EVOLUTIONARY ECOLOGY OF *ALEXANDRIUM* (DINOPHYCEAE) WITH SPECIAL EMPHASIS ON GENOTYPIC AND PHENOTYPIC VARIATION IN THE TOXIGENIC SPECIES *A. TAMARENSE*

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II. SUMMARY

Harmful algal blooms (HABs) are an issue of increasing importance during the last decades with reports of new toxic phenomena and detection of novel toxins, as well as descriptions of previously unknown causative species and observations of unprecedented occurrences of HABs at new localities. In the context of high species richness in marine phytoplankton the question arises of how at times certain species are able to obtain relative dominance over microalgal communities and even can form dense, almost mono-specific algal blooms. Studies on inter- and intra-species diversity are needed to answer such questions on bloom ecology and enhance our understanding of species dispersal. This thesis addresses these issues by application of population markers that allow assessing phenotypic and genotypic characters of clonal isolates representative for natural populations of microalgae.

Dinoflagellates of the genus *Alexandrium* are auto- or mixotrophic organisms that are capable of HAB formation. In this study, chemical interactions among *Alexandrium* and other marine protists (potential grazers and competitors) were investigated to determine the potential of allelopathy to influence the bloom dynamics of *Alexandrium* populations. Allelopathy was shown to be broadly distributed among all tested species of *Alexandrium* (*A. tamarense*, *A. catenella*, *A. ostenfeldii*, *A. taylori*, *A. minutum*, and *A. lusitanicum*). Allelopathic interactions were shown to be independent of bacteria associated with the dinoflagellates. Relative differences in allelopathic effects on different target organisms (diatom, chlorophyte, cryptophyte, other heterotrophic dinoflagellates and ciliates) indicated that a spectrum of substances with different properties may be responsible for differences in target sensitivity, indicating that co-evolution of donor and target species might have occurred.

These results raised questions regarding intra-species diversity in allelochemical properties that were addressed in population-wide studies in combination with other phenotypic and genotypic population markers on a population sample of *A. tamarense* from the Scottish North Sea. Polymorphic genetic markers (DNA microsatellites and amplified fragment length polymorphisms (AFLP)) were developed for *A. tamarense* and tested for their use in species discrimination and investigations on intra-species diversity within the genus. Only AFLP proved to be suitable for inter-species genetic analyses, providing a measure of genome divergence for *A. tamarense*, *A. tamutum* and *A. ostenfeldii*. Both genetic markers, however, enabled analysis of clonal diversity in the population under study. No clonal lineage was sampled twice among more than 80 isolates, indicating high genetic diversity within the clonally reproducing dinoflagellate species. This genotypic diversity was accompanied by high phenotypic diversity as was evident from the cellular toxin content and strain-specific composition of derivatives of the neurotoxin saxitoxin, responsible for paralytic shellfish poisoning (PSP). Allelochemical activity towards the cryptophyte *Rhodomonas salina* and the dinoflagellate *Oxyrrhis marina* differed widely in the population under study but only two of the multiple strains tested were not obviously allelopathic. Detailed studies on a subset of isolates from the population sample showed that expression of allelochemical properties and the presumed associated production of allelochemically active secondary metabolites does not imply a reduction of vegetative growth rates of isolates with high allelochemical activity.

Bayesian analysis of the molecular data showed that the population from the Scottish North Sea displayed significant genetic population substructure when AFLP was used as genotypic marker, but not for microsatellites. These incongruent results were explained by the better genome coverage of AFLP with over 20 times more loci. AFLP was also more powerful in yielding stable ordination patterns of *A. tamarense* isolates by pairwise genetic dissimilarity. Both genetic marker systems, however, showed differentiation of population subgroups as identified by AFLP. The hitherto unrecognised existence of genetic population structure within a single population of *A. tamarense* partly explained significant linkage disequilibrium within the population sample obtained for both markers. Linkage disequilibrium that remained within population subgroups for AFLP was interpreted as a consequence of shifts in the frequency of clonal lineages during vegetative population growth as a signature of clonal selection.

A conceptual population genetic model for *A. tamarense* is presented in this study, able to explain all observed genetic peculiarities by integrating the life cycle characteristics of this meroplanktonic species. The model predicts that vegetative populations differentiate genetically and phenotypically under annually distinct prevailing selective forcings due to selection on clonal lineages. Consequently, sexual cysts formed in each year in planktonic populations reflect this genetic and phenotypic differentiation to the same degree. Since benthic cyst beds contain viable cysts from several consecutive years they give rise to a genetically structured population of recruits seeding the next planktonic population. Cellular PSP toxin content as a phenotypic measure of population differentiation supported the conceptual model; PSP toxin profiles and allelochemical potency did not. According to the presented model, this indicates that the latter two factors were not under strong selection in the years when cyst populations giving rise to the actual planktonic population were formed.

A comparison of populations from different regions of the globally distributed "North American" ribotype of *A. tamarense*, however, provided information on phenotypic population differentiation. PSP toxin profiles from isolates from the North West Atlantic (Bay of Fundy) were markedly different from those from three North Sea populations. Populations from the different regions were also differentiated genetically as manifested by AFLP and microsatellites. Both markers were also tested for their capacity to correctly assign individuals to the respective source populations in Bayesian analyses, for which microsatellites performed slightly better than AFLP. Given the unresolved origin of newly recognised blooms of harmful species, the methods applied herein enable a probabilistic analysis of source populations of new blooms and open up ways to distinguish between recent (perhaps human-mediated) invasion of species or natural dispersal and range extension. First results on genetic population differentiation among globally distributed populations of the *A. tamarense* North American ribotype indicate that Japanese populations are more closely related to European populations than are those from North America. A scenario of natural dispersal with a Pacific origin of the ribotype and consecutive dispersal from that region seems most probable.

The application of genotypic and phenotypic population markers in this study yielded a better understanding of population development and differentiation on ecological and evolutionary time-scales and provided fundamental insights into ecologically significant factors for bloom formation and global spreading of toxic HAB species.

III. ZUSAMMENFASSUNG

Toxische Algenblüten (engl. ‚*harmful algal blooms'*; HABs) gewinnen in den letzten Jahrzehnten zunehmend an Bedeutung durch neuartige Vergiftungserscheinungen und Giftstoffe, sowie durch die Entdeckung von bisher unbekannten Algenblüten-bildenden Arten und das Auftreten von Algenblüten in zuvor unbelasteten Gebieten. Im Zusammenhang mit hoher Artenvielfalt im marinen Phytoplankton stellt sich die Frage, wie bestimmte Arten Mikroalgengemeinschaften zeitweise dominieren und sogar monospezifische Algenblüten ausbilden können. Um Fragen in Bezug auf die Ökologie der Algenblüten und ihrer Verbreitung zu beantworten, ist sowohl inner- als auch zwischenartliche Diversitätsforschung zwingend notwendig. In der vorliegenden Arbeit werden diese Fragestellungen mit Hilfe von Populationsmarkern bearbeitet, die es ermöglichen, phänotypische und genotypische Merkmale klonaler Isolate zu erfassen und dabei deren natürliche Popualtionen zu charakterisieren.

Dinoflagellaten der Gattung *Alexandrium* sind auto- oder mixotrophe Organismen, die toxische Algenblüten ausbilden können. Ein Aspekt dieser Arbeit konzentriert sich auf die chemisch-vermittelten Interaktionen von Mitgliedern der Gattung *Alexandrium* mit anderen marinen Protisten (möglichen Fraßfeinden und Konkurrenten), um den möglichen Einfluss von Allelopathie auf die Algenblütendynamik zu erfassen. Die Untersuchungen zeigen, dass Allelopathie bei allen untersuchten Organismen der Gattung *Alexandrium* (*A. tamarense*, *A. catenella*, *A. ostenfeldii*, *A. taylori*, *A. minutum*, und *A. lusitanicum*) weit verbreitet ist. Dabei stehen allelopathische Interaktionen in keiner Abhängigkeit zu den mit den Dinoflagellaten assoziierten Bakterien. Zwischen den untersuchten Organismen (Diatomeen, Chlorophyten, Cryptophyten, heterotrophen Dinoflagellaten und Ciliaten) lassen sich relative Unterschiede in allelopathischen Effekten aufzeigen. Ein breites Substanzspektrum an Toxinen mag für diese Sensitivitätsunterschiede verantwortlich sein, was auf die Möglichkeit von ko-evolutiven Prozessen zwischen den allelochemische Substanzen produzierenden Organismen und den Zielorganismen hinweist.

Die in diesem Zusammenhang auftauchenden Fragen zur innerartlichen Diversität der allelochemischen Eigenschaften wurden anhand von Populationsstudien an der Art *A. tamarense* aus der Schottischen See in Kombination mit phäno- und genotypischen Populationsmarkern untersucht. Polymorphe genetische Marker (DNA-Mikrosatelliten und ‚*amplified fragment length polymorphisms*' (AFLP)) wurden für *A. tamarense* entwickelt und auf ihre Eignung hinsichtlich der Artunterscheidung sowie der innerartlichen Diversitätsanalyse innerhalb der Gattung *Alexandrium* untersucht. Lediglich AFLP erwies sich als für die zwischenartlichen genetischen Analysen geeignet, und bietet ein Maß der Genomdivergenz von *A. tamarense*, *A. tamutum* und *A. ostenfeldii.* Beide genetische Marker ermöglichten jedoch die Analyse der klonalen Diversität innerhalb der untersuchten Population. Keine der klonalen Linien wurde innerhalb der über 80 Isolate zweimal beprobt, was auf eine hohe genetische Diversität der sich klonal reproduzierenden Dinoflagellaten hindeutet. Diese genotypische Diversität ging mit hoher phänotypischer Diversität einher, wie aus Untersuchungen des zellulären Gehalts an Algengiften und der Zusammensetzung der für die paralytische Muschelvergiftung (engl., *paralytic shellfish poisoning*'; PSP) verantwortlichen

neurotoxischen Saxitoxin-Derivate ersichtlich wurde. Die allelochemische Aktivität gegenüber dem Cryptophyten *Rhodomonas salina* und dem Dinoflagellaten *Oxyrrhis marina* unterschied sich deutlich innerhalb der untersuchten Population, allerdings waren nur zwei der getesteten klonalen Isolate nicht offensichtlich allelopathisch. Detailliertere Untersuchungen eines Teils der Population zeigten, dass die Expression der allelochemischen Eigenschaften und die damit vermutlich einhergehende Produktion von allelochemisch aktiven Sekundärmetaboliten nicht notwendigerweise eine verringerte vegetative Wachstumsrate der Isolate mit hoher allelochemischer Aktivität zur Folge hat. Die Bayesische Analyse der molekularen Daten zeigte, dass mit Hilfe von AFLP eine signifikante genetische Unterstruktur der Population aus der Schottischen See nachzuweisen ist, während dies mit den Mikrosatelliten nicht möglich war. Diese widersprüchlichen Ergebnisse lassen sich durch die bessere Genomabdeckung (> 20-mal so viele Genorte) der AFLP-Marker erklären. AFLP erweist sich zudem als besser geeignetes Markersystem, indem es erlaubt, stabile Ordinationsmuster von *A. tamarense* Isolaten gemäß ihrer paarweisen genetischen Dissimilarität zu erhalten. Beide genetischen Markersysteme zeigten jedoch die Differenzierungen in Populationsuntergruppen auf, wie sie durch AFLP identifiziert wurden. Die bis dahin unbekannte Existenz von genetischer Populationsstruktur innerhalb einer einzelnen Population von *A. tamarense* ist geeignet, um das Kopplungsungleichgewicht, welches mit beiden Markern innerhalb der beprobten Population erkannt wurde, zu erklären. Das für AFLP in den Populationsuntergruppen verbleibende Kopplungsungleichgewicht kann als Resultat von Häufigkeitsverschiebungen klonaler Linien während des vegetativen Populationswachstums und somit als Signatur klonaler Selektion interpretiert werden.

In dieser Studie wird ein konzeptionelles genetisches Populationsmodel für *A. tamarense* vorgestellt, mit dem alle beobachteten populationsgenetischen Besonderheiten durch die Integration von Lebenzyklusmerkmalen dieser meroplanktischen Arten erklärt werden können. Dieses Modell erklärt, wie sich vegetative Populationen als Folge von jährlich unterschiedlich selektierenden Faktoren, die auf die klonalen Stränge einwirken, genetisch und phänotypisch differenzieren. Sexuell-gebildete Zysten, wie sie alljährlich in den Planktonpopulationen gebildet werden, zeigen daher in gleichem Ausmaß genetische und phänotypische Differenzierung auf. Da benthische Zystbetten lebensfähige Zysten aus mehreren aufeinanderfolgenden Jahren beinhalten, tragen sie zu einer entsprechend genetisch divers strukturierten Population von Rekruten bei, die die folgende Planktonpopulation bilden. Zellulärer PSP-Toxingehalt unterstützt als Marker für die phänotypische Populationsdifferenzierung das konzeptionelle Modell, während PSP-Toxinprofile und allelochemische Eigenschaften der klonalen Isolate dies nicht tun. Entsprechend dem vorgestellten Modell bedeutet dies, dass die beiden letztgenannten Faktoren keiner strengen Selektion in den Jahren unterlegen haben, in denen sich jene Zysten gebildet haben, aus denen sich die aktuelle Planktonpopulation rekrutiert hat.

Informationen über die phänotypische Populationsdifferenzierung wurden durch den Vergleich von Populationen des global verbreiteten nordamerikanischen Ribotyps der Art *A. tamarense* aus unterschiedlichen Regionen ermittelt. PSP-Toxinprofile von Isolaten aus dem Nordwestatlantik (Bay of Fundy) unterschieden sich deutlich von jenen aus drei Nordsee-Populationen. Die Populationen aus unterschiedlichen Regionen waren auch genetisch differenziert, wie durch Analysen von AFLP und Mikrosatelliten ersichtlich wurde. Beide Markersysteme

wurden an Hand einer Bayesischen Analyse auch auf ihre Eignung getestet, Individuen ihrer entsprechenden Ursprungspopulation korrekt zuzuordnen, wobei die Mikrosatelliten sich im Vergleich zu AFLP als besser geeignet erwiesen.

In Anbetracht des ungeklärten Ursprungs neu auftretender toxischer Algenblüten (HABs) ermöglichen die hier entwickelten und verwendeten Methoden eine probabilistische Analyse der Ursprungspopulationen neu auftretender HAB-Organismen und weisen Wege auf, zwischen nahe zurückliegender Einbürgerung (möglicherweise anthropogen beeinflusst) und natürlicher Verbreitung zu unterscheiden. Erste Ergebnisse bezüglich der Populationsdifferenzierung der global verbreiteten Populationen des nordamerikanischen Ribotypes der Art *A. tamarense* zeigen, dass die japanischen Populationen näher mit europäischen Populationen verwandt sind, als jene aus Nordamerika. Ein Szenario, das die natürliche Verbreitung des untersuchten Ribotyps von einer pazifischen Ursprungspopulation beschreibt, erscheint auf Grund der neuen Ergebnisse am wahrscheinlichsten.

