show that for the ecological success of those species, which manifests in dense and long-lasting blooms, the allelochemical potential through its direct deterrent and/or destructive effects on unicellular grazers, is of great significance.

The results of **publication I** show that the two heterothrophic dinoflagellates *Oblea rotunda* and *Oxyrrhis marina* are seriously negatively affected by several species and strains of the genus *Alexandrium.* The immobilising and lytic effects of the *Alexandrium* spp. were unrelated to PSP toxin content. The toxic effects are caused by extracellular toxins as *O. marina* did not ingest *Alexandrium* and lytic effects were also observed in cell-free culture medium. The study showed that *Alexandrium* strains can contain a large variety of PSP and/or lytic compounds (allelochemicals) so that ecophysiological consequences of allelochemicals may mimic phycotoxin effects. This may explain some of the inconsistencies in reports on interactions of PSP toxin-producing algae and copepodes (reviewed in Turner and Tester 1997). That such effects on crustacean grazers exposed to *Alexandrium* may be caused by substances not associated with PSP have been proposed before (Huntley et al. 1986; Lush and Hallegraeff 1996; Teegarden and Cembella 1996). That copepods are affected by lytic exudates, have been observed with *C. polylepis* and other toxic algae as well (Nielsen et al. 1990; Uye and Takamatsu 1990). In **Publication II**, grazing experiments were conducted with two different clones of *C. polylepis*, which were virtually identically, but differed in toxicity (as defined by their toxic effects on the brine shrimp *Artemia franciscana*) using the heterotrophic dinoflagellate *Oxyrrhis marina* as a grazer. Profound quantitative differences in grazing and growth of *O. marina* were observed when it was fed with either the toxic or the non-toxic clone. The growth rate of *O. marina* was never inhibited with increasing concentrations of the toxic clone. Therefore the negative effects to the grazers are not related

to the algal concentration and hence not to the concentration of a putative toxin released into the water. Rather, grazing inhibition seems to be related to each single feeding process; *O. marina* apparently avoided ingestion of the toxic clone. This might be because of reception of some toxin-related properties of the algal cell, e.g. olfactory or glutatory factors, as chemosensory capabilities are well known among heterotrophic dinoflagellates (Hauser et al. 1975; Spero 1985; Buskey 1997).

For the ecological impact of allelochemicals some points should be discussed. First, the reduction or elimination of -at least- the protozoan grazers may explain why some *Alexandrium* blooms can persist for months (Mortensen 1985; Carreto et al. 1986). Sublethal, long-term negative effects of lower *Alexandrium* cell concentrations on protozoans are poorly known. According to Hansen (1989), *Favella ehrenbergii* is killed by a PSP toxic *A. tamarense* strain at high cell concentrations but showed ingestion and rapid growth when fed low concentrations of the same strain. This suggests that grazing by protozooplankton might be of significance in controlling the development of a bloom if the concentration of algae is low and the concentration of predators is sufficiently high. For *C. polylepis*, allelochemical activity has been described before, whereas (Riegman et al. 1996) ascribed the success of *C. polylepis* in multispecies culture experiments to the ability of *C. polylepis* to outcompete other algae, Schmidt & Hansen (2001) recently showed that toxins released by *C. polylepis* had a direct harmful effect on a whole range of tested algae in mixed cultures. This allelochemical activity, in addition to a reduced grazing pressure, may explain why a bloom like the one in 1988 in the North Sea became essentially monospecific at high cell concentrations. In addition to the eliminating competitors and/or grazers, allelochemical activity may be coupled with mixotrophic nutrition. This was suggested for the haptophytes *Prymnesium patelliferum* (Tillmann 1998) and *Chrysochromulina* (Estep and MacIntyre 1989). *Alexandrium* spp. are generally considered to be mainly autotrophic, but food vacuoles containing ciliates or phytoplankton cells have been observed in *Alexandrium ostenfeldii* (Jacobson and Anderson 1996). Moreover, it was recently shown that *Alexandrium* has the capacity to take up high molecular weight organic molecules and dissolved free amino acids (Carlsson et al. 1998; Legrand and Carlsson 1998; Yasumoto et al. 1996;John and Flynn 1999). It thus might be speculated that *Alexandrium* probably benefits from enhanced concentrations of dissolved organic matter in consequence of its lytic activity. For *Chrysochromulina* spp. (Estep and MacIntyre 1989) have hypothetisized a auxothrophy followed by induced osmosis (dasmothrophy). The toxin might punch holes in (perforate) the cell membrane of their victims without necessarily killing them, this leads in a outflow of nutrients, which

*Chrysochromulina* can utilize. Additionally, zooplankton grazing on algae, preferably other than toxic ones and subsequent DOC release (Strom et al. 1997) might stimulate the growth of bacteria, which are consumed by mixothophic algae.

It may be attractive to ascribe the whole range of observed negative effects on blood cells (haemolytic effects, Edvardsen et al. 1990; Simonsen et al. 1995; Simonsen and Moestrup 1997b;Eschbach et al. 2001a), heterotrophic protozoa (immobilisation/cell lysis, grazer deterrence, Hansen 1989; Nielsen et al. 1990), algae (growth repression/cell lysis. Blanco and Campos 1988; Arzul et al. 1999; Riegman et al. 1996; Schmidt and Hansen 2001) or copepods grazing inhibition, Huntley et al. 1986; Nielsen et al. 1990) to one single chemical compound. However, Arzul et al. (1999) concluded that the allelopathic activity of *Alexandrium* is caused by a complex of chemicals, rather than by a specific substance. Almost nothing is known about the chemical composition of such compounds. The experiments with culture filtrate at least indicate that these substances are water-soluble but labile in culture media, the same was found for the *Chrysochromulina polylepis* toxin by (Simonsen and Moestrup 1997b). Comparable lytic or allelopathic effects caused by several algal species are mainly thought to be due to glycolipids and polyunsaturated fatty acids. For example, digalactosylglycerol and octapentaenoic acid (18:5ω3) isolated by (Yasumoto et al. 1990) have been shown to be both haemolytic (Yasumoto et al. 1990) and inhibitory to diatom growth (Gentien and Arzul 1990; Arzul et al. 1995). However, (Simonsen and Moestrup 1997b) have demonstrated that lytic compounds are common substances in microalgae and especially polyunsaturated fatty acids (PUFAs) are known to be distributed over a wide range of taxa of algae. **Publication II** demonstrated that it is very unlikely that glycolipids and/or PUFAs are involved in the toxic effects of *C. polylepis*.

In summary the presented experiments showed that allelochemicals rather than the PSP toxin (phycotoxins) content of *Alexandrium* strains were involved in immobilisation and lytic effects towards heterotrophic dinoflagellates. In comparative grazing experiments of a toxic and a non toxic clone of *C. polylepis*, *Oxyrrhis marina* apparently avoided ingestion of the toxic clone. That indicates that allelochemicals through deterrent and/ or destructives effects on grazer, could support bloom formation. However up to now little is known about the chemical nature of allelochemicals, with respect to the results of **Publication II** make glycolipids and/or polyunsaturated fatty acids as allelochemicals very unlikely, so that the chemical nature remains speculative.

### *3.2 Toxin production/regulation*

With **publication III** and **IV**, evidence was brought forward that extrinsic environmental factors, i.e. the photoperiod, can influence the rate of production and cell quota ($Q_t$) of the potential allelochemical substance of *C. polylepis* (**Publication III**) and spirolides of *A. ostenfeldii* (**Publication IV**), respectively. The toxicity (haemoloytic activity) of *C. polylepis* was induced through light (**Publication III**), whereas the spirolide $Q_t$ in *A. ostenfeldii* increased during the beginning of the dark period (**Publication IV**). These data were consistent with studies of Taroncher-Oldenburg et al. (1997) and Pan et al. (1999), who showed that the toxin production of dinoflagellates varied throughout the cell cycle and is coupled to defined time periods.

Because of the lack of a direct quantitative analysis for the toxic substance of *C. polylepis*, a erythrocyte lysis assay was used (Eschbach et al. 2001b) for estimation of changes in toxicity (**Publication III**). Haemolytic activity is not necessarily related to toxicity (Simonsen and Moestrup 1997b), but the toxic clone showed a different haemolytic kinetic, compared to the non-toxic clone, so it was assumed that the differences were found in its toxicity. Therefore, cellular toxin content was measured as haemolytic activity of a constant number of *C. polylepis* cells incubated with a constant number of fish erythrocytes. Changes in the measured lytic activity throughout the sampling period were representative for average differences in the allelochemical (haemolytic) content of the single algal cell. With the start of the light period *C. polylepis* cells enter the G1 phase of the cell cycle. During G1 *C. polylepis* grew in size and physiological activities were performed, like chlorophyll synthesis, whereas the haemolytic activity of the toxic strain increased drastically. This may indicate a light dependent replenishment of the toxin cell quota, after its reduction during the end of the dark period when cell division occurred, as shown for *A. fundyense* (Taroncher-Oldenburg et al. 1997). After the third day, haemolysis was no longer light induced and therefore was uncoupled to the cell cycle, probably because the growth rate was reduced and the toxin relevant G1 phase was expanded (Taroncher-Oldenburg et al. 1997).

At the beginning of the dark period total spirolide per cell *A. ostenfeldii* increased dramatically and decreased during the light period, which showed that spirolide biosynthesis was affected by light-dependent metabolic events and indicated a coupling of spirolide production to the photoperiod and cell cycle. In comparison, in *Prorocentrum lima*, the cell quota of the polyketide-derived DSP toxins increased in the light, but also extended through several phases of the cell cycle (Pan et al. 1999). In contrast, although PSP toxin production by *A. fundyense* occurred in the light, synthesis was restricted to the G1 phase (Taroncher-

Oldenburg et al. 1997, 1999). The maximum cell quota at the end of the dark phase, could be interpreted as net spirolide production, if this period also represents the late mitotic phases G2+M, just prior to cytokinesis. In such a case, after the production of spirolides, the dividing cells evenly distributed the phycotoxin to their daughter cells. In contrast, *A. fundyense* cells produced the PSP toxins after the cell division in the early G1-phase (Taroncher-Oldenburg et al. 1997).