Die Anwendung von geno- und phänotypischen Populationsmarkern in dieser Untersuchung führten zu einem besseren Verständnis der Populationsentwicklung und -differenzierung auf ökologischen und evolutionären Zeitskalen, und liefert grundlegende Erkenntnisse der ökologisch signifikanten Faktoren für Blüte und weltweite Verbreitung von toxischen Algenarten.

IV. ABBREVIATIONS

AFLP = amplified fragment length polymorphism
ASP = amnesic shellfish poisoning
BoF = Bay of Fundy
DA = domoic acid
DNA = deoxyribonucleic acid
DSP = diarrhetic shellfish poisoning
DTX = dinophysistoxin
EDAB(s) = ecosystem disruptive algal bloom(s)
FD = fluorescence detection
GTX(s) = gonyautoxins(s)
HAB(s) = harmful algal bloom(s)
HB = Hiroshima Bay
HPLC = high performance liquid chromatography
ITS = internal transcribed spacer
LD = linkage disequilibrium
LSU = large subunit
MED = Mediterranean
NA = North American
NEO = neosaxitoxin
NS = North Sea
NSP = neurotoxic shellfish poisoning
OA = okadaic acid
PbTX(s) = brevetoxins(s)
PCR = polymerase chain reaction
PSP = paralytic shellfish poisoning
RAPD = random amplified polymorphic DNA
rDNA = ribosomal DNA
SPXs = spirolides
SSU = small subunit
STX = saxitoxin
TA = Temperate Asian
TAS = Tasmanian
TROP = Tropical Asian
WE = Western European
YTX(s) = yessotoxins(s)

1. GENERAL INTRODUCTION

1.1. SPECIES DIVERSITY IN PHYTOPLANKTON

Primary production in the oceans contributes to almost half of world's primary production and planktonic eukaryotic microalgae are the major oceanic primary producers (Field et al. 1998). Marine microalgae, despite their ubiquitous role in biogeochemical cycles, are a heterogeneous group of organisms. From an evolutionary perspective many different clades and phylogenetic lineages are subsumed under the term phytoplankton (Simon et al. 2009). This comes along with a vast variety of life forms and ecological strategies or niche requirements (e.g., Margalef 1978; Smayda 1997) among the thousands of described and estimated tens of thousands of yet undescribed extant species of recent phytoplanktonic eukaryotes (Round et al. 1990; Sournia et al. 1991; Graham et al. 2008). During Earth's geological history the evolution of new phylogenetic lineages of phototrophic protists led to shifts in the gross composition of marine phytoplankton communities and the species that dominated them (Falkowski et al. 2004). In modern oceans those phylogenetic lineages harbouring plastids derived from ancestral red algae by secondary endosymbiosis are especially successful in dominating plankton communities in terms of species numbers and biomass (Falkowski et al. 2004). Among these, the diatoms are especially species-rich (Mann 1999), followed by the dinoflagellates and the haptophytes including the coccolithophores as the third species-richest group (Sournia et al. 1991; Falkowski et al. 2004). Here is to note that some members of the division Dinophyta obtained their plastids by a tertiary endosymbiosis by incorporating haptophyte, cryptophyte or diatom cells as symbionts (Cavalier-Smith 1999; Schnepf and Elbrächter 1999).

The – at first sight – unexpectedly high diversity of phytoplankton species in a presumably homogenous environment such as oceanic waters has been the basis for the formulation of the so-called "paradox of the plankton" (Hutchinson 1961). With only a few determining abiotic factors, competitive exclusion had been expected to preclude co-existence of many species with similar responses to or requirements for such abiotic factors. This "paradox" of species rich phytoplankton communities has been on the one hand attributed to species-specific adaptations to certain environmental conditions and on the other hand to a lack of homogenous well-mixed equilibrium conditions (see e.g., Roy 2007 and references therein). In fact, planktonic marine microalgae live in dynamic environments that are characterised by frequent and often sudden changes in ambient conditions. This is especially true for marine environments of coastal regions. Abiotic parameters, such as turbidity, light intensity, salinity and nutrient availability, can vary dramatically within sub-metre spatial scales in the water column, favouring patchy development of algal populations in space and time (Margalef 1958). Consequently, competition for light and nutrients have been proposed as major evolutionary forces (Falkowski et al. 2004; Kooistra et al. 2007). Specific adaptations to these forces include optimisation of the photosynthetic apparatus (e.g., by the

presence of specific accessory pigments) and acquisition of efficient nutrient uptake and internal recycling strategies (see Simon 2009 and references therein). Besides "bottom-up" control of phytoplankton development by intrinsic determinants of population growth such as the relation of resource (i.e., nutrients, light) availability and species-specific requirements, "top-down" factors influence the growth of phytoplankton (see Strom 2002 and references therein). Such "top down" factors for example are given by biotic interactions among microalgal competitors and/or potential grazers or pathogens of particular microalgal species (e.g., Smayda 1997). Predation or grazing pressure evokes the necessity to evolve antipredation defence mechanisms (Smetacek 2001). The stochasticity of the complex set of environmental determinants has been put forward as the best explanation for the absence of strict competitive exclusion even among species with nearly identical niche requirements contributing to the largely unpredictable nature of phytoplankton community development (Huisman and Weissing 2001; Smayda and Reynolds 2003). The complexity of phytoplankton community development may be drastically enhanced by interplay with 'hidden' components of the phytoplankton community, occurring in often overlooked low abundances (Smayda 2007). Furthermore, cryptic diversity at the species-level (i.e. the existence of species complexes that are composed of morphologically undistinguishable, but evolutionarily and genetically separated species) (e.g., van Oppen et al. 1996; Medlin et al. 2007), as well as the existence of distinguishable eco-types within species (Braarud 1951) may contribute to complex and unforeseeable patterns of phytoplankton community development. Additionally, variability in ecologically important traits at the intra-population level must be of significance for population and, consequently, community development (e.g., Brand 1988/1989; Wood and Leatham 1992; Lakeman et al. 2009).

1.1.1. Cryptic Species Diversity in Marine Microalgae

Several studies of marine microalgae, particularly of diatoms (e.g., Gallagher 1980; Medlin et al. 1991; Beszteri et al. 2007; Kooistra et al. 2008; Vanormelingen et al. 2008), but also of haptophytes (e.g., Sáez et al. 2003) and dinoflagellates (e.g., Scholin et al. 1994; Montresor et al. 2003; Gottschling et al. 2005) have revealed a high degree of variation within recognised microalgal species, both phenotypic and genotypic, even within single populations defined geographically. While most researchers recognise these findings as indication of the existence of hitherto unrecognised, reproductively isolated species, others question the validity of these findings. A lack of demonstrated biogeographic distribution patterns of (suggested) sibling species (i.e., relatively recently derived reproductively isolated species of nearly identical morphological appearance and biological characteristics) is one of the main arguments put forward against the validity of cryptic speciation. The claim that "everything is everywhere – the environment selects" is based on the assumption that all planktonic organisms below a certain body size are globally dispersed and together with enormous estimated population sizes, this would prevent cryptic speciation in unicellular microbes (Finlay and Fenchel 2004; Fenchel 2005). Uncontestedly, when compared to other groups of organisms, molecular biogeographical studies on eukaryotic protists such as marine and freshwater microalgae are still rare (see Lundholm and Moestrup 2006).

However, in view of the large body of conclusive molecular (and in cases morphological) evidence, this argument alone does not allow for rejecting hypotheses on the existence of cryptic (i.e., morphologically undistinguishable, but genetically differentiated) and in cases pseudo-cryptic (i.e., assumed cryptic species, in which existing morphological differences have not been recognised) speciation processes. On the contrary, the evidence argues for intensification of research efforts concerning the existence of biogeographies of cryptic species within species complexes.

Molecular divergence of cryptic microalgal species or members of species complexes can exceed that of recognised sibling species in higher plants (Gallagher 1980; Gallagher 1982). These species at the same time very likely have acquired specific adaptations and have evolved different ecological strategies allowing them to occupy different niches. In the "classical" example of *Skeletonema costatum*, in which issues of cryptic and pseudocryptic diversity have been studied for several decades (Kooistra et al. 2008), such adaptations cause, for example, differences in growth rates, cellular chlorophyll content and carbon uptake rates and lead to differences in timing of appearance and population growth over the annual cycle (Gallagher 1980; Gallagher 1982). In some cases cryptic diversity turned out to be 'pseudo-cryptic' in that at a later point of time species-specific morphological features that were first unrecognised could be found (e.g., Sáez et al. 2003; Cohen-Fernandez et al. 2006; Lundholm et al. 2006; Amato and Montresor 2008). In other cases, however, speciation does not go in hand with morphological differentiation (Mann 2001).

The problem of recognition of genetic or morphological varieties as independent species largely depends on the species concept underlying the particular view point of the researcher making this decision. Interpreted according to a morphological, biological or phylogenetic species concept, respectively, the appraisement of the given character information might be very different (for reviews see e.g., Logares 2006; Lundholm and Moestrup 2006). The need for better definition of current species concepts in protists with respect to questions about "cryptic species" has been emphasised long before (e.g., Taylor 1993; Medlin et al. 1995), but current controversies show that this aim is not yet achieved. As a general conclusion, one can ascertain that molecular studies have shown in many cases that phylogenies in eukaryotic protists should not solely be based on morphological taxonomic criteria, but should incorporate molecular evidence for depicting species evolution (Manhart et al. 1992; McManus and Katz 2009). Recognition of species as units of evolution is an indispensable prerequisite when questions on their autecology are addressed, which is an issue of special importance in species or species complexes that are of potential nuisance or danger due to their formation of harmful algal blooms (Taylor 1993).

1.1.2. Intra-Population Diversity

A common feature of life cycles of eukaryotic microalgae is that they comprise certain stages that reproduce asexually. Irrespective of the frequency of occurrence of sexual reproduction this has the consequence that multiple cells of the same clonal origin must be present in any growing population of these species. Accordingly, it is intriguing to question the extent to which vegetative propagation influences the population structure of microalgae and if clonal propagation may lead to populations consisting of only a few or even single clonal lineages. However, despite suspicions about

homogeneous populations of clones of vegetatively dividing microalgae, already before much experimental data shed light on these issues R.W. Doyle (1975) hypothesised that "any natural population of phytoplankton […] will consist of many cell lines [i.e., lineages of clones of the same species]".

As experimental approaches to phenotypic and genotypic diversity in natural populations of marine and freshwater microalgae became more frequently used, this early conception of population organisation in phytoplankton has been widely supported. Though not all studies conducted at the population level showed broad clonal diversity within the population under study, the absence of such was later attributed to the inadequacy of the genotypic or phenotypic marker used to characterise intra-population diversity (e.g., studies on the freshwater diatom *Asterionella formosa* using isozymes by Soudek and Robinson (1983) as cited in Thornton (2002)). In fact, in all studies, in which suitable population markers were applied, the degree of intra-population diversity proved to be extremely high (see Burkholder and Glibert (2006), Lakeman et al. (2009) and Medlin et al. (2000) and references therein). Besides genotypic diversity at presumably neutral gene loci, which could be only assumed to indirectly reflect diversification in the adaptive potential of clonal lineages of asexually dividing microalgae, population level diversity in microalgae was shown for ecologically important features. Such diversity includes the rate of vegetative reproduction (e.g., Brand 1981; Brand et al. 1981; Brand 1982; Gallagher 1982; Bomber et al. 1989; Chinain et al. 1997; Rynearson and Armbrust 2000; Loret et al. 2002), as well as many other biochemical, physiological and morphological traits (see e.g., Brand 1988/1989; Wood and Leatham 1992; Burkholder and Glibert 2006; Lakeman et al. 2009). Views on microalgal population structure were changed dramatically by such studies and in their recent review Lakeman et al. (2009) not only state as generally accepted knowledge that microalgae populations are not clonally organised, but they further pointed out that genetic variation must be expected to arise even in strictly clonal organisms due to mutations, inevitably occurring during DNA replication. Though the majority of acquired mutations may remain without traceable consequences with respect to ecologically important traits, other mutations that affect the fitness of clonally derived descendants are likely to originate and to establish within genomes.

Model predictions on the development of phenotypically diverse natural populations of vegetatively reproducing organisms assume that as an inherent characteristic the existing variability in phenotypic traits will be maintained as a function of change in environmental conditions (Lynch et al. 1991). Sexuality and accompanying meiotic recombination, even if occurring less frequently when compared to predominating asexual propagation, are expected to drastically contribute to enhanced phenotypic variation in multiple traits at a time (Lynch et al. 1991). Here is to note that in many microalgae studied with adequate thoroughness, sexual reproduction was observed and that the absence of its observation cannot be taken as an absolute indication of its nonexistence. Complete life cycles are only known for (and studied in) a small fraction of the species in some microalgal groups (e.g., Elbrächter 2003). As a consequence of variation in ecologically relevant traits, adaptation to changing environmental conditions can be expected to occur in natural populations of microalgae and to widely influence the population-wide variation in ecologically important phenotypic traits (Doyle 1975; Lynch et al. 1991; Lakeman et al. 2009). The same

seems even to happen in clonal cultures even during relatively short periods (maybe only a few hundreds of days or generations) if selective experimental conditions prevail (Lakeman et al. 2009).

If changes in ambient conditions occur, natural populations of microalgae can react by two different mechanisms to maintain net growth or to secure their persistence. Physiological acclimatisation to the new conditions by which all or the majority of individuals of the respective population express a common phenotypic plasticity is one such mechanism allowing the microalgae population to respond in a short time to the environmental change. The other way to cope with altered ambient conditions is by expression of population-wide phenotypic variation, enabling growth of those individuals that express the relatively better-adapted phenotypes for the respective environmental conditions (Doyle 1975). In the first case, strict physiological acclimation, with all individuals growing equally well under changing ambient conditions, would tend to result in more or less phenotypically uniform populations, whereas in the second case, specific adaptation of populations would lead to phenotypic differentiation of populations due to selection for different phenotypic characters. The eventual result is thus population-wide phenotypic diversity due to balancing selection, especially if changes in determining environmental variables (forcing functions) occur frequently (Rieseberg et al. 2002). The amount and patterns of intra-species phenotypic variation are therefore crucial factors determining the ecological and micro-evolutionary fate of each microalgal species and population. Underlying genetic variation, the basis for observed phenotypic variation, is assumed to reflect the ability of a population to adapt to changing environments (Fisher 1930; Barrett and Schluter 2008). For studies aiming on the description of autecological properties of a species, it is therefore essential to capture the degree of intra-specific variability (Gallagher 1982; Wood and Leatham 1992; Burkholder and Glibert 2006; Lakeman et al. 2009).

1.1.3. Inter-Population Diversity

The observation of broad intra-species diversity in eukaryotic microalgae prompted questions on the distribution of this diversity among geographically separated – and therefore presumably reproductively isolated – populations. The necessity to establish large numbers of clonal isolates from several source populations, however, hampered such studies in marine microalgae. Studies that were technically based on phenotypic or genotypic characterisation of isolates were limited to species maintained in culture at the time of the study. The difficulty to representatively sample the species of interest within its distributional range, e.g., due to problems of accessibility of particular water masses and unreliable predictions of the seasonal occurrence of a species (Medlin 2003), also strongly restricted such studies. In fact, there are very few studies on any of the different groups of marine microalgae that address biogeographic aspects and the notation of Medlin et al. (2000) that population genetics in marine microalgae was years behind the development of that field in other groups of organisms still seems to hold true. Pioneering studies in this field include those on aspects of spatial genetic differentiation in the diatoms *Ditylum brightwellii* (Rynearson and Armbrust 2004), the dinoflagellate *Gymnodinium catenatum* (Bolch et al. 1999) and the haptophyte *Emiliania huxleyi* (Iglesias-Rodríguez et al. 2006) or on temporal genetic differentiation of populations

sampled at geographically fixed locations in the diatom *Ditylum brightwellii* (Rynearson and Armbrust 2005) and the dinoflagellate *Prorocentrum micans* (Shankle et al. 2004). As a general conclusion, marine planktonic microalgae, of all phylogenetic lineages, showed population structure and differentiation to some extent, although not all species exhibited strong temporal or spatial differentiation at the scale at which they were studied (e.g., population samples of *Pseudo-nitzschia pungens* in the North Sea (Evans et al. 2005)).

Studies designed to reveal biogeographical patterns of population differentiation are still rare. Two very recent examples of population genetic studies in dinoflagellates, on *Alexandrium tamarense* (Nagai et al. 2007a) and on *Cochlodinium polykrikoides* (Nagai et al. 2009), meet the standard of those in other groups of planktonic and benthic organisms. Both dinoflagellate species were studied in Japanese and adjacent coastal waters and genetic differentiation of their populations could mostly be explained by isolation by distance, although to some extent human activities were thought to have influenced the current day distribution of both species (see paragraph 1.3.5). Whether or not standard models of population structure apply to eukaryotic microalgae, however, remains an open question.

1.2. HARMFUL ALGAL BLOOMS

1.2.1. Harmful Algal Bloom Phenomena

Mass proliferations or algal blooms have been termed “red tides”, since high densities of algal cells often cause discolouration of surface waters. If accompanied by negative consequences to broadly defined human interests, they are referred to as “harmful algal blooms” or HABs. Some HABs are of considerable importance from an ecological perspective in that they disrupt ecosystem processes and impair ecosystem function and then may be referred to as “ecosystem disruptive algal blooms” or EDABs (Sunda et al. 2006). Some HABs even lead to massive fish kills by different mechanisms such as physical clogging of fish gills, toxicity or environmental oxygen depletion (Rensel and Whyte 2003) or to mortalities in marine mammals (Scholin et al. 2000) and sea birds (Work et al. 1993) as a consequence of their toxicity to vertebrates. The accumulation of toxins produced by certain HAB species in marine organisms, especially shellfish, can severely affect human health if contaminated seafood is consumed. The toxin syndromes associated with shellfish contamination by microalgal toxins (phycotoxins) are conventionally classified according to their specific symptomatology in humans, e.g. as paralytic- (PSP), diarrhetic- (DSP), neurotoxic- (NSP), and amnesic- (ASP) shellfish poisoning (Hallegraeff 2003).

With the exceptions of the long-chain polyethers maitotoxin and ciguatoxins, with molecular weight in excess of 3,000 Da, most toxins of dinoflagellate origin are relatively small organic

molecules (typically <1,000 Da). Most dinoflagellate toxins can be structurally classified as linear or macrocyclic polyether toxins, such as pectenotoxins (PTXs), dinophysistoxins (DTXs) and okadaic acid (OA), or ladder-frame polyether toxins, such as yessotoxins (YTXs) and brevetoxins (PbTxs). Another sub-group can be defined as macrocyclic imines, such as spirolides (SPXs) and pinnatoxins (Cembella 2003). Most toxin sub-groups comprise a number of chemically similar analogues ranging from about 10 for SPXs (MacKinnon et al. 2006) to as many as 100 for YTXs (Miles et al. 2005). The tetrahydropurine saxitoxin (STX) and its derivatives, causative of PSP, are of comparatively low complexity, but also occur in approximately two dozen naturally occurring analogues.

Variation in toxin production and composition for several different classes of phycotoxins is well known among closely related dinoflagellate species and even among strains of the same species (Cembella 1998; Wright and Cembella 1998). The function of these phycotoxins in the producing organism is not known, but remains open to many speculations (Cembella 2003). Hypotheses that aim at explaining the production of different phycotoxins have been summarized by Cembella (2003) and include a putative role in grazing defence (for e.g., YTXs, PbTxs, STXs), metabolic scavenging and cellular clean-up (for e.g., PbTXs) or in disposing excess phosphosynthate (e.g., DA). Other explanations involve a function of phycotoxins as osmolytes (for e.g., DA) or pheromones (for e.g., STXs) (Cembella 2003).

Among the thousands of microalgal species around 300 are known for mass proliferations or "red tides" and around one hundred are classified as HAB species, mostly due to production of toxic or noxious substances (see official website of the Intergovernmental Oceanographic Commission of the UNESCO at: http://www.bi.ku.dk/ioc/). Species that are capable of algal bloom formation in marine ecosystems come from many different phylogenetic groups, but within the harmful species certain flagellate taxa and especially dinoflagellates are especially numerous with about 71 taxa (http://www.bi.ku.dk/ioc/). Among the toxigenic HAB species, the species-rich diatoms are currently only represented by 11 species of the genus *Pseudo-nitzschia* and one species of the genus *Nitzschia.*

Harmful algal blooms are prominent and often recurrent events in near-shore and coastal areas throughout the world from tropical to high-latitude ecosystems. Whereas massive simultaneous growth of different diatom species as a periodic feature of the annual cycle (spring bloom) forms the base of the marine food chain of most coastal and upwelling ecosystems, exceptional blooms, most notably of flagellates, can be at times almost mono-specific (Smayda 1997; Smayda 2002). The complex interactions of factors and mechanisms that lead to such bloom development are poorly understood. There is little doubt that favourable initial physico-chemical conditions, such as stable, calm weather, reduced turbulence, increased water column stratification, and sufficient nutrient input are prerequisites for most flagellate blooms (e.g., Paerl 1988). The real enigma with respect to bloom dynamics is related to factors that determine which species will bloom at a given time and place. Current conceptual and experimental approaches to explain the occurrence and persistence of particular HAB phenomena focus on species-specific physiological and ecological traits of the individual HAB taxa (Smayda and Reynolds 2001; Smayda and Reynolds 2003). As a derivation from 'Margalef's Mandala' (Margalef 1978; Margalef et al. 1979), HAB (and non-HAB) dinoflagellates that differ in their ecological niche requirements have been proposed to be

differentially adapted to an environmental gradient as provided across the range of habitats from onshore to offshore waters (Smayda and Reynolds 2001; Smayda and Reynolds 2003). In their model, Smayda and Reynolds (2001; 2003) distinguish among nine types of dinoflagellate bloom and vegetative life forms with ecological niches that are mainly defined by their nutrient availability, water turbulence and irradiance characteristics. Dinoflagellates life forms and species selection during seasonal development of phytoplankton communities has been concluded to follow a hierarchical taxonomic pathway, making the prediction of bloom taxa at a higher taxonomic level possible according to specific rules of assembly. At a lower hierarchical level, e.g., at the species level, in contrast, selection of a species might be unpredictable (Smayda and Reynolds 2003).

As a general distinction, HAB flagellates tend to have lower nutrient uptake affinities and maximum growth rates than diatoms (Banse 1982; Smayda 1997), thus they are not ideal competitors for limiting inorganic macronutrients, especially in turbulent environments. Smayda (1997) suggested that HAB flagellates have evolved four major strategies to offset the ecological disadvantages of their low nutrient affinity: (1) nutrient retrieval migrations; (2) mixotrophic tendencies; (3) allelochemically enhanced inter-species competition; and (4) allelochemical, antipredation defence mechanisms. The latter two proposed adaptations, collectively summarised as chemical defence, are mediated by the production and release of secondary metabolites. These allelochemicals are specific metabolites that stimulate or suppress growth of other organisms, or elicit other physiological responses in target cells.

1.2.2. The Role of Allelopathy in the Ecology of Bloom Forming Dinoflagellates

The term “allelopathy” was coined by Molisch (1937) to describe inhibitory and stimulatory interactions among plants that are mediated by secondary metabolites. Etymologically the term “allelopathy” is a combination of the words “allelon” (meaning “mutual” or “reciprocal”) and “pathos” (meaning “suffering” or “harm”). Allelochemicals in this context refer to the respective secondary metabolites that mediate allelopathic effects. After Molisch’s formal introduction of allelopathy as a specific aspect of ecological research, allelopathic interactions have been extensively studied in terrestrial plant ecology (Rice 1974; Rice 1984). Whereas the comparative straightforwardness of studying such interactions among neighbouring plants in terrestrial systems is evident, allelopathic interactions in phytoplankton ecology have only rather lately received attention (e.g., see Legrand et al. (2003), Gross (2003), Cembella (2003) and Tillmann et al. (2008b) for reviews).

Although different definitions for allelopathy have included both negative and positive effects of the allelochemically active species on other species, but is used herein to refer only to negative effects elicited by the producer (and releaser or donor) of the allelochemical on a recipient (or target) organism (as already suggested in Rice (1974)). There is a high diversity in nutritional modes among evolutionarily closely related groups of unicellular organisms, ranging from strict autotrophy, heterotrophy, or mixotrophy and including facultative capabilities in many cases. It is therefore difficult to restrict the term allelopathy in the planktonic realm to “microalgae-microalgae” or plant-microbe (e.g., “microalgae-micrograzer” or “microalgae-parasite”)

interactions. This becomes even more evident if one considers that biochemically similar substances or identical modes of action might be involved in the inhibition of competitors or the deterrence of grazers. Allelopathy in the context of marine protists is considered herein to include all inhibitory and negative interactions exerted by eukaryotic protists – independent of their own nutritional mode – if the respective interaction is mediated by secondary metabolites.

In the case of allelopathic interactions among phototrophic organisms the ecological advantage might be inhibition of a resource competitor (e.g., for nutrients or light). Mixotrophic microalgae potentially benefit indirectly from damage or lysis of competing microalgae, when bacterial growth is stimulated by the released algal metabolites. The absorption of algal metabolites may also supplement the mixotrophic nutrition of some allelopathic microalgae. In microalgae–micrograzer interactions, the ecological advantage for the allelopathic active microalgae can be by directly harming their grazers, thus reducing grazing rates on the allelopathically active species. Additionally, deterrence of grazers without causing severe harm can result in: first, release from grazing pressure, and second, to an enhanced grazing on other competing microalgae, leading again to better resource availability for the allelopathic species. In the latter case, the distinction between ecological strategies such as strict allelopathy (only benefit from inhibition of competitors) and chemically mediated phagotrophy may be a continuum rather than being clearly separable.

Allelopathic properties have been studied in different taxonomic groups of eukaryotic microalgae, such as diatoms, dinoflagellates, haptophytes, raphidophytes, chlorophytes (e.g., see compilations in Legrand et al. (2003) and Tillmann (2004) for references). In total, many different autotrophic, mixotrophic or heterotrophic target organisms have been selected to describe the allelopathic capacity of the allelochemical-producing organism (the “donor species”). In most experiments, allelochemical activity of the donor species was inferred by their acute or medium term effects on the target organisms (e.g., lysis or inhibition of growth).

In some cases, it was possible to trace the effect to a specific substance, the putative allelochemical, or at least to a specific mode of action. Some allelochemicals were suggested to act by specific inhibition of enzymatic activities of bacteria and other microalgae (e.g., by okadaic acid (OA) and dinophysistoxin-1 (Windust et al. 1996)). Nevertheless, such attribution to allelochemicals is controversial, since Sugg and VanDolah (1999) concluded that OA is not the main allelopathic agent produced by the benthic marine dinoflagellate *Prorocentrum lima*. Other dinoflagellate toxins have been considered as allelochemical by acting to impair membrane functions (e.g., prymnesin-1 and prymnesin-2 (Igarashi et al. 1998) or karlotoxins (Adolf et al. 2006; Deeds and Place 2006)), but the detailed mode of action in most instances remains unknown (summarised in Cembella (2003) and Legrand et al. (2003)). In most cases, the allelopathic active compounds were distinct from the known phycotoxins and their structure and mode of action still remains to be elucidated (Legrand et al. 2003).

Most studies on allelopathic interactions among planktonic eukaryotes were restricted to the description of allelopathic effects in experimental set-ups with two species systems. These studies were able to unravel important aspects concerning the nature of allelopathy in the investigated microalgae, such as species- or strain-specific differences in allelopathic effects or differential sensitivity of target organisms, and were used to develop more complex models relating to the

ecological significance of allelopathy in larger experimental systems (mesocosms) or natural communities.

An allelopathic strain of *A. tamarense* was shown to provoke drastic alterations in experimental natural plankton community following addition of the culture media of the allelopathic strain (Fistarol et al. 2004b). On the basis of such studies, the idea that allelopathy is of great ecological significance and even may play a crucial role in the development and persistence of almost mono-specific algal blooms formed by some HAB species is now widely accepted (Cembella 2003; Legrand et al. 2003; Granéli and Hansen 2006; Tillmann et al. 2008b). Nevertheless, variation in allelopathic potency among species or within natural populations of the same species, and its significance in accounting for the ecological success and evolution of species remain largely unexplored. Generally, phenotypic traits, such as expression of allelochemical properties against competitors or grazers, are expected to be under directional selection if the prevailing environmental conditions favour the growth and reproduction of those individuals that display these phenotypes. The study of variation in allelopathic potency can help to understand the balance between selective processes, such as adaptive evolution or balancing selection, which promote either the fixation of a certain phenotype for this trait or result in the maintenance of broad phenotypic variation within natural populations, respectively (see paragraph 1.1.2).

1.2.3. Global Spreading of Harmful Algal Blooms

Although there are numerous anomalies and regional exceptions, it is generally accepted that a general global expansion, and an increasing frequency and extent of harmful algal blooms (HABs), is in progress (Smayda 1990; Hallegraeff 1993). This increase in documented HABs in recent decades has led to the formulation of possible explanations for this phenomenon (Anderson 1989; Hallegraeff 1993) as well as to an intensification of research efforts to unravel the causes (Hallegraeff 2003). Monitoring efforts at sites where algal blooms have frequently occurred have intensified over the last few decades, for reasons such as the development of the aquaculture industry and exploitation of new seafood resources in regions that were formerly not surveyed for the occurrence of HAB events and associated phycotoxins (see Hallegraeff (2003) and Anderson (1989) and references therein). This *apparent* increase in HAB events is thus somewhat dependent upon increased awareness and monitoring efforts, thereby increasing the proportion of detected versus undetected bloom events and the total number of events recorded world-wide.