In summary, light or dark (photoperiod) dependent metabolic events influence the rate of production and cell quota ($Q_t$) of the toxin of *Chrysochromulina polylepis* and spirolides of *Alexandrium ostenfeldii*. Whereas the haemolytic activity in *C. polylepis* increased during the light phase, the spirolide cell quota of *A. ostenfeldii* increased at the beginning of the dark period. A hypothetical reason for *Chrysochromulina polylepis* to start the toxin production with beginning of the light period is that this could be to replenish the toxin cell quota of the divided cells. On the other hand, *Alexandrium ostenfeldii* seem to produce spirolides before cell cytokinesis probably using storage products as energy source, so that dividing cells evenly distribute the phycotoxin to its daughter cells.

### *3.3. Phylogeny, biogeographic distribution and evolution*

The phylogenetic analysis of several species and strains of *Alexandrium*, demonstrated that the 18S rRNA analysis could be used to investigate the interspecific relationship within the genus *Alexandrium* because the resolution between major species is appropriate for the comparisons needed. The D1/D2 region should be used only when finer resolution between strains is needed because it is evolving at a much higher rate. The phylogenetic analysis of the SSU of the rRNA sequences was consistent with those of previous studies (Saunders et al. 1997; Walsh et al. 1998; Litaker et al. 1993), and generally agreed with the classification of the dinoflagellates by Fensome et al. (1993) (**Publication V**). Through the analysis of the D1/D2 region of the LSU, a new ribotype within the *Alexandrium tamarense* species complex was discovered (**Publication V)**. Moreover, the phylogenetic analysis demonstrates, as known from literature (Scholin et al. 1994; Medlin et al. 1998; Adachi et al. 1996), that the *A. tamarense* species complex is separated into clades that reflect geographic areas and not the three morphotypes *A. tamarense*, *A catenella*, and *A. fundyense*. The four isolates of the Mediterranean Sea formed a sister clade to the North American clade, well supported due to bootstrap and posterior probability values (**Publication V**). However, this may not be the last discovery of a new ribotype within the *A. tamarense* species complex because of new reports

of a *A. tamarense* species complex from the southern hemisphere (reviewed by Taylor (1987) and Gayoso (2001) and references therein).

Because of the few base substitution among the clades of the *Alexandrium* species complex, the analysis of the D1/D2 region gave no information about the genetic diversity inside clades and populations. To study further the biodiversity inside the species complex, the fingerprint technique of Amplified Fragment Length Polymorphisms (AFLPs) was established for use in this species complex (**Publication VI**). It was demonstrated that AFLPs was a powerful method to investigate the biodiversity within a distinct geographic region or among the cells of an algal bloom, but the genetic variation across the entire *A. tamarense*/*catenella*/*fundyense* species complex is too extensive to allow for comparison of different clades by this technique, if only single representatives of each location are used. The life cycle and the population dynamics of *Alexandrium* could be the reason for such high genetic diversity. *Alexandrium* species are meroplanktonic; they spend only few weeks in the plankton where they undergo vegetative growth and form cysts after gametogamesis. Blooms develop of cysts from several year classes of cysts, which increases the genetic pool available for the next gametogenesis (Dale 1977; Wyatt and Jenkinson 1997; Anderson et al. 1998).

To my knowledge, for the first time a molecular clock was applied to the dinoflagellate sequence data set to calculate the divergence of the genus and the *Alexandrium* species complex (**Publication V**). Based on the calculation and according to the data set analyzed, the average age of the genus *Alexandrium* is around late Cretaceous and early Paleogene (77 Ma) with its earliest possible age at the middle of Cretaceous (119 Ma). The *A. tamarense* species complex potentially diverged around the early Neogene (23 Ma) with a possible first appearance estimated from the 95 % confidence level in the late Paleogene (45 Ma). At that time the water masses could still circulate around the world from Eastern Indian Ocean over the Tethys Sea and Pacific Ocean, whereas counter currents could distribute organisms anti-clockwise. This circulation pattern permitted a global distribution of the species complex. Two hypothetical scenarios were assumed that may explain the global distribution and divergence within the *Alexandrium tamarense* species complex. Both scenarios start with a globally distributed ancestral population.

1. As the oceans became more thermally structured from 40 to 70 MA, this caused divergent populations with different preferences for environmental conditions, such as temperature. This north/south structuring left ancestral populations in the warm Tethys Sea (south), in the North Atlantic (north) and in the western Pacific (north). The warm adapted population was dispersed westwards with the ongoing currents from the Tethys Sea over the

Atlantic Ocean through the Central American Seaway. After the closure of the Tethys Sea and uplifting of Panama, the warm water population was divided into one group in the Mediterranean and another group on the Pacific and the Atlantic side of North America. However, dramatic changes in the water circulation and the climate in this sector of the Atlantic Ocean and Caribbean might explain the extinction of populations on the Atlantic side of North America. The populations on the Pacific side began to migrate northward into those cold regions where they now exist. Regional changes in abiotic and biotic factors might have caused this process. As the Bering Strait opened, they eventually migrated around Canada, down the eastern seaboard of the United States, and then finally were carried back to Europe by the Gulf Stream.

2. In the case of the second scenario the first divergence involves an east/west separation with ancestral populations occupying the Pacific. The Pacific population diverges into populations occupying the eastern and western parts of the Pacific. The eastern Pacific population remains connected to populations in the eastern Atlantic over Panama and the Central American Seaway and its counter currents, whereas the western Pacific population remains connected to western Europe via the Tethys Sea. With the closure of the Tethys Sea, the western Pacific population diverges into the Temperate Asian clade and the West European clade. With the uplifting of Panama, the connection between the eastern Pacific and West Europe is closed leaving ancestral populations in the Mediterranean and in the eastern Pacific. Scholin (1998) mentioned an isolate from the Kamchatka Peninsula, which has an intermediate genotype between both the Temperate Asian and Northern American strains, which may support this initial east/west separation in the Pacific. The North American eastern coastal population might, as suggested in (Scholin et al. 1995), have originated from an ancestral population from the west coast, which migrated after the opening of the Bering Strait over the Arctic Ocean to the Labrador Sea through the Canadian Archipelago or as (Medlin et al. 1998) assumed over the Fram Strait and following the Greenland currents.

In summary, the phylogenetic analysis of the SSU and LSU rRNA were consistent with previous records in the literature. However the Mediterranean isolates of *Alexandrium tamarense* formed a new ribotypes within the *A. tamarense* species complex. The application of a molecular clock gave a probable age of the genus *Alexandrium*. of 77-119 MA and the *A. tamarense* species complex diverges potentially about 23 to 45 MA ago. Two hypothetical scenarios were assumed that may explain the historical and recent global distribution and divergence of the *Alexandrium tamarense* species complex. The fingerprint method Amplified Fragment Length Polymorphisms (AFLPs) was successfully established to investigate the genetic diversity of the *A. tamarense* species complex inside a geographic clade or population and showed blooms of *Alexandrium tamarense* to be highly diverse.

### *3.4. Monitoring*

A common problem found in phytoplankton field ecology is that the species of interest can only be a minor component of the planktonic community and that the identification of species, strains or morphotypes, which are morphological very similar is often very complex (Parsons et al. 1999). The world wide increasing problem of harmful algae blooms leads to a growing number and intensity of mandatory monitoring programs, which then generate large numbers of samples for cell analysis (Hallegraeff et al. 1995). The species composition of those field samples, generally, have to examined by taxonomic experts using light and electron microscopy. To overcome the problems of time, cost, and labor intensive analysis for clear identification, **Publications VI** and **VII** demonstrated the successful design of specific probes for each *A. tamarense* ribotype present in European and North American waters and *A. ostenfeldii*, respectively. All probes can be used to identify clearly their target species or strains in DNA dot blots and whole-cell hybridization. This is interesting because toxicity has been correlated with specific ribotypes, e.g., with the temperate Asian and the North American clade, and these ribotypes co-occur with non-toxic strains in European waters (**Publication VI**). Parsons et al. (1999) have shown that the oligonucleotide probes have a great potential as tools for routine monitoring of HABs even when there are still some technical problems which has to be solved, like e.g., empty thecae, broken and unhealthy cells, resulting in fewer ribosomes and therefore in reduced fluorescence. **Publication VII** presented the first large-scale integrated field observations of the co-distribution of *Alexandrium ostenfeldii* and *A. tamarense*, and their respective toxins, spirolides and PSP toxins. Presumably, the reason why spirolides have never been detected before in European waters might be due to that there have not been any previous efforts to analyse plankton or

shellfish samples for the presence of these compounds. In most toxic phytoplankton monitoring programs, *A. ostenfeldii* has been of marginal interest because of its typically weak ability to produce PSP toxins. Now that the spirolide producing capability of this species has been clearly demonstrated along both the European and North American Atlantic coasts, the requirements for monitoring *A. ostenfeldii* have changed. It was shown that the molecular probes have the capability to distinguish taxa in complex field plankton assemblages. The cell concentrations determined by FISH for each *Alexandrium* species were positively correlated to those enumerated by the classic Utermöhl technique. The total cell counts by the latter method were up to an order of magnitude higher possibly because of non-homogeneous distribution of the cells on the filter; combined with the fact that only a portion of the filter was scanned for labelled cells. However, at two stations *A. ostenfeldii* was detected by means of FISH, corresponding with spirolide detection, whereas by using the Utermöhl approach no cells were detected. This shows an advantage of the FISH technique, especially when the cell concentration of the target species in field samples is low even the biomass of other taxa is large, because even events as rare as a single cell generate a clear visible fluorescent signal.

In summary, the studies demonstrate the design of species and strain specific molecular probes for *Alexandrium ostenfeldii* and the *A. tamarense* species complex, respectively. The application of those probes have shown that both species co-occur at the Scottish east coast. In all field samples where *A. ostenfeldii* was observed, spirolides were detected for the first time in European waters. Hierarchical rRNA probes are a promising tool for field studies and monitoring programs of phytoplankton composition and bloom formation, without the necessary need to be a taxonomist.

### *3.5. Perspectives for future research.*

The investigations of allelochemical grazer defense of *Alexandrium* species and strains upon heterothrophic dinoflagellates leads to suggestion that grazing by protozooplankton might be of significance in controlling the development of a bloom if the concentration of algae is low and the concentration of predators is sufficiently high, but extensive investigations on potential long-term effects of sublethal concentrations of allelochemicals on protozoan grazers still have to be carried out. The avoidance of the toxic clone as compared with the non-toxic clone of *Chrysochromulina polylepis* bz *Oxyrrhis marina* was obvious, but it could not be demonstrated that *O. marina* prefer to feed on the non-toxic clone when also the toxic clone was offered as food. We attempted to label selectively one strain over the other but the chosen vital stain was expelled from the algae. Because the vital staining approach failed, a new method has to be worked out to somehow label the toxic or non toxic clone to permit simultaneous feeding experiments with both clones.