While this certainly is part of the explanation for the increase of recorded HABs, there is also abundant evidence of actual increases in the frequency, duration, magnitude and biogeographical distribution of these events over the last several decades. Detection of novel HAB species and their effects even at localities that have been intensively monitored (Lilly et al. 2002) or which have a long and complete anecdotal history, indicating that these areas were formerly free of such events, has led to the development of the "*Global Spreading Hypothesis*" (Wyatt 1995). More controversially, documentation of human-assisted means of introduction of HAB species to new areas, indicates that recent range extensions of some bloom-forming species may not only be due to naturally occurring dispersal, but in many cases are the consequence of human activities (Smayda

2007). Increasing intercontinental ship traffic, with the associated discharge of ship ballast water, and the translocation of infested seafood stock such as aquacultured shellfish are proposed as major vectors for human-mediated translocation of bloom-forming microalgal species, including those causative of HABs (Smayda 2007). In other cases, resident populations of harmful species may have been present at low cell concentration but remained undetected as a hidden component of the local phytoplankton community. Anthropogenically driven or natural regime changes in prevailing environmental conditions (e.g., enhanced nutrient input or elevated sea surface temperatures) may favour the development and expansion of hidden populations of these harmful species, resulting in sudden mass occurrences of HABs (for a deeper discussion on the topic of colonisation vs. hidden flora species see Smayda (2002)).

Strategies for avoidance of future introductions of HAB species and mitigation of their effects must include consideration of possible routes and means of translocation of potentially harmful species. Determining the causes and effects of *apparent* and/or *actual* spreading of HABs requires access to techniques to track the inherent diversity and biogeography of the implicated taxa at the cellular and population level and recent natural- and human-assisted dispersal must be clearly distinguished from long existing but hidden local populations of harmful species.

1.3. THE TOXIGENIC DINOFLAGELLATE *ALEXANDRIUM TAMARENSE*

1.3.1. The Genus *Alexandrium*

Alexandrium is a marine gonyaulacoid dinoflagellate genus comprising about three dozen species defined by morphological characters, of which 28 are currently considered to be of certain taxonomic status (see e.g., http://www.algaebase.org and references provided there for current opinions on synonymy of morphological defined *Alexandrium* species). Some *Alexandrium* species have been involved in rare events of massive fish kills (Mortensen 1985; Cembella et al. 2002) and marine mammal mortalities and morbidity (Durbin et al. 2002; Doucette et al. 2006) Among HAB forming dinoflagellates, the genus *Alexandrium* is one of the most significant to humans, as some *Alexandrium* species are the source of toxins associated with paralytic shellfish poisoning (PSP), a neurological affliction that has caused human illness for centuries (Prakash et al. 1971) via consumption of contaminated shellfish (see paragraph 1.3.3). In total, eleven *Alexandrium* species are reported to express toxicity due to the production of PSP toxins in seven species or spirolides (only *A. ostenfeldii*) or to cause other deleterious effects in their aquatic habitats. The distribution of many *Alexandrium* species is best characterised as "modified latitudinal cosmopolitanism" (Taylor and Pollingher 1987), as many species are present in similar climatic zones of either hemisphere, particularly along temperate and subtropical coasts (e.g., Hansen et al. 2003; John et al. 2003; Lilly

et al. 2005; Lilly et al. 2007). As a meroplanktonic dinoflagellate with haploid vegetative cells and diploid motile and non-motile zygotes *Alexandrium* depends on suitable cyst bed habitats in nearshore or shelf waters (Wyatt and Jenkinson 1997; Anderson 1998) from which the incipient blooms of vegetative cells are directly derived, but may also be subject to longshore or offshore advective transport and mixing of populations.

Life history characteristics in *Alexandrium* were first studied in *A. tamarense* (Wyatt and Jenkinson 1997; Anderson 1998), but other species seem to share most of the life cycle features. Planktonic populations of *A. tamarense* are initiated by hatching of diploid planomeiocytes from resting hypnozygotes from sea floor cyst beds in coastal marine waters at the beginning of the annual growth cycle. Meiotic divisions of diploid cells lead to the formation of haploid cells that reproduce asexually by vegetative cell division and form part of the marine phytoplankton (Wyatt and Jenkinson 1997; Anderson 1998). The formation of gametes is essential to complete the annual life cycle at the end of the planktonic population growth since durable hypnozygotes that over-winter in sea-floor cyst beds only are formed after fusion of gametes and maturation of the resulting quadriflagellate planozygote (Wyatt and Jenkinson 1997). Hypnozygotes pass through an obligatory dormancy period before they acquire the competence to hatch again. The duration of this obligatory dormancy period, the long term viability of hypnozygotes and the necessary physico-chemical conditions that trigger cyst hatching probably is genetically determined and may vary among individuals and populations. Hypnozygotes, however, remain viable for several years (Keafer et al. 1992). From this general life cycle, deviations such as the formation of temporary pellicular cysts may occur (Anderson and Wall 1978), but their adaptive significance in natural populations is unclear. Furthermore, direct transition from the planozygote to the planomeiocyte stage could possibly also occur in the plankton without prior formation of hypnozygotes, as it has been described in other *Alexandrium* species (Figueroa et al. 2006; Figueroa et al. 2007). Formation of gametes and consecutive events of sexual reproduction are restricted in time and presumed to be triggered by certain changes in environmental conditions such as depletion of nutrients (Anderson et al. 1984; Anderson and Lindquist 1985) and/or internal mechanisms (e.g., “endogenous clock”) (Anderson 1998), predominantly occurring towards the end of the planktonic bloom phase, when optimal growth conditions are no longer present. The interpretation of the benthic cyst stage as a mechanism to escape suboptimal conditions for population growth is in accordance with the postulated need of dinoflagellates for such an adaptive strategy to compensate for the low probability of encounter of the niche space required for their planktonic proliferation and persistence during the whole year (Smayda 2002).

With regard to their ecological niche requirements, members of the genus *Alexandrium* have been assigned to different life form types (Smayda and Reynolds 2001). *Alexandrium tamarense* for example has been placed in the ‘Type IV’ life form category, which contains frontal zone taxa with moderate shear stress and nutrient stress tolerance. The closely related (perhaps conspecific) taxon *Alexandrium fundyense*, however, has been categorised as a coastal current entrained dinoflagellate or ‘Type VI’ life form, with slightly greater adaptive capabilities to inaccessible nutrients and higher shear stress tolerance due to physical processes (Smayda and Reynolds 2001). In an onshore-offshore gradient both species, however, may form part of nearshore, coastal or shelf phytoplankton communities (Smayda and Reynolds 2001). The general validity of such strict categorisation has

been questioned, as for the same species populations with different adaptive strategies and niche requirements seem to exist (Anderson and Rengefors 2006).

Although *Alexandrium* spp. are often considered to be "background" taxa, in that they often are outnumbered by co-occurring phytoplankton (Anderson 1998), high-biomass blooms that even discolour the water ("red tides") do occur, including those of *A. minutum* in south Australia (Hallegraeff et al. 1988), or dense blooms (even $>1 \times 10^6 \cdot L^{-1}$) of *A. tamarense* in the Argentine Sea (Carreto et al. 1986), coastal Nova Scotia (Cembella et al. 2002) and the lower St. Lawrence estuary (Fauchot et al. 2005). Especially in the Mediterranean, dense blooms of different *Alexandrium* species are repeatedly observed (Vila et al. 2001a; Vila et al. 2001b), and references therein). In confined areas, such as harbours or embayments along the Mediterranean coast, *Alexandrium* may achieve cell concentration up to $6 \times 10^6 \cdot L^{-1}$ (Vila et al. 2001a; Vila et al. 2001b).

Mass proliferations of different *Alexandrium* species that occur at the same locations seem to be triggered by species-specific adaptations to variable environmental factors as they often occur in different seasons (e.g., Ogata et al. 1982; Bravo et al. 2008). Blooming of *Alexandrium* species is not only the consequence of achieving species-specific niche requirements for planktonic growth (Bravo et al. 2008), but might also arise due to timing differences in cyst hatching and inoculum levels of planktonic populations (Smayda and Reynolds 2001). Differences in exogenous or endogenous factors that trigger cyst hatching might be responsible for interannual variability of development of blooms of *Alexandrium* species, as the strength of recruitment from benthic cyst beds has been suggested a major factor in initiation of bloom populations (Anderson and Rengefors 2006).

Principally, *Alexandrium* shows all the ecological traits that have been suggested by Smayda (1997) as major adaptations for bloom forming flagellates (see paragraph 1.2.1). Diel downward migration of *Alexandrium* spp. are generally explained by the need for nutrient acquisition in nutrient rich waters (mostly at or below the pycnocline), whereas upward migration is needed to reach waters with irradiance levels that allow for efficient photosynthesis (Anderson and Stolzenbach 1985; MacIntyre et al. 1997; Townsend et al. 2005a) although not all strains respond equally to nutrient limitation (Poulton 2001). Since *Alexandrium* species possess chloroplasts they can rely solely on photosynthesis for energy production, but mixotrophy has been shown for several species. *Alexandrium* species were observed to feed on prey such as bacteria (Nygaard and Tobiesen 1993), other dinoflagellates (Jacobson and Anderson 1996), prymnesiophytes, cryptophytes and raphidophytes (Jeong et al. 2005), as well as on diatoms (Du Yoo et al. 2009).

With respect to the remaining two adaptations suggested by Smayda (1997), allelochemical activity towards potentially competing microalgae and towards potential heterotrophic grazers has been documented in almost all *Alexandrium* species tested. Allelochemical effects on eukaryotic protists have been mostly observed and quantified as immobilisation of target cells followed by their lysis or cyst formation (Tillmann and John 2002; Fistarol et al. 2004a). Protists shown to be sensitive to *Alexandrium* allelochemical activity include diatoms, haptophytes, cryptophytes, chlorophytes, ciliates and even other dinoflagellates, among the latter group including obligate autotrophic or heterotrophic as well as mixotrophic species (Blanco and Campos 1988; Hansen 1989; Arzul et al. 1999; Matsuoka et al. 2000; Tillmann and John 2002; Tillmann et al. 2007).

Effects on diverse planktonic protists in most cases have been assayed in two species co-incubation experiments, leaving open the question on the universality of the ecological significance of allelopathy in *Alexandrium*. Nevertheless, the potential of allelopathic *Alexandrium* to alter composition of plankton communities has been demonstrated. Experiments on an allelopathically active strain of *A. tamarense* showed that the addition of filtered culture medium of such an allelopathic strain to a natural plankton assemblage provoked drastic alterations in the experimental plankton community and especially a marked reduction of ciliate micrograzers (Fistarol et al. 2004b). However, allelopathic properties seem not to be omnipresent or effective in all *Alexandrium* populations, as even in extremely dense blooms grazing by a tintinnid ciliate contributed to bloom termination (Sorokin et al. 1996).

Regarding allelopathy in *Alexandrium*, neither the mode of action in the diverse target organisms nor a specific self-protection mechanism is known. Furthermore, neither the chemical nature and biosynthetic pathway of the allelopathic compounds nor their potential cellular localisation, storage and excretion mechanisms have yet been elucidated. Membrane targeting biological activity of *Alexandrium* allelochemicals, however, has been assumed due to the observed effects on target organisms (Hansen 1989). A complex of chemicals rather than one specific substance has been suggested to account for allelopathic effects in *Alexandrium* (Arzul et al. 1999).

1.3.2. The '*Alexandrium tamarense* Species Complex'

Balech (1985) re-described the genus *Alexandrium* Halim 1960 and transferred a group of gonyaulacoid dinoflagellates with previous genus designations *Protogonyaulax* and *Gonyaulax* into this genus. His decision was based on morphological characters, such as absence of horns and spines or well developed lists, as well as a typical tabulation of thecal plates after Kofoidian notation. In his view, the "tamarensis group" is clearly differentiated from other dinoflagellates of the genus *Gonyaulax* (Balech 1985). Assignment of species to the genus *Alexandrium sensu* Balech was contentious, which is best illustrated by the vast number of genus designations, including *Goniodoma*, *Gonyaulax*, *Gessnerium*, *Protogonyaulax*, *Triadinium*, *Heteraulacus* and *Pyrodinium*, proposed for some or all members in the past (Balech 1995).

Prominent species of the genus *Alexandrium* known to cause paralytic shellfish poisoning events in many regions of the world are *A. tamarense* (Lebour) Balech, *A. catenella* (Whedon et Kofoid) Balech and *A. fundyense* Balech. The morphological criteria to distinguish among these three species are that the two 'tamarensoid' forms tend to occur as unicells or short chains of up to 4 cells. The tamarensoid cells are typically longer than wide and bear an ovoid or subrectangular apical pore complex (APC). A ventral pore on the first apical plate (1') may be present (*A. tamarense*) or absent (*A. fundyense*) (Balech 1995). The only 'catenelloid' species, *A. catenella*, usually forms chains of more than eight cells. The cells are antero-posteriorly compressed and have a complex "lamb-chop" shaped apical pore. They have no pore on 1' plate (Balech 1995). Whereas morphological criteria to discriminate the three morphospecies are clearly defined, their value as a taxonomic criterion has been questioned (Cembella et al. 1987; Cembella et al. 1988a). On one hand, they display some morphological plasticity: e.g., absence of a ventral pore in *A. catenella*

(Kim et al. 2002) is not necessarily stable in clonal isolates, 'catenelloid' cells show frequently a tendency to cease chain formation and to become rather isodiametrical (Cembella et al. 1987), and intermediate forms of the morphospecies *A. tamarense* and *A. catenella* are frequently observed in boundary zones of the distribution areas as e.g., in British Columbian waters (Taylor 1984; Cembella and Taylor 1985; Cembella and Taylor 1986). On the other hand, authors such as Leaw et al. (2005) argue that some morphological criteria on which taxonomic decisions have been based in the genus *Alexandrium* (e.g., possession of a ventral pore or the position of the connecting pore in the apical pore complex) are homoplastic (i.e., appeared independently in separate evolutionary lineages) characters. In another group of closely related *Alexandrium* species, the '*Alexandrium minutum* group', where it distinguishes between the morphospecies *A. minutum* and *A. angustitabulatum*, the possession of a ventral pore proved to be a rather variable character and its value for taxonomic decisions in the genus was therefore questioned (Hansen et al. 2003). A determination of this character by multiple genes seems most likely (Anderson et al. 1994) , but the genetic basis for expression of this trait thus should be the same in all members of the genus. In this case, only differences in homologous genes determine if a pore is formed or not.

In addition to morphological criteria, phenotypic and genetic characters could give support for the validity of three morphological defined species (*A. tamarense*, *A. catenella* and *A. fundyense*), but no such characters could be found in any of the detailed studies on mating compatibility (Sako et al. 1990; Anderson et al. 1994), toxin composition (Cembella et al. 1987; Anderson et al. 1994), surface immunogenicity (Adachi et al. 1993; Sako et al. 1993), bioluminescence (Schmidt and Loeblich III 1979a; Anderson et al. 1994), isozymes (Cembella and Taylor 1985; Cembella and Taylor 1986; Cembella et al. 1988a; Hayhome et al. 1989), and nuclear ribosomal genes (Scholin et al. 1994; Scholin and Anderson 1994; Adachi et al. 1996). On the contrary, genetic characters support the existence of several phylogenetic lineages that contain one or more morphotypes (Scholin et al. 1994). Since morphotype and ribotype do not form the same groups, the hypothesis that ribotypes rather than morphotypes represent true "biological species" was formulated (Scholin et al. 1994). The morphospecies *A. tamarense*, *A. fundyense* and *A. catenella*, therefore, form part of a phylogenetically unresolved group, the '*A. tamarense*/*catenella*/*fundyense* species cluster' (Scholin et al. 1994) or for short the '*A. tamarense* species complex' (John et al. 2003).

Five phylogenetic lineages were previously defined according to their LSU rDNA sequences or ribotypes (John et al. 2003). After the regions, from which samples of these ribotypes were first obtained, they were termed North American (NA), Western European (WE), Temperate Asian (TA), Tasmanian (TAS) (Scholin et al. 1994) and Mediterranean ribotype (MED) (John et al. 2003). One additional ribotype, the Tropical Asian (TAS) ribotype, recognised by Scholin et al. (1994) was later observed to stand outside the species complex by John et al. (2003) and now is seen as belonging to the closely related species *A. tropicale* Balech 1985 (Lilly et al. 2007).

In addition to their genetic differentiation, ribotypes differ consistently by the presence or absence of neurotoxins associated with paralytic shellfish poisoning (PSP): The North American and Temperate Asian ribotypes are exclusively toxic, whereas the Western European, Tasmanian and Mediterranean ribotypes are exclusively nontoxic (Scholin et al. 1994; John et al. 2003; Lilly et al. 2007). Paralytic shellfish poisoning toxin production in the *A. tamarense* species complex and other species complexes (e.g., the '*A. minutum* species complex') or clades of the genus

Alexandrium most likely has evolved from a common predisposition and the loss or the reacquisition of this trait might have occurred repeatedly in the evolutionary history of species. However, PSP toxin production does not serve especially well as a phylogenetic character as it is observed also in another, rather distantly related naked dinoflagellate of the genus *Gymnodinium* (Mee et al. 1986; Oshima et al. 1991) and even is known from non-dinoflagellate organisms such as cyanobacteria (Jackim and Gentile 1968).

Due to the fact that the distribution ranges of ribotypes of the *A. tamarense* species complex extend beyond their eponymous geographical areas, in a recent paper Lilly et al. (2007) proposed a group numbering scheme. This step, however, will not help to settle the discussion on the validity of either morphological characters or ribotypes as base for assignment of species status. Most likely, this discussion will only be laid down as better arguments are found to support either view (e.g., from studies on mating compatibility or incompatibility of regional populations from different ribotypes and or morphospecies). Though not carried out for all combinations of ribotypes, first results from such studies indicate that indeed ribotypes not morphotypes represent species according to a biological species concept (Brosnahan et al. in press).

Molecular studies on the relationship of the genera *Alexandrium* and *Pyrodinium* Plate 1906, based upon large subunit rDNA sequences, place *Pyrodinium* within the genus *Alexandrium* (Leaw et al. 2005), clearly at odds with evidence from gross morphology, ecophysiology and cyst characteristics. Acknowledging this demonstration of polyphyly of *Alexandrium* would urge for a major revision of the taxonomy of the genus as *Pyrodinium* is the older genus name. Such a step, however, can only be taken, if consistent molecular support by other phylogenetic markers can be obtained. Taxonomic decisions on the sole basis of molecular characters, however, are rare in dinoflagellate systematics and it is unlikely that such steps will be performed without additional support by structural characteristics as may be obtained from studying the ultrastructural details of the respective taxa. The assignment of the genus name *Pyrodinium* to the members of the *A. tamarense* species complex, however, would be a step back in the history of taxonomic decisions on members of the genus, since long ago Taylor (1976) placed the closely related species, which we now know as *A. minutum* in the genus *Pyrodinium*. Shortly thereafter however, he reconsidered this step, based on cyst morphology (spiny in *Pyrodinium* and smooth in *A. monilatum*, another closely related species), and suggested the acceptance of *A. minutum* as the valid species name (Taylor 1979).

1.3.3. Paralytic Shellfish Poisoning Toxins in *Alexandrium* and the *A. tamarense* Species Complex

Investigations on the cause of toxicity in shellfish established a link between the occurrence of *A. catenella* (referred to as *Gonyaulax catenella* in the respective publications) in the water column and shellfish toxicity at the Pacific coast (Whedon and Kofoid 1936; Sommer and Meyer 1937; Riegel et al. 1949) and for *A. tamarense* (referred to as *Gonyaulax tamarensis* in the respective publications) at the Atlantic coast of North America (Needler 1949; Prakash 1963; Prakash et al.

1971). From cultures of *A. catenella* eventually a single toxic substance was characterised (Schantz et al. 1966), then named saxitoxin (STX) after the Alaska butter clam *Saxidomus giganteus* from which the toxin had previously been isolated (Mold et al. 1957; Schantz et al. 1957)). Subsequently, saxitoxin and analogues were also shown to be the cause of toxicity caused by *A. tamarense* (Schantz et al. 1975a; Shimizu et al. 1975a; Shimizu et al. 1975b).

The specific mode of action of saxitoxin by effectively blocking voltage gated sodium channels of nervous cells was discovered shortly after its characterisation (Hille 1968; Narahashi and Moore 1968). The cellular mode of action helps to explain the paralysis of the neuromuscular system observed in humans after consumption of contaminated shellfish, which include a tickling sensation of lips, numbness of extremities, gastrointestinal problems and difficulty in breathing as summarised in Shimizu (2000).

Additional fractions containing neurotoxins other than STX were later separated from *A. tamarense* cell extracts (Shimizu et al. 1975a; Shimizu et al. 1975b). Structural elucidation showed that all these molecules are derivatives of STX with the same tetrahydropurine structure and modifications occurring in three positions (Schantz et al. 1975b). In *Alexandrium*, these modifications of the saxitoxin molecule and their combinations allow for formation of various derivatives: *N*-1-hydoxyl-substitution in saxitoxin e.g., leads to neosaxitoxin (NEO) (Shimizu et al. 1978)), 11-hydoxysulfate-substitution to the α- and β-epimers gonyautoxins II and III (or GTX2 and GTX3), respectively (Boyer et al. 1978), and 21-*N*-sulfo-substitution to toxin B1 (Hall et al. 1980; Wichmann et al. 1981). The derivatives of NEO with 11-hydoxysulfate-substitution are the α- and β-epimers GTX1 and GTX4, respectively, and the *N*-sulfocarbamoyl derivatives of NEO, GTX2, GTX3, GTX1 and GTX4 are called B2, C1, C2, C3 and C4 (as summarised in Shimizu (2000)). Specific enzymatic activities required to perform some of these biotransformations have been detected and characterised in proteinaceous fractions from e.g., *Alexandrium catenella* and *Gymnodinium catenatum* (Ishida et al. 1998). Decarbamoylisation of saxitoxin and its derivatives leads to further derivatives (dc-STX, dc-NEO, dc-GTX1-4), but are usually not found in *Alexandrium* cell extracts and are thought to rather represent products of biotransformation of toxins occurring in e.g., shellfish, from which they first have been isolated (Ghazarossian et al. 1976).

Investigations on the biological activity of STX and its derivatives showed that the carbamate toxins are generally much more toxic than those of the low potency *N*-sulfocarbamoyl group (Genenah and Shimizu 1981; Sullivan et al. 1985; Oshima 1995b).

It was shown that different isolates of toxic *Alexandrium* species produce different relative amounts of the naturally occurring saxitoxin derivatives and that the relative composition of PSP toxins is a quite stable phenotypic trait and changes significantly only under rather extreme differences in growth conditions in batch and semi-continuous cultures (Hall 1982; Boyer et al. 1987; Cembella et al. 1987; Ogata et al. 1987a; Boczar et al. 1988; Anderson et al. 1990a). The general ability, however, to produce a certain suite of toxins seems to be genetically fixed for each clonal strain (see e.g., Anderson 1990 and Cembella (1998) and references therein). Further, toxin profiles exhibit a biparental inheritance that is consistent with Mendelian segregation, which implies that the expression of a specific toxin profile is regulated by nuclear genes (Sako et al. 1992; Sako et al. 1995). Experiments with antibiotic treated and non-treated control cultures

indicate that expression of toxin profiles and cellular toxin content to some extent are influenced by the presence of bacteria (or the related treatment) (Hold et al. 2001). Further investigations on the biotransformatory capacity of bacteria associated with toxic (and non-toxic) strains of *A. tamarense* and other dinoflagellates showed that some of the bacteria were able to transform certain STX derivatives by oxidase activity and other unknown biotransformation processes (Smith et al. 2002). Such bacterial biotransformatory activity, though not related to the initial production of PSP toxins, might be the explanation for observed effects of antibacterial treatments on the toxin profiles of PSP toxin producing dinoflagellates (Doucette et al. 1998).

Cellular toxin content, however, is a less stable phenotypic character of a clonal isolate than its toxin profile. Average toxin content of *Alexandrium* isolates has been found to vary considerably in different phases of growth in batch cultures. Typically, an increase of toxin content is observed during early exponential phase, whereas a decrease occurs when cultures shift from late exponential phase to the stationary phase (Boczar et al. 1988). The cellular toxin content during stationary phase remains more or less stable with about half of that during its peak in early exponential phase (Boczar et al. 1988).

When first evidence was given that other PSP toxins than saxitoxin were produced by *A. tamarense* (Shimizu et al. 1975a; Shimizu et al. 1975b) and *A. catenella* (Shimizu et al. 1975b; Shimizu et al. 1977; Hall et al. 1979) (Shimizu 1979; Noguchi et al. 1983) the idea arose to use the specific expression of certain toxin derivatives chemotaxonomically for the purposes of differentiation of the different morphotypes. Various attempts in this direction have been made to interpret toxin composition chemotaxonomically (Alam et al. 1979; Shimizu 1979; Hall 1982; Oshima et al. 1982a; Cembella and Taylor 1985; Boyer et al. 1986), but early studies were restricted by the sensitivity of the detection method for PSP toxins or they suffered from low sample sizes for the different morphospecies from a geographic region. With the advent of HPLC, more reliable analyses of the content of certain PSP toxins in *Alexandrium* isolates were possible. Important for the interpretation of PSP composition data is to acknowledge that only some transitions from one derivative to the other seem to be enzymatically mediated (and therefore represent genetic differences in those isolates that vary in relative amounts for certain toxins), whereas others such as transitions between alpha and beta epimers occur spontaneously (Hall 1982; Oshima et al. 1991).

Early studies that focused on the diversity of cellular toxicity or toxin profiles within local populations of a certain *Alexandrium* species were carried out with populations of *Alexandrium* from the North-West (Hall 1982; Cembella and Taylor 1986; Cembella et al. 1987) and the North-East coast (Maranda et al. 1985; Hayhome et al. 1989) of North America. Some of these studies were carried out in conjunction with analyses of isozymes in the same isolates (see paragraph 1.3.2). By using several clonal isolates from specific sampling locations and times, these studies already showed that considerable inter-population variation exists not only between the different locations studied, but also intra-population variation, i.e. among isolates coming from one geographically defined population. Differences in toxicity were not only attributable due to differing amount of cellular toxin content, but also due to the relative composition of saxitoxin derivatives and a relatively higher content of highly potent carbamate toxins (Anderson et al. 1994). Multivariate statistical techniques applied to toxin composition data from regionally separated

populations showed that in some cases regional populations of *Alexandrium* can clearly be distinguished from others by toxin profiles (Cembella et al. 1987; Anderson et al. 1994; Cembella and Destombe 1996).

As an example might serve the comparison of PSP toxin composition of field samples of planktonic *Alexandrium tamarense* populations from different sampling sites in the St. Lawrence estuary, eastern Nova Scotia and the Bay of Fundy (Cembella and Destombe 1996). Populations from the Bay of Fundy display a considerable homogeneity in the relative amounts of PSP toxins, whereas the other populations are characterised by larger differences in toxin composition. Using toxin profiles as a chemotaxonomic character at the population level, this implies that the St. Lawrence populations on one hand are well mixed and presumably are seeded from the same cyst beds at the northern shore of the estuary. On the other hand they are distinct from the other eastern Canadian population, which indicates the existence of a geographic separation that leads to reproductive isolation of populations (Cembella and Destombe 1996).

Two possible explanations for the development of inter-population differences in PSP toxin composition among *Alexandrium tamarense/fundyense* (Group I / North American ribotype) populations at the North American Atlantic coast were formulated in Anderson et al. (1994): One explanation is that environmental factors favour the selection of certain phenotypically differentiated individuals originating from a common cyst bed. Such locally differing selection during development of vegetative growing populations could lead to the establishment of phenotypically differentiated bloom populations after dispersal to different regions. Alternatively, dispersal of *Alexandrium* populations from different centres of origin was taken into consideration to explain inter-population differences in the relative composition of PSP toxins. While the first mechanism is based on the idea of short term differentiation of planktonic populations, the second implies long-term processes, which might be enhanced by e.g., prevailing current patterns.

Though PSP toxin production in *Alexandrium* spp. has been studied quite intensively with respect to its physiology, the ecological and evolutionary significance of PSP toxins to the producing dinoflagellate is not well understood (Cembella 2003). Hitherto, attention was focused on several hypotheses, one of them being that PSP toxins function as predator/grazer defence compounds, e.g., against major grazers such as copepods and tintinnids. However, the equivocal and often contradictory results obtained in grazing studies with various copepod species on *Alexandrium* strains of differing PSP toxicity (reviewed by e.g., Turner et al. 1998) have cast doubt upon this hypothesis as a simple generalisation (Cembella 2003). Also, the lytic activity of *Alexandrium* spp. strains towards other planktonic protists (microalgae and heterotrophs) is unrelated to the PSP toxin production ruling out a function in allelopathic interactions (Tillmann and John 2002). Further functional roles of saxitoxin and its derivatives that have been speculated about are functions in chromosome organisation (Anderson and Cheng 1988), cellular homoeostasis (Wyatt 1990), or as pheromones (Wyatt and Jenkinson 1997). Since up to now, no conclusive physiological or ecological function for PSP toxins in any of the producing organisms has been found, the biological meaning of its production remains obscure.

1.3.4. Distribution and Range Extension of the *A. tamarense* Species Complex

Among the three now recognised morphospecies, *A. tamarense* is the most widely distributed (see Table 1.1). Besides its wide distribution in European waters, it is found along the Atlantic coast of the North American continent. In the Northern Pacific, this species is also reported from the Eastern and the Western coasts, spanning from Japan via the Aleutian chain to Alaska and then southward of British Columbia. Furthermore, the morphospecies occurs in Atlantic waters of South America and is found in the southwestern Pacific. More recent records include South Africa and the Antarctic, and sub-antarctic islands. All available information support the view that *A. tamarense* (as a morphospecies) is widely cosmopolitan and virtually globally distributed.

In contrast, *A. fundyense* is much more limited in distribution and is almost exclusively found along the Atlantic coast of North America (Balech 1985; Anderson et al. 1994). While massive blooms of this morphospecies seem to be restricted to that region, there is just one additional reported occurrence each from the west coast of North America (Scholin 1998) and from Australia (Lilly et al. 2007). However, there are no reports on the morphospecies in the Pacific causing massive blooms comparable to those in the Gulf of Maine region.