This study has provided significant insights into the light-dependence of the *C. polylepis* toxin and spirolide production, but little information is available on the biosynthesis of allelochemicals and especially polyketide-derived metabolites by prymnesiophyceae and dinoflagellates. Further effort will be directed towards the use of cell synchronisation techniques coupled with studies of gene expression of putative biosynthetic genes for allelochemicals (*Chrysochromulina* toxin) and spirolides. Molecular techniques, such as differential display, subtraction hybridisation, and cDNA libraries, have already been established and applied to identify genes, e.g. polyketide synthases, that are involved in the toxin biosynthesis (John, U. and Medlin, L.K., unpublished data). An EU project (EUKETIDES) has now advanced to the contract negiotiation stage which will investigate the production and synthesis of these genes.

The phylogenetic studies of *Alexandrium* spp. have shown that in the future several revisions of the classification of Balech (1995) could be expected as already mentioned in (Taylor and Fukuyo 1998). Some morphological distinguishable species appear rather as morphotypes than species, as shown for the *A. tamarense* species complex or as unexpectable closely related sister group as in the case of *A. affine* and *A. concavum* or *A. tamaranse* and *A. cohorticula*. We have started to collect many different non-sequenced *Alexandrium* species to include them in our phylogenetic studies to get a more profound overview about the sustainability of the 29 described species and their phylogenetic relationship.

In order to identify the origin of algal isolates on a basis of bloom membership or from which bay or port the strain derives, rRNA probes have their limits. The use of specific PCR markers developed from AFLP bands that are common for strains from a distinct geographic area or a bloom (**Publication VI**) may be used in the future to identify the strain's origin (McLenachan et al. 2000). The AFLP-derived PCR markers might help to identify the source of the introduced strains and to hold perpetrators accountable for the effects of HABs. Also, many isolates of a bloom can be characterized genetically by AFLPs and those results compared with the isolates' chemical properties, e.g., toxin profile, and temperature, salinity tolerance and grazer defense. These kind of data may can then be used to feed artificial neural networks to model prediction for bloom formation of toxic and non-toxic species.

DNA dot blots, DNA micro-arrays or DNA-chips hybridisation with hierarchical probes offer possibilities to study algae composition even in large scale monitoring programs, without taxonomical expertise. For a further automation of the FISH methodology and the increase of the probability to encounter a rare event instrument, such as a solid phase cytometer (Chemunex) can be used to scan automatically the hybridised field sample filter. FISH technology also can be combined with flow cytometry to give online data about the species composition at the sample stations. Early warning systems using disposable microchips are underway. Results have been obtained with the probes for *Alexandrium tamarense* and *A. ostenfeldii*. Probes for *A. minutum* are under development. Flow cytometry in combination with *in situ* hybridisation offers the opportunity to measure microalgal diversity on a larger scale and with greater precision, even in real time on board research vessels. In addition, algal cells can be sorted automatically and can be used for further studies or verification, for example, by sequencing of genes (Lange et al. 1996;Simon et al. 1997).

## *4. References*

Adachi, M., Sako, Y. and Ishida, Y. 1996. Analysis of *Alexandrium* (Dinophyceae) species using sequences of the 5.8S ribosomal DNA and internal transcribed spacer regions. Journal of Phycology 32:424-32.

Adams, J.A., Seaton, D.D., Buchanan, J.B. and Longbotton, M.R. 1968. Biological observations associated with the toxic phytoplankton bloom off the East coast. Nature 220:24-25.

Anderson, D.M. 1990. Toxin Variability in *Alexandrium* Species. *In* Graneli, E. Sundström, B., Edler, L. and Anderson, D.M., [Eds.] Toxic Marine Phytoplankton. Elsevier Science Publishing Co., Inc. Amsterdam. Pp. 41-51

Anderson, D.M. 1998. Physiology and bloom dynamics of toxic *Alexandrium* species, with emphasis on life cycle transitions. *In* Anderson, D.A., Cembella, A.D., and Hallegraeff, G.M., [Eds.] Springer, Berlin, pp. 29-48

Anderson, D. M., Cembella, A. D., and Hallegraeff, G. M. 1998. Physiological Ecology of Harmful Algal Blooms Springer, Berlin, Germany.

Anderson, D.M., Kulis, D.M., Sullivan, J.J. and Hall, S. 1990. Toxin Composition Variations in one Isolate of the Dinoflagellate *Alexandrium fundyense*. Toxicon 28:885-893.

Arzul, G., Gentien, P., Bodennec, G., Toularastel, F., Youenou, A. and Crassus, M.P. 1995. Comparison of toxic effects in *Gymnodinium* cf. *nagasakiense* polyansaturated fatty acids. *In* Lassus, P., Arzul, G., Gentien, P., & Marcaillou, C., [Eds.] Harmful Marine Algal Blooms. Lavoisier, Intercept Ltd. Pp. 395-400

Arzul, G., Seguel, M., Guzman, L. and Erard Le Denn, E. 1999. Comparison of allelopathic properties in three toxic *Alexandrium* species. Journal of Experimental Marine Biology and Ecology 232:285-295.

Falconer, I. R. 1993. Algal Toxins in Seafood and Drinking Water Academic Press, London, 87-104 pp.

Balech, E. 1995. The Genus *Alexandrium* Halim (Dinoflagellata) Sherkin Island Marine Station, Sherkin Island Marine Station, Ireland.

Balech, E. and Tangen, K. 1985. Morphology and taxonomy of toxic species in the tamarensis group (Dinophyceae): *Alexandrium excavatum* (Braarud) *comb. nov*. and *Alexandrium ostenfeldii* (Paulsen) *comb. nov.* Sarsia 70:333-343.

Barrett, B. A. and Kidwell, K. K. 1998. AFLP-based genetic diversity assessment among wheat cultivars from the Pacific Northwest. Crop Science 38:1261-1271.

Bates, S.S. 1998. Ecophysiology and Metabolism of ASP Toxin Production. *In* Anderson, D.M., Cembella, A.D., & Hallegraeff, G.M., [Eds.] Physiological Ecology of Harmful Algal Blooms Springer, Heidelber Berlin, Germany, pp. 405-426

Bates, S.S., Bird, C.J., de Freitas, A.S.W., Foxall, R., Gilgan, M., Hanic, L.A., Johnson, G.R., McCulloch, A.W., Odense, P., Pocklington, R., Quilliam, M.A., Sim, P.G., Smith, J.C., Subba Rao, D.V., Todd, E.C.D., Walter, J.A. and Wright, J.L.C. 1989. Pennate diatom *Nitzschia pungens* as the primary source of domoic acid, a toxin in shellfish from Eastern Prince Edward Island, Canada. Can. J. Fish Aquatic Sci. 46:1203-1215.

Berdalet, E. and Estrada, M. 1993. Effects of turbulence on several dinoflagellate species. *In* Smayda, T.J. & Shimizu, Y., [Eds.] Toxic phytoplankton blooms in the sea. Elsevier Science Publishers. Pp. 737-740

Blanco, J. and Campos, M.J. 1988. The effect of water conditioned by a PSP-producing dinoflagellate on the growth of four algal species used as food for invertebrates. Aquaculture 68:289-298.

Botana, L.M., Rodruiguez-Vieytes, M., Alfonso, A. and Louzao, M.C. 1996. Phycotoxins: paralytic shellfish poisoning and diarrhetic shellfish poisoning. Food Sci. Technology 77:1147-1169.

Botana, L. M. and MacGlashan, D. W. Jr. 1993. Effect of okadaic acid on human basophil secretion. Biochem. Pharmacol. 45:2311-15.

Brussaard, C.P.D., Riegman, R., Noordeloos, A.A.M., Cadée, G.C., Witte, H., Kop, A.J., Nieuwland, G., van Duyl, F.C. and Bak, R.P.M. 1995. Effects of grazing, sedimentation and phytoplankton cell lysis on the structure of a coastal pelagic food web. Marine Ecology Progress Series 123:259-271.

BURKHOLDER, J. M., GLASGOW, H. B., and HOBBS, C. W. Fish kills linked to a toxic

ambush-predator dinoflagellate: distribution and enviromental conditions. Marine Ecology Prog. Series 124, 43-61. 95.

Burkholder, J.M., Glasgow, H.B. and Steidinger, K.A. 1995. Stage transformation in the complex life cycle of an ichtyotoxic "ambush-prdator" dinoflagellate. *In* Lassus, P., Arzul, G., Erard, E., Gentien, P., & Marcaillou, C., [Eds.] Harmful Marine Algal Blooms. Lavoisier, Intercept Ltd, pp. 567-572

Buskey, E.J. 1997. Behavioral components of feeding selectivity of the heterotrophic dinoflagellate *Protoperidinium pellucidum*. Mar. Ecol. Prog. Ser. 153:77-89.

Carlsson, P., Edling, H. and Béchemin, C. 1998. Interactions between a marine dinoflagellate (*Alexandrium catenella*) and a bacterial community utilizing riverine humic substances. Aquat. Microb. Ecol. 16:65-80.

Carreto, J.I., Benavides, H.R., Negri, R.M. and Glorioso, P.D. 1986. Toxic red-tide in the Argentine Sea. Phytoplankton distribution and survival of the toxic dinoflagellate *Gonyaulax excavata* in a frontal area. J. Plankton Res. 8:15-28.

Cembella, A.D. 1988. Electrophoretic characterization of esterase isozymes in *Protogonyaulax tamarensis* . Journal of Phycology 24 :14.

Cembella, A.D. 1998. Ecophysiology and metabolism of paralytic shellfish toxins in marine microalgae. *In* Anderson, D.M., Cembella, A.D., & Hallegraeff, G.M., [Eds.] Physiological Ecology of Harmful Algal Blooms. Springer Verlag, Berlin, pp. 381-403

Cembella, A.D., Lewis, N.I. and Quilliam, M.A. 1999. Spirolide composition of micro-extracted pooled cells isolated from natural plankton assemblages and from cultures of the dinoflagellate *Alexandrium ostenfeldii.* Natural Toxins 7:197-206.

Cembella, A.D., Lewis, N.I. and Quilliam, M.A. 2000. The marine dinoflagellate *Alexandrium ostenfeldii* (Dinophyceae) as the causative organism of spirolide shellfish toxins. Phycologia 39:67-74.