The distribution range of *A. catenella* includes the Eastern and Western North Pacific as well as temperate regions of the Southern Pacific and its transition to the Southern Indian Ocean. Furthermore, this morphospecies has been reported from the Southwest Pacific and its transition to the Southwest Atlantic and the Atlantic coast of South Africa, as well as from the Mediterranean. With the observed range extension of morphospecies of the *A. tamarense* species complex an increasing frequency of bloom events has been observed during the last decades (Anderson 1989; Hallegraeff 1993; Hallegraeff 2003). Due to the recognised difficulties with a morphological species concept in the *A. tamarense* species complex it seems more plausible to investigate the distribution patterns and their origins under acknowledgement of ribotypes as biological entities of species evolution rather than morphotypes.

Scholin et al. (1995) already suggested that disjunct populations of the *A. tamarense* species complex have arisen as a result of dispersal and subsequent vicariance. On base of the estimation that the species complex existed for at least tens of millions of years in many parts of the world these authors speculated that changes in sea level, climate, continental drift and other factors must have contributed to the formation of reproductively isolated endemic populations. Such disjunct populations then evolved to the ribotypes present today. Despite this plausible theory of evolution of the ribotypes, a complex distribution pattern for the majority of ribotypes is observed. With the exception of the Tasmanian and the Mediterranean ribotype, which are still only observed in their name giving region, all other ribotypes are dispersed to several continents (Lilly et al. 2007): The Western European ribotype for example is known from the Atlantic coasts of Western Europe, including the Baltic proper, from the Mediterranean and from East Asia. The Temperate Asian ribotype is known from the North West Pacific and Australia as well as from the Mediterranean (Lilly et al. 2007). With respect to the most widely distributed toxic ribotype, the North American (NA) ribotype, Scholin et al. (Scholin et al. 1995) suggest that populations from the North American East and West coast are descendants from a common population with a distribution range spanning all North American coasts at geological times when these coasts were of temperate

Table 1.1. Reported occurrences of morphospecies of the *Alexandrium tamarense* species complex.

Species	Oceanic region	Country or region (Reference)
A. tamarense	Eastern North Atlantic	British Isles (Lebour 1925; Parke and Dixon 1976; Medlin et al. 1998), Scandinavia (Braarud 1945; Moestrup and Hansen 1988; Persson et al. 2000), Russian Barents Sea (Okolodkov 2005), Mediterranean (John et al. 2003)
	Western North Atlantic	Bay of Fundy (Needler 1949), St. Lawrence Estuary (Prakash et al. 1971; Cembella et al. 1988b), U.S. East coast (Anderson and Morel 1979; Anderson et al. 1994)
	Eastern North Pacific	British Colombia (Taylor 1975), Alaska (Hall 1982)
	Western North Pacific	Japan (Ogata et al. 1982; Oshima et al. 1982b), South Korea (Han et al. 1992), Russia (Orlova et al. 2007)
	Atlantic South America	Venezuela (Reyes-Vásquez et al. 1979) Brazil (Persich et al. 2006) Argentina (Carreto et al. 1986)
	Southwestern Pacific	Australia (Leaw et al. 2005), New Zealand (MacKenzie et al. 2004), Tasmania (Hansen et al. 2003)
	Western South Atlantic	South Africa (Ruiz Sebastián et al. 2005)
	Antarctic waters	Antarctic and the subantarctic islands (McMinn and Scott 2005)
A. fundyense	Western North Atlantic	US coast and Canada (Balech 1985; Anderson et al. 1994)
	Eastern North Pacific	California (Whedon and Kofoid 1936; Scholin 1998)
	Transition between South Pacific and southern Indian Ocean	Australia (Lilly et al. 2007)
A. catenella	Eastern North Pacific	California (Whedon and Kofoid 1936), British Columbia (Prakash and Taylor 1966)
	Western North Pacific	Japan (Toriumi and Takano 1979), Korea (Lee et al. 1993), China (Qi and Qian 1994)
	Southern Pacific and transition to southern Indian Ocean	New Zealand (Hansen et al. 2003; MacKenzie et al. 2004), Australia (Hallegraeff et al. 1988)
	Western South Pacific and transition to western South Atlantic	Chile (Lagos 1998), Argentina (Benavides et al. 1995)
	Mediterranean	Spain (Margalef and Estrada 1987), France (Lilly et al. 2002)
	Eastern South Atlantic	South Africa (Ruiz Sebastián et al. 2005)

climate (i.e., about three million years ago during the Pliocene). Scholin et al. (1995) reasoned that independent evolution of the regional East and West coast populations was only possible after restriction of gene flow between them after climate cooling, restricting the passage of Arctic waters. In their study, Medlin et al. (1998) analysed *A. tamarense* morphotype isolates collected at the Orkney Islands North of Scotland and concluded that these European populations belonged to the North American ribotype. These findings not only led to an extension of the then known distribution area of this ribotype to another continent, but also opened the possibility for refining models concerning the mechanism of range extension from locally derived endemic populations to almost globally distributed species. Medlin at al. (1998) precluded a recent introduction from the distribution area of the ribotype along the North American East coast based on sequence divergence among strains from Europe and the American East coast. Introduction by humans e.g., via ballast water transport or translocation by shellfish stocks therefore was explicitly ruled out (Medlin et al. 1998). These authors put forward the idea of an alternative route of natural dispersal of the ribotype by first colonising North European region after passage through the waters between Greenland and Svalbard and followed by spreading to the North American East coast. Their data, however, did not allow for rigorous testing the hypothesis that European *A. tamarense* of the NA ribotype are ancestral to the North American Atlantic populations.

John et al. (2003), supported by refined analyses of a molecular clock model, developed a scenario of allopatric evolution of disjunct natural populations of the *A. tamarense* species complex as a consequence of plate tectonics, past global climate change and paleoceanograpy in accordance with the hypothesis put forward by Scholin et al (1995). The confirmation of NA ribotype populations in the Western Pacific Ocean north of Japan, from the Russian Pacific coast, have been interpreted as further evidence for the natural dispersal of the ribotype by natural means (Orlova et al. 2007). Lilly et al. (2007) summarise the present day distribution of the North American ribotype of the *A. tamarense* species complex (in this study referred to as "Group I") as known from the western and eastern coasts of the temperate to sub-polar North Pacific, as well as the Western coast of the Northern Atlantic and Northern European waters and from the Pacific and the Atlantic coasts of temperate South America and from South Africa. Whereas for this ribotype no recent introductions by direct human activity could be confirmed by molecular data (Lilly et al. 2007), recent expansion of originally endemic populations of this and other ribotypes to new regions might have occurred. Besides ongoing environmental change, e.g., due to coastal eutrophication and/or climate warming (Sellner et al. 2003; Lilly et al. 2007), direct human assisted dispersal (e.g., by dislodgement of cysts from non-endemic species with ballast water of cargo ships (Hallegraeff et al. 1990; Hallegraeff and Bolch 1991)) and subsequent colonisation of the new habitat might have contributed to a recent range extension of some *A. tamarense* species complex ribotypes. Such a scenario was presented by Scholin et al. (1994) for toxic TA ribotype populations to Australia, which these authors assumed to have its origin in Northern Asia or specifically waters around Japan. Furthermore, proof for the possibility of transfer of *Alexandrium* species by movement with ship's ballast water was demonstrated by the presence of viable cysts of the Temperate Asian and the North American ribotype in ships sampled at Australian ports with their ballast water derived from Japanese or Korean waters (Scholin et al. 1994). Whereas populations of the NA ribotype are not yet observed in Australian waters, the observation of TA ribotypes in Australia with identical

sequence signatures as those from Japan, were taken as an indication of modern time translocation and subsequent population establishment (Scholin et al. 1995). However, the possibility of natural dispersal of the TA ribotype, e.g., in the direction from Japan to Australia during the last Pleistocene glacial maximum, which might have provided the environmental settings enabling such dispersal, was not ruled out as a possibility of natural dispersal of the TA ribotype (Scholin et al. 1995).

In contrast to the Australian situation, an anthropogenic introduction, presumably by ballast water of cargo ships, is that of the TA ribotype in the Mediterranean (Lilly et al. 2002). Absence of the species from monitoring data as well as phylogenetic placement of LSU ribosomal gene sequences within strains with origin in Japanese waters (Western Pacific Ocean) support the theory that this ribotype has only recently started to spread through the Mediterranean after its first appearance in the 1980ies (Penna et al. 2005; Penna et al. 2008).

Despite their usefulness in delineating ribotype clades, the genes of the ribosomal operon did not serve especially well in analysing the fine scale population structure within a ribotype. Such studies, however, might allow a more detailed analysis of evolutionary processes and the accompanying biogeography in ribotypes or species. Molecular markers with higher resolving power at the population level might proof the right tools for such approaches that aim at the distinction between native and non-native populations of a certain ribotype and natural versus anthropogenically mediated or facilitated dispersal and range extension. Very recently, Masseret et al. (2009) in a study characterising populations of the TA ribotype from the Mediterranean and Japanese waters by highly polymorphic microsatellite markers reported on the comparatively low genetic diversity of isolates from a Mediterranean population. Despite the possibility that local environmental conditions in the sampled Mediterranean lagoon influenced population size and genetic diversity, these results were taken as supportive for the hypothesis of a small founder population, recently introduced to the Mediterranean (Masseret et al. 2009).

The comprehensive study of Nagai et al. (2007a) on genetic differentiation of Japanese populations of the North American ribotype indicates that human activities such as translocation of shellfish stocks are the most plausible explanation for deviations of population differentiation from a strict isolation by distance model. This study hitherto is the only study showing differentiation of *A. tamarense* populations on a small geographical scale. A pending question, however, is how genetic population differentiation relates to the development of individual populations of the same ribotype and morphospecies over annual and inter-annual cycles.

1.4. MOTIVATION FOR THIS THESIS

The genus *Alexandrium* is one of the most important harmful algal bloom genera in terms of associated health risks for humans as consumers of potentially contaminated seafood. Besides its significance for humans, production of PSP toxins in several, but not all members of the genus (and different ribotypes within the *A. tamarense* species complex) by itself is an intriguing feature, as its ecological relevance for the toxigenic species itself is not understood. The aim of this thesis is to enhance our current understanding of ecologically relevant phenotypic characters in *Alexandrium* to better understand mechanisms of population development and bloom formation. An evolutionary perspective on allelopathic properties shall be provided by inter- and intra-species analyses of this trait, which is presumed of ecological relevance in bloom formation and maintenance. The establishment and application of state-of-the-art genotyping methodologies addresses questions on the applicability of novel methodologies for determination of species boundaries and in-depth analysis of species evolutionary divergence, as well as to survey and analyse genotypic diversity within *Alexandrium tamarense*.

Alexandrium tamarense, in particular the North American ribotype, was chosen, since it provides an interesting model organism for an integrative study on population characteristics of a bloom forming dinoflagellate. First, this species has a wide geographical distribution range and a well monitored bloom history in many regions (e.g., for Scottish waters see Smayda (2006)). Second, the physiology, growth characteristics and environmental tolerance have been well studied (e.g., Prakash 1967; Brand 1981; Watras et al. 1982; Glibert et al. 1988; Parkhill and Cembella 1999; Yamamoto and Tarutani 1999). Third, many strains isolated from natural populations produce potent neurotoxins associated with paralytic shellfish poisoning (PSP) (e.g., Ghazarossian et al. 1974; Shimizu et al. 1975b; Oshima et al. 1977; Alam et al. 1979; Hall 1982; Cembella et al. 1987; Anderson et al. 1994; Ichimi et al. 2002) and/or allelochemical substances causing lysis of co-occurring protists (e.g., Arzul et al. 1999; Tillmann and John 2002; Fistarol et al. 2004b), which both can be used as phenotypic population markers. Fourth, this species has high relevance because of its role in outbreaks of PSP due to the accumulation of these toxins in the food chain. Fifth, the life cycle characteristics of different ecotypes of *A. tamarense* have been described in detail (see Wyatt and Jenkinson (1997) and Anderson (1998) and references therein). Finally, and perhaps most importantly, phylogenetic (Scholin et al. 1994; Scholin and Anderson 1994) and genotypic (John et al. 2004; Nagai et al. 2004) population markers have been developed with specific reference to the species and are now at hand. Taken together, this represents an exceptionally sound base of knowledge for further, in-depth analyses on specific aspects of the evolutionary ecology of these dinoflagellates by combining different fields of research such as toxinology and studies of chemical mediated species interactions with phylo- and population genetic methods.

1.5. METHODOLOGICAL CONSIDERATIONS

1.5.1. Analysis of Paralytic Shellfish Poisoning Toxins

Differences in PSP toxicity of clonal isolates of *Alexandrium* species can be caused by different processes. First, the production rates of PSP toxins may vary considerably between different isolates under the same environmental or experimental conditions. Second the cell quota of PSP toxins depends on the rate of cellular growth and cell size – cells that divide faster at a smaller cell size accumulate less PSP toxins per cellular volume than cells that grow slower and attain larger cells sizes, even when both isolates produce PSP toxins at the same rate per cellular volume. Third, toxicity of a certain isolate does not only depend on the content of PSP toxin, but also on the relative composition of the different saxitoxin derivatives due to their differences in specific potency (see paragraph 1.3.3). Fourth, processes such as cellular degradation of PSP toxins or cellular leakage and release of PSP toxins to the surrounding media can counteract the accumulation of PSP toxins, even if PSP toxin production rates are maintained at equal levels (Anderson et al. 1990b). Especially in experimental cultures when senescence of the culture is reached these processes are thought to play an important role in explaining the phenomenon of decreasing toxicity in ageing cultures. Finally, all these processes can be influenced by one or more environmental variables and differences in PSP toxin content within an *Alexandrium* isolate can be caused by diverse factors, such as temperature, light intensity, salinity, nutrient availability and growth stage in batch culture (e.g., see Anderson (1990) and references therein) and other, chemically mediated, biotic cues (Selander et al. 2006; Bergkvist et al. 2008; Selander et al. 2008). Though all these influencing factors already have been studied and well characterised in a limited number of isolates, it is very probable that a considerable physiological diversity in PSP toxin production can be expected with respect to variations in each of the above listed variables, when different clonal isolates from a specific population are studied.

However, though environmental factors such as temperature, light intensity and salinity – factors which also cause differences in cellular growth rates and cell division rates in cultures – have been shown to influence the rates of toxin production in *Alexandrium* in experimental cultures, it also has been shown that the relative composition of PSP toxins under such culture conditions essentially remains constant under a wide range of experimental conditions (Anderson 1990). In the present study, to avoid conditions that lead to alterations in the relative composition of PSP toxins in a clonal isolate, all isolates were maintained in nutrient replete growth media and harvested during exponential growth phase where toxin production is assumed a rather stable trait. Moreover, comparisons on toxin content were only carried out among groups of isolates that were haphazardly sampled precluding any systematic sampling bias.