Cembella, A. D., Quilliam, M. A., Lewis, N., Bauder, A. G., and Wright, J. L. C. 1998. Identifying the planktonic origin and distribution of spirolides in coastal Nova Scotian waters. *In* Reguera, B., Blanco, J., Fernandez, M. L., and Wyatt, T. [Eds.] Harmful Algae, Xunta de Galicia and IOC/UNESCO, Vigo, pp. 481-484

Cembella, A.D., Taylor, F.J.R. and Therriault, J.C. 1988. Cladistic analysis of electrophoretic variants within the toxic dinoflagellate genus Protogonyaulax . Botanica Marina [BOT. MAR.] 31:39-51.

Cembella, A.D., Lewis, N.I. and Quilliam, M.A. 2000. The marine dinoflagellate *Alexandrium ostenfeldii* (Dinophyceae) as the causative organism of spirolide shellfish toxins. Phycologia 39:67-74.

Cho, H.J. and Matsuoka, K. 2000. Cell lysis of a phagotrophic dinoflagellate, *Polykrikos kofoidii* feeding on *Alexandrium tamarense*. Plankton Biol. Ecol. 47:134-136.

Dahl, E., Lindahl, O., Paasche, E. and Throndsen, J. 1989. The *Chrysochromulina polylepis* bloom in Scandinavian waters during spring 1988. *In* Cosper, E.M., Bricelj, V.M., & Carpenter, E.J., [Eds.] Novel Phytoplankton Blooms: causes and impacts of recurrent brown tides and other unusual blooms. Springer Verlag, Berlin, pp. 383-405

Dale, B. 1977. Cysts of the toxic red-tide dinoflagellate *Gonyaulax excavata* (Braarud) Balech from Oslofjorden, Norway. Sarsia 63:29-34.

Dale, B. and Yentsch, C.M. 1978. Red tide and paralytic shellfish poisoning. Oceanus 21:41-49.

Donaghay, P.L. and Osborn, T.R. 1997. Towards a theory of biological-physical control of harmful algal bloom dynamics and impacts. Limnol. Oceanogr. 42:1238-1296.

Donaldson, S.L., Chopin, T. and Saunders, G.W. 1998. Amplified Fragment Length Polymorphism (AFLP) as a source of genetic markers for red algae. Journal of Applied Phycology 10:365-370.

Donaldson, S.L., Chopin, T. and Saunders, G.W. 2000. An assessment of the AFLP method for investigating population structure in the red alga *Chondrus crispus* Stackhouse (Gigartinales, Florideophyceae). Journal of Applied Phycology 12:25-35.

Edvardsen, B. 1993. Toxicity of *Chrysochromulina* species (Prymnesiophyceae) to the brine shrimp, *Artemia salina*. *In* Smayda, T.J. & Shimizu, Y., [Eds.] Toxic Phytoplankton Bloom in the Sea. Elsevier Science Publisher B.V. pp 681-686

Edvardsen, B., Moy, F. and Paasche, E. 1990. Hemolytic activity in extracts of *Chrysochromulina polylepis* grown at different levels of selenite and phosphate. *In* Graneli,

E., Sundström, B., Edler, L., & Anderson, D.M., [Eds.] Toxic Marine Phytoplankton. Elsevier, New York, pp. 284-289

Edvardsen, B. and Paasche, E. 1992. Two motile stages of *Chrysochromulina polylepis* (Prymnesiophyceae): morphology, growth, and toxicity. J. Phycol. 28:104-114.

Edvardsen, B. and Paasche, E. 1998. Bloom dynamics and physiology of *Prymnesium* and *Chrysochromulina*. *In* Anderson, D.M., Cembella, A.D., & Hallegraeff, G.M., [Eds.] Physiological Ecology of Harmful Algae Blooms. Springer Verlag, Belin, Germany, pp. 193-208

Edvardsen, B. and Vaulot, D. 1996. Ploidy analysis of the two motile forms of *Chrysochromulina polylepis* (Prymnesiophyceae). J. Phycol. 32:94-102.

Eschbach, E., Reckermann, M., John, U. and Medlin, L.K. 2001a. A simple and highly efficient fixation method for *Chrysochromulina polylepis* (Prymnesiophytes) for analytical flow cytometry. Cytometry 44:126-132.

Eschbach, E., Scharsack, J.P., John, U. and Medlin, L.K. 2001b. Improved erythrocyte lysis assay in microtitre plates for sensitive detection and efficient measurement of haemolytic compounds from ichthyotoxic alga. Journal of Applied Toxicology 21:513-519.

Estep, K.W. and MacIntyre, F. 1989. Taxonomy, life cycle, distribution and dasmotrophy of *Chrysochromulina*: a theory accounting for scales, Haptonema, muciferous bodies and toxicity. Mar. Ecol. Prog. Ser. 57:11-21.

Falkowski, P. G., Barber, R. T. and Smetacek, V. 1998. Biogeochemical controls and feedbacks on ocean primary production. Science 281:200-206.

Fensome, R.A., Taylor, F.J.R., Norris, G., Sarjeant, W.A.S., Wharton, D.I. and Williams, G.L. 1993. A classification of living and fossil dinoflagellates. Micropaleontology Special Publication Number 7.

Gayoso, A.M. 2001. Observations on *Alexandrium tamarense* (Lebour) Balech and other dinoflagellate populations in Golfo Nuevo, Patagonia (Argentinia). J. Plankton Res. 23:463-468.

Gentien, P. and Arzul, G. 1990. A theoretical case of competition based on the ectrocrine production by *Gyrodinium* cf. *aureolum*. *In* Graneli, E., Sundström, B., Edler, L., &

Anderson, D.M., [Eds.] Toxic Marine Phytoplankton. Elsevier Science Publisher, New York, pp. 161-164

Geraci, J.R., Anderson, D.M., Timperi, R.J. and et al. 1989. Humpback whales (Megatera novaeangliae) fatally poisoned by dinoflagellate toxin. Canadian Journal of Fisheries and Aquatic Sciences 46:1895-1898.

Gjøsæter, J., Lekve, K., Stenseth, N.C., Leinaas, H.P., Christie, H., Dahl, E., Danielssen, D.S., Edvardsen, B., Olsgard, F., Oug, E. and Paasche, E. 2000. A long-term perspective on the *Chrysochromulina* bloom on the Norwegian Skagerrak coast 1988: a catastrophe or an innocent incident? Mar. Ecol. Pog. Ser. 207:201-218.

Graneli, E., Paasche, E. and Maestrini, S.Y. 1993. Three years after the *Chrysochromulina polylepis* bloom in Scandinavian waters in 1988: some conclusions of recent research and monitoring. *In* Smayda, T.J. & Shimizu, Y., [Eds.] Toxic Phytoplankton Bloom in the Sea . Elsevier Science Publisher B.V. pp 23-32

Green, J. C. and Leadbeater, B. S. C. 1994. The Haptophyte Algae. Published for the Systematics Association by Clarendon Press, Oxford, UK.

Hallegraeff, G.M. 1993. A review of harmful algal blooms and their apparent global increase. Phycologia 32:79-99.

Hallegraeff, G. M., Anderson, D. M., and Cembella, A. D. 1995. Manual on Harmful Marine Microalgae IOC-UNESCO, Paris, 1-24 pp.

Hallegraeff, G.M. 1998. Transport of toxic dinoflagellates via ships' ballast water: bioeconomic risk assessment and efficacy of possible ballast water management strategies. Marine Ecology Progress Series 168:297-309.

Hansen, P.J. 1989. The red tide dinoflagellate *Alexandrium tamarense*: effects on behaviour and growth of a tintinnid ciliate. Mar. Ecol. Prog. Ser. 53:105-116.

Hansen, P. J., Cembella, A. D. and Moestrup, O. 1992. The marine dinoflagellate *Alexandrium ostenfeldii*: Paralytic shellfish toxin concentration, composition, and toxicity to a tintinnid ciliate. Journal of Phycology. 28:597-603.

Hasle, G.R., Lange, C.B. and Syvertsen, E.E. 1996. A review of *Pseudo-nitzschia*, with special reference to the Skagerrak North Atlantic, and adjacent waters. Helgoländer wiss.

Meeresunters. 50:131-175.

Hasle GR 1995. Pseudo-nitzschia pungens and P. multiseries (bacillariophyceae) – nomenclatural history, morphology and distribution. Journal of Phycology 31:428-435.

Hauser, D.C.R., Levandowski, M., Hunter, S.H., Chunosoff, L. and Hollwitz, J.S. 1975. Chemosensory responses by the heterotrophic dinoflagellate, *Crypthecodinium cohnii*. Microb. Ecol. 1:246-254.

Higman, W.H., Stone, D.M. and Lewis, J.M. 2001. Sequence comparisons of toxic and non-toxic *Alexandrium tamarense* (Dinophyceae) isolates from UK waters. Phycologia 40:256-262.

Hu, T., Burton, I. W., Cembella, A. D., Curtis, J. M., Quilliam, M. A., Walter, J. A. and Wright, J. L. C. 2001. Characterization of spirolides A, C, and 13-desmethyl C, new marine toxins isolated from toxic plankton and contaminated shellfish. J. Nat. Prod. 64:308-312.

Hu, T., Curtis, J.M., Oshima, Y., Quilliam, M.A., Walter, J.A., Watson-Wright, W.M. and Wright, J.L.C. 1995. Spirolides B and D, two novel macrocycles isolated from the digestive glands of shellfish. J. Chem. Soc., Chem. Commun. 2159-2161.

Hu, T., Curtis, J.M., Walter, J.A. and Wright, J.L.C. 1996. Characterization of biologically inactive spirolides E and F: identification of the spirolide pharmacophore. Tetrahedron Lett. 37:7671-74.

Huntley, M., Sykes, P., Rohan, S. and Marin, V. 1986. Chemically-mediated rejection of dinoflagellate prey by the copepod *Calanus pacificus* and *Paracalanus parvus*: mechanism, occurence and significance. Mar. Ecol. Prog. Ser. 28:105-120.

Huss, V.A.R. and Sogin, M.L. 1990. Phylogenetic position of some Chlorella species with the Chlorococcales based upon complete small-subunit ribosomal RNA sequences. J Mol. Evol. 31:432-442.

Imai, I., Yamaguchi, M. and Watanabe, M. 1998. Ecophysiology, Life Cycle, and Bloom Dynamics of *Chattonella* in the Seto Inland Sea, Japan. *In* Anderson, D.M., Cembella, A.D., & Hallegraeff, G.M., [Eds.] Physiological Ecology of Harmful Algal Blooms Springer, Berlin Heidelberg, Germany, pp.