1.5.2. Study of Allelopathic Properties

Several criteria have been defined by Willis (1985) that ideally should be fulfilled for a complete assessment of all aspects related to allelopathy. First, the inhibiting species (the donor) must produce a specific secondary metabolite, the allelochemical compound, for which the specific biosynthetic pathway and production rates should be identified. Second, the inhibition of the target species by the allelopathic organism must follow a consistent pattern that can not be explained by other factors (e.g., alteration of the environmental conditions by increase of pH). Finally, a mechanism for release of the allelochemical compound should be known as well as the means of uptake of the allelochemical by the donor species, i.e. the mechanism of the allelochemical interaction should be clarified. In their review, Legrand et al. (2003) surveyed the existing literature on allelochemical interactions among phytoplankton and concluded that in the majority of studies all these requirements have rarely been met. More specifically, previous studies of allelochemical effects of *Alexandrium* species on potential competitors and grazers have also not advanced to the state where all the above requirements have been fulfilled. While the mode of action might consistently be explained by a mechanism targeting membrane structures, the identity of the responsible chemical is not sufficiently well uncovered to establish a mechanism for the nature of allelopathy in *Alexandrium*. For this reason, it is especially necessary to establish the independence of the allelopathic effects from other factors such as e.g., limitation of nutrients or pH effects, to not confound unspecific factors with allelopathic properties (Schmidt and Hansen 2001). Therefore, strictly controlled experimental set-ups with clonal isolates of two species – the donor and the target – are the preferred method for comparative assessment of allelochemical activity among donor strains. In one approach, whole cell cultures of the donor are used to assess the effect after co-incubation of donor and target (e.g., Tillmann and John 2002); another approach makes use of only the cell-free culture filtrate – putatively enriched with allelopathic compounds (e.g., Arzul et al. 1999; Fistarol et al. 2004b). Both these strategies were followed in this thesis in a comparative way. A multifunctional nature of the allelochemical effective compound, however, can not be excluded until these compounds have been identified and characterised in detail, so that other functions can be excluded. Otherwise, the observed negative effect still might only be a side product without mediating the hypothesised ecological advantage to the putative allelopathic species. However, studies aiming at the demonstration of ecological effects in planktonic communities require thorough preparations and studying allelochemical effects in two-species systems is a first step in approaching ecological studies at more complex levels (e.g., multi-species systems or mesocosms). Nevertheless, the mere absence of one of the required characterisation steps in fully describing the nature of allelopathic interactions among certain species does not support rejection of hypotheses concerning the significance of allelopathy.

1.5.3. Genetic Markers for Inter- and Intra-Species Comparisons in Microalgae

Ribosomal DNA sequences have proven suited for phylogenetic studies on different hierarchical levels from deep phylogenies of major protistan lineages (e.g., Cavalier-Smith 2003; Saldarriaga et al. 2004; Moreira et al. 2007) down to the level of resolution of ribotypes within species complexes of cryptic or pseudocryptic species (e.g., grouping members of the *Alexandrium tamarense* 'species complex' into regional ribotypes (Scholin and Anderson 1994; John et al. 2003) or species within '*Skeletonema costatum* species complex' (Sarno et al. 2005; Sarno et al. 2007; Kooistra et al. 2008)). The wide range of applications of ribosomal genes is explained by the differences in evolution rates of the different genes within the ribosomal operon (18S rDNA, internal transcribed spacer 1 (ITS 1), 5.8S rDNA, ITS 2, and 28S rDNA) and specific regions of these genes. With the 18S rDNA and the hypervariable region (or D1-D2 region) of the 28S rDNA being among the most and the least conserved of the genes that encode ribosomal RNA, respectively (Hillis and Dixon 1991). Both ITS genes are even more variable than the D1-D2 region of the LSU rDNA, making them useful in certain population level applications in marine microalgae (Godhe et al. 2006; Penna et al. 2008; Kremp et al. 2009).

Genotypic markers with even higher resolving power, such as molecular fingerprinting methods, are needed at the population level to assess genotypic diversity within populations of microalgae. In their review from almost a decade ago, Medlin et al. (2000) noted that fingerprinting techniques had only just begun to appear in phytoplankton studies. The development and application of neutral genetic markers in different microalgae species has rapidly advanced. Anonymous fingerprinting techniques such as random amplified polymorphic DNA (RAPD) (Williams et al. 1990) and amplified fragment length polymorphism (AFLP) (Vos et al. 1995) have made their appearance in studies of e.g., diatom (AFLP: (Beszteri et al. 2007), RAPD: (Godhe et al. 2006)), haptophyte (RAPD: (Barker et al. 1994; Medlin et al. 1996)) and dinoflagellate (AFLP: (John et al. 2004), RAPD: (Adachi et al. 1997; Bolch et al. 1999; Shankle et al. 2004; Martínez et al. 2006)) microalgae. Furthermore, the number of species for which DNA microsatellite markers (see e.g., Tautz and Renz (1984) and explanations below) have been established is growing rapidly. Specific microsatellite markers have been developed quite recently for several marine microalgae, including species of diatoms (Rynearson and Armbrust 2000; Evans et al. 2004), haptophytes (Iglesias-Rodríguez et al. 2002), raphidophytes (Nagai et al. 2006b; Nishitani et al. 2007), and dinoflagellates (Santos and Coffroth 2003; Nagai et al. 2004; Renshaw et al. 2006; Nagai et al. 2007b; Pettay and Lajeunesse 2007; Smith 2008; Cho et al. 2009; Nishitani et al. 2009) microalgae. The usefulness of the different types of markers largely depends on the specific objectives of the study and the respective organisms studied. Characteristics of each marker system therefore have to be considered carefully before valid interpretation of genotypic fingerprinting data can be achieved.

Microsatellites are by nature predominantly multiallelic markers. Specific gene loci are usually amplified by PCR techniques and the amplified alleles are distinguished by their size subsequently. Consisting by definition of multiple repeats of short (one to six base pairs long) sequences, microsatellite alleles mostly vary in length by multiples of the repeat unit (Hancock 1999). The mutation of microsatellite loci in most of all cases is the consequence of slippage of

DNA strands during replication of repetitive sequences (Levinson and Gutman 1987). Homoplasy of microsatellite alleles due to back-mutations may occur frequently at loci with high mutation rates, however, this source of homoplasy has been found not to represent a significant problem for many types of population genetic analyses (Estoup et al. 2002). Upstream and downstream flanking regions of the core repetitive sequence are experimentally used to place specific PCR primers for each locus and analyses usually are performed by PCR amplification of alleles of single loci or more elaborate of multiple loci at a time by multiplex PCR. The need for obtaining sequence information and establishing adequate PCR protocols for each locus before experimental allele scoring can be carried out restricts microsatellite analyses in most studies to a limited number (often less then ten) of markers. However, in diploid or polyploid organisms microsatellite data is extremely valuable not only due to the mostly multiallelic nature when compared with biallelic marker systems, but also due to their co-dominant nature, meaning that heterozygotes can be distinguished at each locus by presence or absence of differently sized alleles. Anonymous genotypic markers such as AFLP and RAPD do not yield such information. In the latter two marker systems, amplified fragments are either scored as presence or as absence alleles in a specimen depending if an amplification product of a certain size is obtained or not, respectively. The impossibility to distinguish among heterozygotic individuals, possessing both the 'presence allele' and the 'absence allele', and homozygotes, containing twice the 'presence allele', accounts for their dominant nature in diploid organisms (or life stages). The advantage of these markers, however, is that many loci (up to several dozens) can be amplified in a single PCR reaction. Experimentally the number of amplified loci can be varied by choosing different PCR primers. In AFLP these are used to selectively amplify subsets of the genomic DNA previously digested by two different restriction enzymes. After restriction, specific double stranded oligomer adapters are ligated to the restriction sites (Vos et al. 1995). This allows for subsequent amplification of fragments containing the adapter by primers specific to the sequence of the ligated fragment plus the restriction site of the respective enzyme. By adding additional bases to the 3'-end of a primer the selectivity of the PCR reaction for a subset of the pool of fragment is enhanced (Vos et al. 1995). Selective amplification in AFLP can be carried out in two PCR steps, in which the number of selective bases added to the 3'-end of the primer is increased in the second reaction. In contrast to AFLP, the experimental protocol for RAPD does not include a restriction and ligation step, but relies on specific PCR amplification of a number of genes by different randomly composed oligomers, which are used in PCR reactions on genomic DNA containing a single PCR primer (Williams et al. 1990). Due to the low reproducibility of this method, especially when compared to AFLP (Jones et al. 1997), its use is nowadays discouraged (e.g., see author's guidelines of the Journal Molecular Ecology: http://www.wiley.com/bw/submit.asp?ref=0962-1083&site=1). However, both markers – AFLP and RAPD – have in common that point mutations at primer binding sites are the most likely mechanism of evolution of new alleles. Besides such mutations that determine the amplifiability of a locus, other mutations (e.g., insertions or deletions within an amplified sequence) lead to length polymorphism of the locus (Rieseberg 1996). The latter type of mutations usually is not assessed by the anonymous detection methods used and not accounted for in most population genetic models. However, these mutations are assumed to occur at a much lower frequency and the influence on subsequent analyses should be considerably low (Clark and Lanigan 1993; Rieseberg 1996; Innan

et al. 1999). Homoplasy due to amplification of different loci of the same size is a known problem in anonymous fingerprinting techniques, but can be reduced by e.g., restricting the analysis to larger fragments where homoplasy occurs less frequent (Vekemans et al. 2002).

The amplified fragment length polymorphism (AFLP) technique has been applied to several groups of organisms – including the lower eukaryotes – at the population level (e.g., Mueller and Wolfenbarger (1999) and references therein). As well, AFLP has been employed to discriminate apparently closely related species in inter-species studies, where it has proven useful in determining phylogenetic relationships (e.g., Xu and Sun 2001; Després et al. 2003; Pelser et al. 2003; Althoff et al. 2007; Beszteri et al. 2007).

1.6. OUTLINE OF THE THESIS

The studies comprising the scientific contribution of this thesis are written as seven separate research articles of which five are already published at the time of submission of the thesis, one is accepted for publication and in press and the last is prepared for submission to a scientific journal. An overview of the conception of the seven publications is provided here:

Qualitative and quantitative aspects of allelopathic properties within the genus *Alexandrium* against different protistan targets were approached by an inter-species comparison. These include the study of target-specificity and qualitative differences as well as of independence of production of causative substances from associated bacteria, **(Publication 1)**: For this purpose six species of *Alexandrium* were included in short-term (24h) bioassays in combination with different marine protists such as other microalgae as potential competitors and heterotrophic dinoflagellates and a ciliate as potential grazers. Allelopathic effect strength of an *Alexandrium* strain was quantified by assessing the number of remaining intact cells of the target species. Testing for dependence of the allelopathic phenotype on bacteria externally associated with an *Alexandrium* strain was achieved by comparing cultures treated with antibiotics to untreated control cultures (and control of bacteria numbers in both).

The author of this thesis together with the other authors of the published research article developed the conceptual frame of this study and selected the different strains of donor and target species and planned the different experiments. He conducted all short-term incubation experiments together with U.T. The author of this thesis was the major contributor in preparing the manuscript of the published article.

Genotypic markers for in-depth population genetic analyses based on population-wide gene frequency distributions or on individual genotypes needed to be developed for the North American ribotype of *A. tamarense* **(Publication 2)**: For this purpose, genomic fragments of an *A. tamarense* strain of the NA ribotype were enriched for repeats and used for construction of a sequence library. Sequencing of about 200 clones from this library and subsequent screening for DNA microsatellites resulted in identification of different repeat types. Primers for auspicious polymorphic microsatellite loci were selected and tested on a number of strains from the NA ribotype. Additionally, the same primers were used to test their capacity for cross-amplification of microsatellite loci in other ribotypes of the species complex and in other *Alexandrium* species. The clonal strains of the NA ribotype used for polymorphism testing of the microsatellite loci were obtained during a sampling cruise along the Scottish North Sea coast.

The author of this thesis screened the enriched sequence library for repetitive sequences and developed PCR primers and protocols for selected microsatellite loci. He conducted all PCR experimentation for the establishment of PCR primers and reaction conditions. The author of this thesis established and maintained the clonal isolates of *A. tamarense* needed for characterisation of microsatellite markers during a research cruise on RV Heincke in May/June 2004. The author of

this thesis wrote the manuscript of this research article and prepared it for publication.

The second genotypic marker, amplified fragment length polymorphism (AFLP), to be used in population level studies was tested for its applicability at the inter-species level as a mean to support sequence based phylogenies and also to reveal genetic substructure at the population level **(Publication 3)**: For this purpose, all available clonal isolates from different *Alexandrium* species from the most diverse sampling station of the same sampling cruise in Scottish waters were genotyped by several AFLP primer combinations. An evolutionary model for mutations at combined restriction and selective primer binding sites was adopted for the estimation of evolutionary genome divergence (the rate of nucleotide substitutions over whole genomes) among clonal strains and species.

The idea of adopting the specific evolutionary model to AFLP based phylogenetic studies of *A. tamarense* was developed by the author of this thesis. He established the cultures of different *Alexandrium* species during the same cruise. The author of this thesis conducted all laboratory and subsequent phylogenetic analyses. The author of this thesis coordinated and drafted the manuscript and prepared it for publication.

The largest sample of *A. tamarense* NA isolates obtained from the Scottish North Sea was to be used for further in-depth analyses at the intra-population level. In the first study, the focus was directed on the degree of genotypic and phenotypic diversity among clonal isolates from a single *Alexandrium tamarense* population and the relation of the two **(Publication 4)**: Genotyping of a multitude of isolates was performed with AFLP and with microsatellites to approach the issue of intra-populational genotypic diversity. Reliability of patterns of genotypes resulting from ordination according to pairwise genetic dissimilarities was tested to estimate the relative performance of the two marker systems in this study. Phenotypic diversity of the sampled population was assessed as expression of PSP toxin profiles, cellular PSP toxin content and allelopathic effects towards *Oxyrrhis marina* and *Rhodomonas salina*, a heterotrophic dinoflagellate and a phototrophic cryptophyte, respectively. The relation of genotype and phenotype expression was analysed by a global correlation analysis for PSP toxin profiles and by individual loci for allelochemical potency and PSP toxin content. Together these analyses aimed at providing a broad picture of the distribution of phenotypic and genotypic characters within natural populations of *A. tamarense*, helpful in understanding processes of population development and bloom formation.

The author of this thesis developed the idea on intra-population level studies on *A. tamarense* together with the co-authors of this research article. Field sampling and establishment of *A. tamarense* cultures were carried out by the author of this thesis. He conducted the experiments on allelopathic potency of *A. tamarense* strains. The author of this thesis conducted all molecular analyses and evaluation of phenotypic and genotypic data. He wrote the complete manuscript and prepared it for publication.

A more detailed study was conducted with a subset of ten clones from the same large population sample from the Scottish North Sea with the aim to fully quantitatively cover the determination of allelochemical properties in these clones and to address questions on potential

qualitative differences in allelopathic effects and metabolic costs of allelochemical production **(Publication 5)**: For this purpose detailed dose-response curves were obtained for the ten selected clonal isolates with the same two target organisms as in the previous study, *O. marina* and *R. baltica*. Additionally, growth rate under non-nutrient limiting conditions were determined for all ten clones and related to the observed strength in allelopathic potency.