Inoye, I. and Kawachi, M. 1994. The Haptonema. *In* Green, J.C. & Leadbeater, B.S.C., [Eds.]

The Haptophyte Algae Clarendon Press., Oxford, U.K., pp.73-90

Jacobson, D.M. and Anderson, D.M. 1996. Widespread phagocytosis of ciliates and other protists by mixotrophic and heterotrophic thecate dinoflagellates. J. Phycol. 32:279-285.

Janssen, P., Coopman, R., Huys, G., Swings, J., Bleeker, M., Vos, P., Zabeau, M. and Kersters, K. 1996. Evaluation of the DNA fingerprinting method AFLP as a new tool in bacterial taxonomy. Microbiology 142:1881-1893.

Johansson, N. and Graneli, E. 1999. Influence of different nutrient conditions on cell density, chemical composition and toxicity of *Prymnesium parvum* (Haptophyta) in semi-continuous cultures. J. Exp. Mar. Biol. Ecol. 239:243-258.

John, E. H. and Flynn, K. J. 1999. Amino acid uptake by the toxic dinoflagellate *Alexandrium fundyense*. Marine Biology 133:11-19.

Johnsen, T.M. and Lomsland, E.R. 1990. The culmination of the *Chrysochromulina polylepis* (Manton & Parke) bloom along the western coast of Norway. *In* Graneli, E., Sundström, B., Edler, L., & Anderson, D.M., [Eds.] Toxic Marine Phytoplankton. Elsevier, New York, pp. 177-182

Karp, A., Seberg, O. and Buiatti, M. 1996. Molecular techniques in the assessment of botanical diversity. Ann. Bot. 78:143-149.

Kat, M. 1979. The occurrence of of *Prorocentrum* species and coincidental gastroinstestinal illness of mussel consumers. *In* Taylor, D. and Seliger, H. H. [Eds.] Toxic Dinoflagellate Blooms, Elsevier, Amsterdam, pp. 215-220

Kat, M. 1979. The occurrence of *Prorocentrum* species and coincidental gastrointestinal illness of mussels consumers. *In* Taylor, D.L. & Seliger, H.H., [Eds.] Toxic Dinoflagellate Blooms. Elsevier, Amsterdam, pp. 215-220

Kim, M.C., Yoshinaga, I., Imai, I., Nagasaki, K., Itakura, S., Uchida, A. and Ishida, Y. 1998. A close relationship between algicidal bacteria and termination of *Heterosigma akashiwo* (Raphidophyceae) blooms in Hiroshima Bay, Japan. Mar. Ecol. Prog. Ser. 170:25-32.

Kooistra, W.H.C.F., Olsen, J. L., Stam, W. T. and van den Hoek, C. 1993. Problems relating to species sampling in phylogenetic studies: An example of non-monophyly in Cladophoropsis and Struvea (Siphonocladales, Chlorophyta). Phycologia 32:419-428.

Lackey, J.B. Notes on plankton flagellates from the Scioto River. Lloydia 2:128-143.

Lam, C. W. Y. and Ho, K. C. 1989. Red tides in Tolo Habour, Hong Kong. *In* Okaichi, T., Anderson, D. M., and Nemoto, T. [Eds.] Red tides: Biology, Environmental Science and Toxicology. Elsevier Science Publishing, New York, pp. 49-52

Lancelot, C., Keller, M.D., Rousseau, V. and Smith, W.O.Jr. 1998. Autecology of the Marine Haptophyte *Phaeocystis* sp. *In* Anderson, D.M., Cembella, A.D., & Hallegraeff, G.M., [Eds.] Physiological Ecology of Harmful Algae Blooms. Springer, Belin, Germany, pp. 209-224

Lange, M., Guillou, L., Vaulot, D., Simon, N., Amann, R.I., Ludwig, W. and Medlin, L.K. 1996. Identification of the Class Prymnesiophyceae and the Genus *Phaeocystis* with Ribosomal RNA-targeted Nucleic Acid Probes detected by Flow Cytometry. Journal of Phycology 32:858-868.

Larsen, A. 1999. *Prymnesium parvum* and *P. patelliferum* (Haptophyta) - one species. Phycologia 38:541-543.

Lebour, M. V. 1925. The dinoflagellates of the northern seas. Mar. Biol. Ass. U.K., Plymouth

Lee, R. E. 1999. Phycology. Cambridge University Press, Cambridges, UK.

Watson, D. 1998. Natural Toxicants in Food Sheffield Academic Press, Sheffield, UK, pp.182-224

Legrand, C. and Carlsson, P. 1998. Uptake of high molecular weight dextran by the dinoflagellate *Alexandrium catenella*. Aquat. Microb. Ecol. 16:81-86.

Lindahl, O. 1983. On the development of a *Gyrodinium aureolum* occurrence on the Swedish west coast in 1982. Mar. Biol. 77:143-150.

Lipp, E. and Rose, J. 1997. The role of seafood in foodborne diseases in the United States of America. REVUE-SCIENTIFIQUE-ET-TECHNIQUE-DE-L-OFFICE-INTERNATIONAL-DES-EPIZOOTIES 16:620-640.

Litaker, W., Duke, C.S., Kenney, B.E. and Ramus, J. 1993. Short-term environmental variability and phytoplankton abundance in a shallow tidal estuary. II. Spring and fall. Mar. Ecol. Prog. Ser. 94:141-154.

Lopez-Garcia, P., Rodriguez-Valera, F., Pedros-Alio, C. and Moreira, D. 2001. Unexpected diversity of small eukaryotes in deep-sea Antarctic plankton. Nature 409:603-607.

Lush, G.J. and Hallegraeff, G.M. 1996. High toxicity of the red tide dinoflagellate *Alexandrium minutum* to the brine shrimp *Artemia salina*. *In* Yasumoto, T., Oshima, Y., & Fukuyo, Y. [Eds.] Harmful and toxic algal blooms. UNESCO. pp. 389-392

Mackenzie, L., Rhodes, L., Till, D., Chang, F. H., Kaspar, H., Haywood, A., Kapa, J., and Walker, B. 1995. A *Gymnodinium* sp. bloom and the contamination of shellfish with lipid soluble toxins in New Zealand, Jan-April 1993. *In* Lassus, P., Arzul, G., Erard, E., Gentien, P., and Marcaillou, C. [Eds.] Harmful Marine Algal Blooms, Lavoisier, Intercept Ltd., Paris, pp. 795-800

Mackenzie, L., White, D., Oshima, Y. and Kapa, J. 1996. The resting cyst and toxicity of *Alexandrium ostenfeldii* (Dinophyceae) in New Zealand. Phycologia 35:148-155.

Maclean, J.L. 1989. Indo-pacific red tides, 1985-1988. Marine Polution Bulletin 20:304-310.

Maestrini, S.Y. and Graneli, E. 1991. Environmental conditions and ecophysiological mechanisms which led to the 1988 *Chrysochromulina polylepis* bloom: an hypothesis. Oceanologica Acta 14:397-413.

Manton, I. and Parke, M. 1962. Preliminary observations on scales and their mode of origin in *Chrysochromulina polylepis* sp. nov. J. Mar. Biol. Ass. U.K. 42.

Margulis, L. and Bermudes, D. 1985. Symbiosis as a mechanism of evolution: status of cell symbiosis theory. Symbiosis 1:101-24.

Matsuoka, K., Cho, H.J. and Jacobsen, D.M. 2000. Observation of the feeding behaviour and growth rates of the heterotrophic dinoflagellate *Polykrikos kofoidii* (Polykrikaceae, Dinophyceae). Phycologia 39:82-86.

McLenachan, P.A., Stöckler, K., Winkworth, R.C., McBreen, K., Zauner, S. and Lockhart, P.J. 2000. Markers derived from amplified fragment length polymorphism gels for plant ecology and evolution studies. Molecular Ecology 9:1899-1903.

Medlin, L., Lange, M., Wellbrock, U., Donner, G., Elbrächter, M., Hummert, C. and Luckas, B. 1998. Sequence comparison links toxic European isolates of *Alexandrium tamarense* from the Orkney Islands to toxic north American stocks. Europ. J. Protistol. 34:329-335.

Medlin, L. K., Kooistra, W. H. C. F., Potter, D., Saunders, G. W. and Andersen, R. A. 1997. Phylogenetic relationships of the 'golden algae' (haptophytes, heterokont chromophytes) and their plastids. Plant Systematics & Evolution187-219.

Medlin, L.K., Kooistra, W.H.C.F., Gersonde, R. and Wellbrock, U. 1996. Evolution of the diatoms (bacillariophyta). 2. Nuclear-encoded small-subunit rRNA sequence comparisons confirm a paraphyletic origin for the centric diatoms. Molecular Biology & Evolution 13:67-75.

Mortensen, A.M. 1985. Massive fish mortalities in the Faroe Islands caused by a Gonyaulax excavata red tide. *In* Anderson, D.M., White, A.W., & Baden, D.G., [Eds.] Toxic Dinoflagellates. Elsevier, New York, pp. 165-170

Nielsen, T.G., Kiorboe, T. and Bjornsen, P.K. 1990. Effects of a *Chrysochromulina polylepis* subsurface bloom on the planktonic community. Mar. Ecol. Prog. Ser. 62:21-35.

Ogata, T., Koike, K., Nomura, S. and Kodama, M. 1996. Utilisation of organic substances for growth and toxin production by *Alexandrium tamrense*. *In* Yasumoto, T., Oshima, Y., & Fukuyo, Y., [Eds.] Harmful and Toxic Algal Blooms. Intergovernal Oceanographic Commision of UNESCO, Paris, pp. 343-346

Okaichi, T. 1989. Red tide problems in the Seto Island Sea, Japan. *In* Okaichi, T., Anderson, D. M., and Nemoto, T. [Eds.] Red tides: Biology, Environmental Science and Toxicology, Elsevier, New York, pp. 137-142

Paasche, E., Edvardsen, B. and Eikrem, W. 1990. A possible alternate stage in the life cycle of *Chrysochromulina polylepis* Manton et Parke (Prymnesiophyceae). Nova Hedwigia Beih. 100:91-99.

Pan, Y. and Cembella, A. D. 1998. Flow cytometric determination of cell cycles and growth rates of *Prorocentrum* spp. *In* Reguera, B., Blanco, J., Fernandez, M. L., and Wyatt, T. [Eds.] Harmful Algae, Xunta de Galicia and IOC/UNESCO, Vigo, pp. 173-176

Pan, Y., Cembella, A.D. and Quilliam, M.A. 1999. Cell cycle and toxin ptoduction in the benthic dinoflagellate *Prorocentrum lima*. Mar. Biol. 134:541-549.

Pan, Y., Cembella, A.D. and Quilliam, M.A. 1999. Cell cycle and toxin production in the benthic dinoflagellate Prorocentrum lima. Marine Biology 134:541-549.

Parke, M., Manton, I. and Clarke, B. 1955. Studies on marine flagellates II. Three new species of *Chrysochromulina*. J. Mar. Biol. Ass. U.K. 34:579-609.

Parkhill, J.P. and Cembella, A.D. 1999. Effects of salinity, light and inorganic nitrogen on growth and toxigenicity of the marine dinoflagellate *Alexandrium tamarense* from northeastern Canada. Journal of Plankton Research 21:939-955.

Parsons, M.L., Scholin, C.A., Miller, P.E., Doucette, G.J., Powell, C.L., Fryxell, G.A., Dortch, Q. and Soniat, T.M. 1999. *Pseudo-nitzschia* species (Bacillariophyceae) in Louisiana coastal waters: Molecular probe field trials, genetic variability, and domoic acid analyses. Journal of Phycology 35:1368-1378.

Parsons, T. R., Takahashi, M., and Hargrave, G. 1984. Biological Oceanographic Processes Pergamon Press, New York

Paulsen, O. 1904. Plankton-investigations in the waters round Iceland in 1903. *In* Meddelelser fra Kommision for Havundersoegelser Koebenhaven Serie Plankton. Pp. 1-40

Paulsen, O. 1949. Observations on Dinoflagellates. K. Danske Vidensk. Selsk. Biol. 6:1-67.

Pawlowski, J., Bolivar, I., Fahrni, J.F., Vargas, C.d., Gouy, M. and Zaninetti, L. 1997. Extreme Differences in Rates of Molecular Evolution of Foraminifera Revealed by Comparison of Ribosomal DNA sequences and the Fossil Record. Mol. Biol. Evol. 14:498-505.

Pérez, T., Albornoz, J. and Dominguez, A. 1998. An evaluation of RAPD fragment reproducibility and nature. Mol. Ecol. 7:1347-1358.

Peters, E. and Thomas, D.N. 1996. Prolonged darkness and diatom mortalityI: Marine Antartic species. J. Exp. Mar. Biol. Ecol. 207:25-41.

Pimm, S.L., Russell, G.J., Gittleman, J.L. and Brooks, T.M. 1995. The future of biodiversity. Science 269:347-350.

Raven, J.A. and Falkowsky, P.G. 1999. Oceanic sinks for atmospheric CO2. Plant, cell and Environment 22:741-755.

Riegman, R., de Boer, M. and de Senerpont Domis, L. 1996. Growth of harmful marine algae in multispecies cultures. J. Plankton Res. 18:1851-1866.

Rosenberg, R., Lindahl, O. and Blanck, H. 1988. Silent spring in the sea. Ambio 17:289-90.

Saunders, G.W., Hill, D.R.A., Sexton, J.P. and Andersen, R.A. 1997. Small-subunit ribosomal RNA sequences from selected dinoflagellates: testing classical evolutionary hypotheses with molecular systematic methods. *In* Bhattacharya, [Eds.] Origins of Algae and their Plastids Springer, Wien, pp. 115-138

Schmidt, L.E. and Hansen, P.J. 2001. Allelopathy in the prymnesiophyte Chrysochromulina polylepis: effect of cell concentration, growth phase and pH. Marine Ecology-Progress Series 216:67-81.

Scholin, C.A. 1998. Morphological, genetic, and biogeographic relatioships of the toxic dinoflagellates *Alexandrium tamarense*, *A. catenella*, and *A. fundyense*. *In* Anderson, D.M., Cembella, A.D. & Hallegraeff, G.M., [Eds.] Physiological Ecology of Harmful Algal Blooms. Springer-Verlag, Berlin, pp. 13-27

Scholin, C.A., Gullard, F., Doucette, G.J., Benson, S., Busman, M., Chavez, F.P., Cordarol, J., DeLong, R., de Vogelaere, A., Harveys, J., Haulena, M., Levebre, K., Lipscomb, T., Loscutoff, S., Lowenstine, L.J., Ill, R.M., Miller, P.E., McLellan, W.A., Moeller, P.D.R., Powell, C.L., Rowles, T., Silvagni, P., Silver, M., Spraker, T., Trainer, V. and van Dolah, F.M. 2000. Mortality of sea lions along the central California coast linked to a toxic diatom bloom. Nature 403:80-84.

Scholin, C.A., Hallegraeff, G.M. and Anderson, D.M. 1995. Molecular evolution of the *Alexandrium tamarense* species complex (Dinophyceae)-dispersal in the North American and West Pacific regions. Phycologia 34:472-485.

Scholin, C.A., Herzog, M., Sogin, M, and Anderson. D,M, 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. Journal of Phycology 30:999-1011.

Scoging, A.C. 1998. Marine biotoxins. Journal of Applied Microbiology 84:41S-50S.

Seki, S., Agresti, J.J., Gall, G.A.E., Taniguchi, N. and May, B. 1999. AFLP analysis of genetic diversity in three populations of ayu Plecoglossus altivelis. Fisheries Science 65:888-92.

Shaw, B.A., Andersen, R.J. and Harrison, P.J. 1997. Feeding deterrent and toxicity effects of apo-fucoxanthinoids and phycotoxins on a marine copepod (*Tigriopus californicus*). Mar. Biol. 128:273-280.

Shimizu, Y. 1996. Microalgal metabolites: A new perspective. Annual Review of Microbiology 50:431-465.

Shimizu, Y., Gupta, S. and Chou, H. N. 1990. Biosynthesis of red tide toxins. Mar. Toxins, ACS Symp. Ser. 418:21-8.

Shimizu, Y., Gupta, S., Masuda, K., Maranda, L., Walker, C. K. and Wang, R. 1989. Dinoflagellate and other microalgal toxins: chemistry and biochemistry. Pure Appl. Chem 61:513-16.

Shirota, A. 1989. Red tide problem and countermeasures (1). Int. J. Aquat. Fish. Technol. 1:25-38.

Simon, N., Brenner, J., Edvardsen, B. and Medlin, L.K. 1997. The identification of *Chrysochromulina* and *Prymnesium* species (Haptophyta, Prymnesiophyceae) using fluorescent or chemoluminescent oligonucleotide probes: a means for improving studies on toxic algae. Eur. J. Phycol. 32:393-401.

Simonsen, S. and Moestrup, O. 1997. Toxicity tests in eight species of *Chrysochromulina* (Haptophyta). Can. J. Bot. 75:129-136.

Simonsen, S., Moller, B.L., Larsen, J. and Ravn, H. 1995. Haemolytic activity of *Alexandrium tamarense* cells. *In* Lassus, P., Arzul, G., Gentien, P., & Marcaillou, C., [Eds.] Harmful Marine Algal Blooms. Lavoisier, Intercept Ltd. Pp. 513-517

Smayda, T.J. 1990. Novel and nuisance phytoplankton blooms in the sea: evidence for a global epidemic. *In* Graneli, E., Sundström, B., Edler, L., & Anderson, D.M., [Eds.] Toxic Marine Phytoplankton. Elsevier, New York, pp. 29-40

Smayda, T.J. 1997a. Harmful algal blooms: Their ecophysiology and general relevance to phytoplankton blooms in the sea. Limnology and Oceanography 42:1137-1153.

Smayda, T.J. 1997b. What is a bloom? A commentary. Limnol. Oceanogr. 42:1132-1136.

Smayda, T.J. 2000. Ecological features of harmful algal blooms in coastal upwelling

ecosystems. South African Journal of Marine Science/Suid-Afrikaanse Tydskrif vir Seewetenskap 22:219-253.

Sommer, U. 1994. Planktologie. Springer Verlag, Berlin

Sournia, A. 1995. Red-tide and toxic marine phytoplankton of the world ocean: An inquiry into biodiversity. *In* Harmful marine algal blooms. Lavoisier pp. 103-112

Sournia, A., Chretiennot-Dinet, M.J. and Ricard, M. 1991. Marine phytoplankton: how many species in the world ocean? J. Plankton Res. 13:1093-1099.

Spero, H.J. 1985. Chemosensory capabilities in the phagotrophic dinoflagellate *Gymnodinium fungiforme*. J. Phycol. 21:181-184.

Strom, S.L., Benner, R., Ziegler, S. and Dagg, M.J. 1997. Planktonic grazers are a potentially important source of marine dissolved organic carbon. Limnology and Oceanography 42:1364-1374.

Takezaki, N., Rzhetsky, A. and Nei, M. 1995. Phylogenetic Test of the Molecular Clock and Linearized Trees. Mol. Bol. Evol. 12:823-833.

Taroncher-Oldenburg, G., Kulis, D. M. and Anderson, D. M. 1999. Coupling of Saxitoxin Biosynthesis to the G sub(1) Phase of the Cell Cycle in the Dinoflagellate Alexandrium fundyense: Temperature and Nutrient Effects. Natural Toxins. 7:207-219.

Taroncher-Oldenburg, G., Kulis, D.M. and Anderson, D.M. 1997. Toxin variability during the cell cycle of the dinoflagellate Alexandrium fundyense. Limnol. Oceanogr. 42:1178-1188.

Taylor, F. J. R. 1984. Toxic dinoflagellates: taxonomic and biogeographic aspects with emphasis on protogonyaulax. *In* Ragelis, E. P. [Eds.] Seafood Toxins, American Chemical Society, Washington, D.C., pp. 77-97

Taylor, F.J.R. 1987. General and marine ecosystems. *In* Taylor, F.J.R., [Eds.] The Biology of Dinoflagellates Blackwell, Oxford, pp. 399-502

Taylor, F.J.R. and Fukuyo, Y. 1998. The Neorotoxigenic Dinoflagellate Genus *Alexandrium* Halim: General Introduction. *In* Anderson, D.M., Cembella, A.D., & Hallegraeff, G.M., [Eds.] Physiological Ecology of Harmful Algal Blooms. Springer, Berlin, pp. 3-12

Teegarden, G. J. 1999. Copepod grazing selection and particle discrimination on the basis of PSP toxin content. Marine-Ecology-Progress-Series 181:163-176.

Teegarden, G. J. and Cembella, A. D. 1996. Grazing of toxic dinoflagellates, *Alexandrium* spp, by adult copepods of coastal Maine: implications for the fate of paralytic shellfish toxins in marine food webs. J. Exp. Mar. Biol. Ecol. 196:145-176.

Tillmann, U. 1998. Phagotrophy of a plastidic haptophyte, *Prymnesium patelliferum*. Aquatic Microbial Ecology 14:155-160.

Tillmann, U. and Reckermann, M. submitted. Dinoflagellate grazing on the raphidophyte *Fibrocapsa japonica* Toriumi & Takano. Aquat. Microb. Ecol.

Turner, J.T. and Tester, P.A. 1997. Toxic marine phytoplankton, zooplankton grazers, and pelagic food webs. Limnology and Oceanography 42:1203-1214.

Uye, S. and Takamatsu, K. 1990. Feeding interactions between planktonic copepods and red tide flagellates from Japanese coastal waters. Mar. Ecol. Prog. Ser. 59:97-107.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Vandelee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. and Zabeau, M. 1995. AFLP – a new technique for DNA fingerprinting. Nucleic Acids Research 23:4407-4414.

Walsh, D., Reeves, R.A., Saul, D.J., Gray, R.D., MacKenzie, L., Bergquist, P.R. and Bergquist, P.L. 1998. Heterogeneity of SSU and LSU rDNA sequences of *Alexandrium* species. Biochem. Syst. Ecol. 26:495-509.

Woese, C.R. 1987. Bacterial Evolution. Microbiol. Rev. 51:221-271 .

Woese, C.R., Kandler, O. and Wheelis, M.L. 1990. Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. Proc. Natl. Acad. Sci. 87:4576-4579.

Woloszynska, J. and Conrad, W. 1939. *Pyrodinium phoneus* n. sp., agent de la toxicité des moules du canal maritime de Bruges à Zeebruge. Bull. Mus. Roy. Hist. nat. Belg. 15:15.

Work, T. M., Beale, A. M., Fritz, L., Quilliam, M. A., Silver, M., Buck, K. and Wright, J. L. C. 1993. Domoic acid intoxication of brown pelicans and cormorants in Santa Cruz, California. Dev. Mar. Biol. (Toxic Phytoplankton Blooms in the Sea) 3:643-9.

Wyatt, T. and Jenkinson, I. R. 1997. Notes on *Alexandrium* population dynamics. J. Plankton Res. 19:551-575.

Yasumoto, T., Oshima, Y., Sugahara, K., Fukuyo, Y., Oguri, H., Igarishi, T. and Fujita, N. 1980. Identification of *Dinophysis fortii* as the causative organism of diarrhetic shellfish poisoning. Bull. Jpn. Soc. Sci. Fish. 46:1405-1411.

Yasumoto, T., Underal, B., Aune, T., Hormazabal, V., Skulberg, O. M., and Oshima, Y. 1990. Sreening for hemolytic and icthyotoxic components of Chrysochromulina polylepis and Gyrodinium aureolum from Norwegian coastal waters. *In* Graneli, E., Sundstrom, B., Edler, L., and Anderson, D. M. [Eds.] Toxic Marine Phytoplankton (Proc. 4th Int. Conf. on Toxic Marine Phytoplankton), Elsevier, NY, pp. 436-40

Zingone, A. 1995. The role of viruses in the dynamics of phytoplankton blooms. Giorn. Botanico Italiano 129:415-423.

## 5. *Summary*

In order to clarify the role of PSP toxins and other substances in *Alexandrium*/protozoa interactions, sixteen strains of the dinoflagellate genus *Alexandrium* were tested for their short-term effects on the heterotrophic dinoflagellates *Oblea rotunda* and *Oxyrrhis marina*. Some *Alexandrium* strains regardless of whether they produce PSP-toxins or not, caused loss of motility and cell lysis of heterotrophic dinoflagellates. This clearly indicates that the toxic effects are not due to PSP toxins. The observed effects are caused by extracellular substances, because *Oxyrrhis* did not ingest *Alexandrium* and lytic effects were also found in cell-free culture medium. Two clones of the ichtyotoxic prymnesiophyceae *Chrysochromulina polylepis* were used in a comparative grazing experiment with *O. marina*. Both clones were similar in size and shape, but differed in their toxicity, as demonstrated by an *Artemia* bioassay. When *O. marina* was fed with the toxic clone, vacuoles within *O. marina* cells contained fewer food particles and the cells grew slower, compared to those fed the non-toxic clone. Profound differences in ingestion, clearance, cell division, and gross growth efficiency of *O. marina* were apparent when fed with the two clones of *C. polylepis*. The toxin acts as a grazer deterrent. Even at high algal concentrations, *O. marina* was not killed by the presence or ingestion of toxic *C. polylepis*. In view of the discussion that grazing is one of the major factors in regulation of bloom formation, this led to the suggestion that the allelochemicals of some *Alexandrium* species and *C. polylepis* could support bloom formation through deterrent and/ or destructive effects on grazer.

In addition to grazing experiments, lipid classes and fatty acids of both algal clones were analysed and compared to test the hypothesis that toxicity of *C. polylepis* is caused by liposaccharides, lipids, or fatty acids. However, the chemical composition with respect to lipid classes and fatty acids of both clones were similar, making it unlikely that these substances are involved in the toxicity towards *Artemia* and *O. marina*.

To study the regulation and factors which might influence toxin production of *C. polylepis*, batch cultures were synchronized. The G1 phase lasted approximately 19-22 hours, including the entire light phase, the S phase around 4 hours and G2+M phase between 1 and 2 hours. Analysis of the toxicity of *C. polylepis* was conducted using an erythrocyte lysis assay (ELA), in which fish erythrocytes were incubated with cells of *C. polylepsis.* Cellular toxin content was measured as haemolytic activity and presented (displayed) as percentage of lysed erythrocytes. The toxicity of *C. polylepis* was induced by light and increased during the first hours of the light phase (G1 phase). The effects of physiological status on spirolide production were studied in nutrient-replete batch cultures of a toxic strain of the dinoflagellate

*Alexandrium ostenfeldii*. Analysis of spirolides by liquid chromatography coupled with mass spectrometry (LC-MS) showed that the toxin profile did not vary significantly over the L/D cycle, and consisted primarily of a des-methyl-C derivative. The total spirolide concentration per unit culture volume was directly related to the concentration of cells, but there was a dramatic increase in cell quota of spirolides at the beginning of the dark phase and a corresponding decrease in the light. Hypothetically, *A. ostenfeldii* produces spirolides before cell cytokinesis, probably using storage products as energy source, so that dividing cells evenly distribute the phycotoxin to their daughter cells. *C. polylepis* seems to initiate the toxin production at the beginning of the light period. This could be to replenish the toxin cell quota of the divided cells.

The existence of toxic and non-toxic species and strains of the genus Alexandrium required a closer look at its phylogeny. The phylogenetic analysis of several strains of the *Alexandrium tamarense/catenella/fundyense* species complex on LSU rDNA sequences confirmed the separation into clades according to geographic locations and not by the three different morphotypes. In addition, a new clade representing non-toxic strains from the Mediterranean Sea was discovered. Fossil records of dinoflagellates from the Peridiniales (~190 Ma), Gonyaulacaceae (~180 Ma) and the Ceratiaceae (145 Ma) were used to calibrate a molecular clock. Based on the calculation and according to the data set analyzed, the average age of the genus *Alexandrium* is 77 Ma (around late Cretaceous and early Paleogene) with its earliest possible age at the middle of Cretaceous (119 Ma). The *A. tamarense* species complex potentially diverged around the early Neogene (23 Ma) with a possible first appearance in the late Paleogene (45 Ma). Two hypothetical scenarios were assumed which may explain the global distribution and divergence within the *A. tamarense* species complex, based on a globally distributed ancestral population.

For further genetic diversity studies, the fingerprint technique of Amplified Fragment Length Polymorphism (AFLP) was established for the species complex. The experiments show that AFLP is a powerful method to investigate the genetic diversity inside a distinct geographic region or among the cells of an algal bloom, but for the species complex as a whole it shows too much polymorphism because of the extensive genetic variability within the species.

The phylogenetic trees of the SSU and LSU rRNA sequences of *Alexandrium* ssp. were used to develop specific molecular probes for the entire species complex and three different geographic clades (North America/Orkney Islands, Western Europe, Mediterranean Sea) which can be used to identify these strains even in mixed populations and field samples by

DNA dot blot and *in situ* hybridization. These probes are a first step towards the development of an early warning system for this species, as *Alexandrium* species are difficult to discriminate accurately by conventional light microscopy. Species-specific rRNA probes based upon 18S and 28S ribosomal DNA sequences were developed and also tested for *A. ostenfeldii*. Determination of spirolides and PSP toxins by instrumental analysis on board ship during a cruise along the Scottish east coast, demonstrated the presence of both toxin groups in plankton assemblages from the Orkney Islands and confirmed the association of *A. ostenfeldii* with spirolides in northern European waters. These experiments also showed that rRNA probes could detect *A. tamarense* and *A. ostenfeldii* and discriminate these species in field studies.

# 6. Zusammenfassung

In dieser Arbeit wurden die Wirkungsweisen und Regulationsmechanismen von Toxinen der Phytoplanktonorganismen *Alexandrium* spp. und *Chrysochromulina polylepis* untersucht. Die Anwendung bestimmter molekularer Techniken ermöglichte dabei einen Einblick in die Phylogenie, Biogeographie und Populationsdynamik des *Alexandrium tamarense* Artenkomplex. Zusätzlich wurden molekulare Sonden entwickelt, welche eine Beobachtung und Identifizierung giftiger Algen im Freiland und im Labor möglich machen.

Verschiedene Arten und Stämme der Dinoflagellaten Gattung *Alexandrium* zeigten in Kurzzeitversuchen einen deutlichen negativen Einfluss auf die heterotrophen Dinoflagellaten *Oblea rotunda* und *Oxyrrhis marina.* In einigen Fällen wurden, unabhängig von der Produktion paralytischer Muschelgifte (PSP-Toxine), die betroffenen Dinoflagellaten nachhaltig in ihrer Motorik gestört und/oder es kam zur Zellysis. Hierdurch konnte eindeutig gezeigt werden, dass die toxischen Effekte von einigen *Alexandrium* Stämmen nicht mit PSP-Toxinen zusammenhängen.

In weiteren Fraß-Experimenten wurden zwei Klone (B11 & B1511) der ichtyotoxischen Prymnesiophyceae *Chrysochromulina polylepis* mit *O. marina* gehältert. Beide Klone waren in Bezug auf Größe, Gestalt sowie Kohlenstoff- und Stickstoffgehalt nahezu identisch, allerdings zeigte im *Artemien* Test nur Klon B1511 toxische Wirkungen. In Ansätzen, in denen *O. marina* mit dem giftigen Klon (B1511) gefüttert wurde, befanden sich weniger *C. polylepis* Zellen in den Futtervakuolen und das Wachstum von *O. marina* war in diesen Ansätzen geringer. Das Gift wirkt scheinbar als Abschreckung vor Predatoren, da *O. marina* auch bei sehr hohen *C. polylepis* Konzentrationen nicht getötet wurde. Da der Fraßdruck auf Phytoplankter einen der entscheidenen Faktoren zur Kontrolle von Algenblüten darstellt, kann vermutet werden, daß die allelochemischen Substanzen von *Alexandrium* und *C. polylepis* durch ihre abschreckende und/oder tödliche Wirkung auf potentielle Fraßfeinde die Blütenbildung von diesen Arten unterstützen.

Die toxischen Wirkungen von *C. polylepis* wurden in früheren Untersuchungen mit Lipiden und Fettsäuren in Verbindung gebracht. Durch Vergleiche der Lipidgruppen und der Fettsäurezusammensetzung des giftigen (B1511) und des ungiftigen Klons (B11) von *C. polylepis* konnte diese Hypothese jedoch als unwahrscheinlich verworfen werden.

Die Faktoren und Regulationsmechanismen der Toxinproduktion wurden mit Hilfe synchronisierter *C. polylepis* Kulturen untersucht: Die G1 Phase des Zellzyklus erstreckte sich über ungefähr 20 Stunden und startete mit der Lichtphase. Die anschließende S-Phase

benötigte rund 4 Stunden und die G2+M Phase dauerte 1-2 Stunden. Mittels eines Erythrocyten Lysis Assay (ELA) konnten Aussagen über die Toxizität von *C. polylepis* getroffen werden. Hierzu wurden Fischerythrocyten mit einem *C. polylepis* Extrakt inkubiert und die zelluläre Toxinquote aus der hämolytischen Aktivität ermittelt. Es zeigte sich, dass die Toxizität von *C. polylepis* durch Licht induziert wird und während der ersten Stunden der G1 bzw. Lichtphase stark zunimmt.

Die Spirolidproduktion wurde in *Alexandrium ostenfeldii* Kulturen untersucht. Das Phycotoxin konnte mittels Massenspektrometrie dedektiert werden. Das Toxinprofil der einzelnen Spirolidderivate veränderte sich über die Licht-Dunkel Zyklen nicht und des-Methyl-C Spirolid stellte die Hauptkomponenten dar. Die Spirolidkonzentration pro Kulturvolumen war direkt von der Zellkonzentration abhängig, aber die Spirolidzellquote verdoppelte sich nahezu zu Beginn der Dunkelphase. Möglicherweise produziert *A. ostenfeldii* die Spirolide aus Reservestoffen kurz vor der Zellteilung, um den Tochterzellen die nötige Spirolidmenge mitzugeben. Dagegen scheint *Chrysochromulina polylepis* die Toxinproduktion mit dem Beginn der Lichtphase zu starten. Die Tochterzellen könnten so die Toxinzellquote wieder auffüllen.

Durch die Existenz von giftigen und ungiftigen Arten und Stämmen der Gattung *Alexandrium* erschien eine genauere Studie der Phylogenie nötig. Die phylogenetischen Analysen von LSU-rDNA Sequenzen einiger *Alexandrium tamarense/catenella/fundyense* Stämme bestätigten deren geographische Aufteilung und ergaben keine Differenzierung zwischen den drei Morphotypen. Es wurde außerdem eine neue mediterrane Gruppe, bestehend aus ungiftigen Stämmen entdeckt. Mittels Fossilien der Peridiniales (~190 Mio. Jahre), der Gonyaulacaceae (~180 Mio. Jahre) und der Ceratiaceae (~145 Mio. Jahre) konnte eine "Molekulare-Uhr" kalibriert werden. Basierend auf dem analysierten Datensatz wurde die Entstehung der Gattung *Alexandrium* für die späte Kreide oder das frühe Paleogen (77 Mio. Jahre) mit einem möglichen Auftreten in der mittleren Kreide (119 Mio. Jahre) datiert. Der *Alexandrium tamarense* Artenkomplex entstand wahrscheinlich im frühen Neogen (23 Mio. Jahre) mit einer möglichen früheren Aufteilung im späten Paleogen (45 Mio. Jahre). Es wurden zwei Modelle entwickelt, um die heutige Verteilung des Artenkomplexes zu erklären.

Um die genetische Diversität von *Alexandrium* Stämmen genauer zu untersuchen, wurden *Amplifizierte Fragment Längen Polymorphien* (AFLP) als molekulare Fingerabdruck-Methode etabliert. Die Experimente zeigten, dass AFLP hochauflösend genug ist, um die genetische Diversität von begrenzten Gebieten oder einer Algenblüte zu untersuchen.

Allerdings war die Auflösung zu groß, um verschiedene Populationen miteinander zu vergleichen.

Zur Entwicklung von molekularen Sonden wurden die phylogenetischen Stammbäume der SSU und LSU rDNA Sequenzen der Gattung *Alexandrium* herangezogen. Die rRNA gerichteten Sonden waren für den gesamten Artenkomplex spezifisch sowie für einige geographischen Gruppen/Ribotypen (Nordamerika/Schottische Küste, Westeuropa, Mittelmeer). Mittels der Sonden konnten die Stämme auch in gemischten Kulturen und in Feldproben durch DNA Dot-Blot und *in situ* Hybridisierung unterschieden und identifiziert werden. Außerdem wurden artspezifische rRNA Sonden, basierend auf SSU und LSU rRNA Sequenzen für *A. ostenfeldii* entwickelt und getestet. Spirolide und PSP Toxine konnten während einer Forschungsfahrt an der schottische Ostküste an Bord gemessen und ersrmalig gemeinsam mit *A. tamarense* und *A. ostenfeldii* in Planktonproben der nördlichen Nordsee nachgewiesen werden. Mit Hilfe der rRNA Sonden konnten *A. tamarense* und *A. ostenfeldii* unterschieden und bestimmt werden.

Da *Alexandrium* Arten und Stämme durch konventionelle Lichtmikroskopie nur schwer zu identifizieren sind, stellen spezifische rRNA Sonden eine mögliche Grundlage für ein Frühwarn- und Monitoringsystem dar

# 7. *Danksagung*

Mein erster Dank gilt Linda Medlin. Sie gab mir die Gelegenheit an sehr vielseitigen Projekten mitzuarbeiten und die Möglichkeit zu promovieren. Ich danke Linda für die fördernde Betreuung, das große Vertrauen und ihre immerwährende Begeisterung. Ich hoffe ich konnte ihr während meiner Zeit in ihrem Labor einiges zurückgeben, danke.

Auch möchte ich Prof. Dr. Gunter O. Kirst und Prof. Dr. Victor Smetacek für ihre Bereitschaft meine Arbeit zu begutachten danken. Dem Fachbereich und der Sektion mit Prof. Bathmann und den weiteren Angehörigen möchte ich für den Spaß und insbesondere für die Hilfe danken. Mein besonderer Dank geht natürlich an die Arbeitsgruppe und ihren „Freundeskreis“. Es hat viel Spaß gemacht, auch wenn die Treffen im Alt-Bremerhaven immer zu früh angesetzt waren. Ich hoffe René hat keine chronischen Albträume von den diversen Korrekturen bekommen und kann immer noch gut schlafen. Ich danke auch Alberto, Bank, Gundula, Helga, Katja, Katrin, Kerstin, Klaus und Sabine. Vielen Dank für die kompetenten Tipps und die unzähligen Denkanstöße und das Schaffen eines netten Arbeitsklimas.

Allan Cembella möchte ich für die Einführung in Algenphysiologie, Toxikologie und für die reichlichen und sehr interessanten Denkanstöße danken. Auch möchte ich ihm für die gute Zusammenarbeit während meiner Aufenthalte in Kanada, die vielen Diskussionen und die herzliche Gastfreundschaft mit unvergessenen BBQs im Alten Fritz danken. Danke auch für die freundschaftliche Betreuung per Email und Telefon über einige tausend Kilometer hinweg. Bei Bente Edvardsen in Oslo haben meine ersten Versuche mit *Chrysochromulina* gestartet. Unter anderem für Ihre Gastfreundschaft und Ihre Hilfe möchte ich mich bedanken. Mit Urban Tillmann kam die Grundlage des „gemeinen Zusammenlebens“ in meine Arbeit, das Fressen und Gefressen werden, ich bedanke mich für die nette und lehrreiche Zusammenarbeit. Ich möchte Christian Hummert herzlichst danken. Er hat mir in vielen langen Diskussionen die analytische Chemie näher gebracht, mir in Jena Unterschlupf gewährt und geholfen manch eine frustrierende Phase zu überbrücken.

Für die schöne Zeit in Bremerhaven, sind so einige verantwortlich. Frank und Torsten haben diese Zeit musikalisch bereichert und sich auch für die Förderung des Sportsgeistes vorbildlich eingesetzt. Beim Thema Sport, möchte ich Thomas für viele Kilometer und super nette und hilfreichen Diskussionen danken. Die Bogenstrassen-Gang inklusive Björn, Eva, Felix, It, Marrittta, Nora und Olaf plus dem ganzen Drumherum danke ich für, sie wissen schon wofür.

Ach ja, die Wichtigsten. Mareile, Klaus, Elf, Timm, Lutz, Großmutti und Andrea ist natürlich am dollsten gedankt, für super viel Geduld und Unterstützung, geschafft! Puh, wurde auch Zeit. Der Hannover (Hang-over) Connection danke für die Nachsicht, und die immer gelungene Zerstreuung, es kommen wieder intensivere Zeiten.

Die Arbeit entstand im Rahmen eines vom Deutschen Ministerium für Bildung und Forschung (BMBF) geförderten und vom Alfred-Wegener-Institut für Polar- und Meeresforschung mitfinanziertem Projekt TEPS (Projekt Nr. 03F0161).