The author of this thesis developed the concept of this study together with U.T. He performed the experiments and data analysis and interpretation together with the co-authors of the research article. The author of this thesis to a large extent contributed to the preparation of the manuscript.

The genotypic and phenotypic data as obtained in the first study on the large population sample from the North Sea (Publication 4) was also used for an analysis of population structure and consequences of vegetative growth of clonal lineages at the intra-population level **(Publication 6)**: Analyses of intra-populational linkage disequilibrium and population structure were conducted to test for traceability of differential selection on clonal lineages and potential effects of life cycle peculiarities. Supportive data from phenotypic analyses was included to develop a conceptual population genetic model for meroplanktonic dinoflagellates such as *A. tamarense*.

The author of this thesis elaborated the conceptual frame of the research for this article and performed all data analyses. He developed the population genetic model and wrote the manuscript of this research article.

With a view towards the observed global range extension of HAB species a study on phenotypic and genetic differentiation in globally dispersed populations of the NA ribotype of the *A. tamarense* species complex was performed. Additionally, the utility of two different neutral markers (microsatellites and AFLP) for population genetic studies in *A. tamarense* across different spatial scales was tested **(Publication 7)**: Genotypic data of isolates of the *A. tamarense* NA ribotype from three regions in the Northern hemisphere (West Atlantic, East Atlantic and West Pacific) was generated by both marker systems. Comparative analyses by multivariate and Bayesian methods were conducted to give evidence for the usefulness of each marker in inter-population level studies and for population assignment in the context of delineating the dispersal history of populations. Additionally, PSP toxin profiles were analysed for isolates from two of the three regions to provide a picture of phenotypic differentiation of globally dispersed populations of the NA ribotype and to test the usefulness of this marker for analyses of its dispersal history.

The author of this thesis planned and conceptualised the research carried out in this article. He performed most of the population sampling and performed all molecular analyses. The author of this thesis processed and interpreted all data, and with the input of co-authors wrote the manuscript of this research article.

2. PUBLICATIONS

Publication 1:

Urban Tillmann, Tilman Alpermann, Uwe John, Allan Cembella (2008) Allelochemical interactions and short-term effects of the dinoflagellate *Alexandrium* on selected photoautotrophic and heterotrophic protists. *Harmful Algae*, 7, 52–64.

Publication 2:

Tilman J. Alpermann, Uwe John, Linda K. Medlin, Keith J. Edwards, Paul K. Hayes, Katharine M. Evans (2006) Six new microsatellite markers for the toxic marine dinoflagellate *Alexandrium tamarense*. *Molecular Ecology Notes*, 6, 1057–1059.

Publication 3:

Tilman J. Alpermann, Bánk Beszteri, Urban Tillmann, Allan D. Cembella, Uwe John (2008) Species discrimination in the genus *Alexandrium* by amplified fragment length polymorphism. In: Moestrup, Ø., et al. [Eds.] *Proceedings of the 12th International Conference on Harmful Algae.* International Society for the Study of Harmful Algae and Intergovernmental Oceanographic Commission of UNESCO, Copenhagen, Denmark, 51–54.

Publication 4:

Tilman J. Alpermann, Urban Tillmann, Bánk Beszteri, Allan D. Cembella, Uwe John (in press) Phenotypic variation and genotypic diversity in a planktonic population of the toxigenic marine dinoflagellate *Alexandrium tamarense* (Dinophyceae). *Journal of Phycology*.

Publication 5:

Tilman J. Alpermann, Bánk Beszteri, Uwe John, Urban Tillmann, Allan D. Cembella (2009) Implications of life history transitions on the population genetic structure of the toxigenic marine dinoflagellate *Alexandrium tamarense*. *Molecular Ecology,* 18, 2122–2133.

Publication 6:

Urban Tillmann, Tilman Alpermann, Rodrigo C. da Purificação, Bernd Krock, Allan Cembella (2009) Clonal variability in allelochemical potency of *Alexandrium tamarense*. *Harmful Algae*, 8, 759–769.

Publication 7:

Tilman J. Alpermann, Katherine M. Evans, Allan D. Cembella, Donald M. Anderson, Satoshi Nagai, Urban Tillmann, Uwe John (in preparation) Genetic and phenotypic differentiation among globally dispersed populations of the North American ribotype of the toxic dinoflagellate *Alexandrium tamarense*. *Applied and Environmental Microbiology*.

2.1. ALLELOCHEMICAL INTERACTIONS AND SHORT-TERM EFFECTS OF THE DINOFLAGELLATE *ALEXANDRIUM* ON SELECTED PHOTOAUTOTROPHIC AND HETEROTROPHIC PROTISTS

2.1.1. Abstract

The marine dinoflagellate genus *Alexandrium* (Halim) Balech contains members that produce highly potent phycotoxins (PSP toxins or spirolides) as well as lytic substances and other allelochemicals of unknown structure and ecological significance. One isolate each of six *Alexandrium* species (*A. tamarense*, *A. ostenfeldii*, *A. lusitanicum*, *A. minutum*, *A. catenella*, *A. taylori*), of the closely related gonyaulacoid dinoflagellate *Fragilidium subglobosum*, and of the peridinioid *Scrippsiella trochoidea* were tested in 24 h co-incubation experiments for their short-term deleterious effects on a diversity of marine protists. Both autotrophs (*Rhodomonas salina*, *Dunaliella salina*, *Thalassiosira weissflogii*) and heterotrophs (*Oxyrrhis marina*, *Amphidinium crassum*, *Rimostrombidium caudatum*) were included as target species. All donor isolates except *S. trochoidea* exhibited lytic effects on at least some target species. Lytic effects were observed with all *Alexandrium* species, for both whole cell samples and culture filtrate (< 10 µm and < 0.2 µm). Antibiotic treated cultures with drastically reduced bacterial numbers did not show any general reduction in lytic capacity, therefore direct involvement of extracellular bacteria in allelochemical production is unlikely. Values of EC_{50}, defined as the *Alexandrium* cell concentration causing lysis of 50 % of target cells, differed by two orders of magnitude depending on the donor/target combination, from 3.1 x 10^3 cells mL^{-1} (*A. minutum*/*O. marina*) down to 0.02 x 10^3 cells mL^{-1} (*A. catenella*/*D. salina*). Within the array of nine donor *Alexandrium*/target combinations, variable ratios in EC_{50} values between donor/target combination cannot be explained by quantitative differences in allelochemical production, but rather indicate qualitative differences in the composition of compounds produced by different *Alexandrium* strains. In conclusion, our study confirms the widespread lytic capacity within the genus *Alexandrium*, although allelochemical effects are not restricted to this genus. Allelochemical interactions mediated by such lytic substances may be significant in explaining the formation and maintenance of *Alexandrium* blooms through direct destructive effects on competing algae or unicellular grazers.

2.1.2. Introduction

Blooms of noxious and/or toxic phytoplankton species are often termed “red tides” where water discolouration is produced via high biomass, or “harmful algal blooms” (HABs) when the bloom effects result in ecological disruption, faunal mortalities, human health risks or other deleterious socio-economic consequences. Many HAB species produce toxins or other noxious substances that can severely affect normal food-web processes and thereby adversely affect marine fauna from

benthic invertebrates to pelagic fish and even mammals and seabirds. Such HAB events are a prominent and often recurrent feature of nearshore and coastal areas throughout the world from tropical to high-latitude ecosystems. Although there are numerous anomalies and regional exceptions, it is generally accepted that a general global expansion, and an increasing frequency and extent of such blooms, is in progress (Smayda 1990; Hallegraeff 1993).

Whereas massive growth of diatoms as a recurrent feature of the annual cycle (spring bloom) forms the base of the marine food chain of most coastal and upwelling ecosystems, exceptional blooms, most notably of flagellates, can be at times almost mono-specific. The complex interactions of factors and mechanisms that lead to bloom development are poorly understood. There is little doubt that favourable initial physico-chemical conditions, such as stable, calm weather, reduced turbulence, increased water column stratification, and sufficient nutrient input are prerequisites for most flagellate blooms (e.g. Paerl 1988). The real enigma with respect to bloom dynamics is related to factors that determine which species will bloom at a given time and place. More precisely, to explain the occurrence and persistence of particular HABs, which are often dominated by a single species, current conceptual and experimental approaches focus on species-specific physiological and ecological traits of individual HAB taxa (Smayda and Reynolds 2001; Smayda and Reynolds 2003).

Most HAB events in marine ecosystems are caused by flagellates, belonging to the dinoflagellates, raphidophytes, prymnesiophytes, or pelagiophytes. Some HABs are also attributable to diatoms (e.g., *Pseudo-nitzschia* spp.) or cyanobacteria, but their ecophysiological characteristics and nutritional modes, such as *N*-fixation in the case of most bloom-forming cyanobacteria, are clearly distinguishable from those of marine flagellates. As a generalization, HAB flagellates tend to have lower nutrient uptake affinities and maximum growth rates than diatoms (Banse 1982; Smayda 1997), thus they are not ideal competitors for limiting inorganic macronutrients, especially in turbulent environments. Smayda (1997) suggested that HAB flagellates have evolved four major strategies to offset the ecological disadvantages of their low nutrient affinity: (1) nutrient retrieval migrations, (2) mixotrophic tendencies; (3) allelochemically enhanced inter-specific competition; and (4) allelochemical, antipredation defence mechanisms. The latter two proposed adaptations, collectively summarised as chemical defence, are mediated by the production and release of secondary metabolites. These allelochemicals are specific metabolites that stimulate or suppress growth of other organisms, or elicit other physiological responses in target cells.

In marine environments, allelochemicals may function in chemical defence as agents capable of incapacitating or even killing competitors and/or deterring grazers (see reviews in McClintock and Baker 2001; Cembella 2003; Legrand et al. 2003). Allelochemicals are probably an important mediator of species-specific biological interactions, including resource competition and predator-prey interactions, and are thus a potentially important adaptive factor in many biotic associations.

Among HAB flagellates, dinoflagellates of the genus *Alexandrium* are among the most ecologically important, as they have been involved in massive fish kills (Mortensen 1985; Cembella et al. 2002) and marine mammal mortalities and morbidity (Durbin et al. 2002; Doucette et al. 2006). Furthermore, *Alexandrium* blooms are the proximal source of toxins associated with

paralytic shellfish poisoning (PSP), a neurological affliction that has caused human illness for centuries (Prakash et al. 1971; Durbin et al. 2002) via consumption of contaminated shellfish.

As potent neurotoxins with Na-channel blocking activity, produced among various populations of *Alexandrium* spp., early attention was focussed on the hypothesis that PSP toxins, including saxitoxin and about two dozen naturally occurring derivatives (Shimizu 1996), functioned as predator defence compounds, e.g., against major grazers such as copepods and tintinnids. Nevertheless, the equivocal and often contradictory results obtained in grazing studies with various copepod species on *Alexandrium* strains of differing PSP toxicity (reviewed by Turner et al. 1998) have cast doubt upon this hypothesis as a simple generalization. Furthermore, there is increasing evidence that within the genus *Alexandrium*, lytic activity of extracellular metabolites and other negative effects upon other microalgae (Blanco and Campos 1988; Arzul et al. 1999; Fistarol et al. 2004b) and towards heterotrophic protists (Hansen 1989; Hansen et al. 1992; Matsuoka et al. 2000; Tillmann and John 2002) is rather widespread, but is unrelated to the cell quota of saxitoxin derivatives. The allelochemical mechanism against other protists, typically immobilization followed by cell lysis or cyst formation in the target species, was shown to be due to unknown extracellular substances – independent of both groups of known phycotoxins, PSP toxins (Tillmann and John 2002) and spirolides (Tillmann et al. 2007), produced among various *Alexandrium* strains. In spite of the detailed and more comprehensive literature on the effects of *Alexandrium* cells on macrozooplankton grazers, qualitative and quantitative information on the allelochemical potency within the genus *Alexandrium* against protistan targets is still comparatively scarce. Therefore, the aim of the present study was to quantify and compare short term effects of a number of different *Alexandrium* species towards a range of both photo- and heterotrophic protists. In addition, experiments were performed to evaluate the potential role of extracellular bacteria in the observed lytic effects.

2.1.3. Materials and Methods

Experimental cultures

Allelochemical activity of one isolate each of six different *Alexandrium* species was compared with that of the related gonyaulacoid dinoflagellate *Fragilidium subglobosum*, and with *Scrippsiella trochoidea* as a non-toxic control (Table 2.1.1). Cultures were grown in K- medium (Keller et al. 1987), supplemented with selenite (Dahl et al. 1989), prepared from sterile-filtered (VacuCap 0.2 µm Pall Life Sciences) natural Antarctic seawater (salinity 34 PSU) in 500 mL Erlenmeyer flasks. Cultures were maintained under controlled conditions at 15 °C under cool-white fluorescent light at a photon flux density (PFD) of 100 µmol m^{-2} s^{-1} on a 16:8 h light-dark photocycle. Prior to sub-sampling, culture flasks were shaken gently by hand to allow for a homogenous cell distribution.

A variety of different photo- and heterotrophic target protists were cultured (K-medium, 0.2 µm filtered natural North Sea water, 32 PSU) for exposure experiments conducted with the above organisms. The heterotrophic dinoflagellates *Oxyrrhis marina* (Göttingen culture collection, strain

Table 2.1.1. Species and strains tested for allelochemical potency.

Species	Strain no.	Origin (year); collector
Alexandrium tamarense	SZNB01	Mediterranean, Gulf of Naples, Italy (1999), M. Montresor
Alexandrium ostenfeldii	BAH136	South Pacific, Timaru, New Zealand; N. Berkett
Alexandrium lusitanicum	BAH91	Iberia, Laguna de Obidos, Portugal (1996)
Alexandrium minutum	AL1T	Mediterranean, Gulf of Trieste, Italy; N. Berkett
Alexandrium catenella	BAH255	Mediterranean, Thau Lagoon, France; M. Delgado
Alexandrium taylori	AY2T	Mediterranean, Lagoon of Marano, Italy; A. Beran
Fragilidium subglobosum	FSUT1	southern North Sea (2001); U. Tillmann
Scrippsiella trochoidea	STUT1	southern North Sea (2001); U. Tillmann

B21.89, isolated from Helgoland, Germany) and *Amphidinium crassum* (isolated from Barcelona harbour, Spain) were maintained at 15° C and at a PFD of 20 µmol m^{-2} s^{-1} and grown with the chlorophyte *Dunaliella salina* or the cryptophyte *Rhodomonas salina*, respectively, as food. The ciliate *Rimostrombidium caudatum*, isolated from Wadden Sea water from the island of Sylt, was maintained under the same light and temperature conditions as the heterotrophic dinoflagellates but with the chrysophyte *Isochrysis galbana* as food. All heterotrophic species were transferred once or twice per week to fresh medium containing food organisms. Cultures of heterotrophic protists for the experiments were grown to high cell concentrations until they became almost deprived of food, as checked by microscopic examination.

Autotrophic test algae used in the experiments, i.e. *Rhodomonas salina* (Kalmar culture collection, KAC 30), the chlorophyte *Dunaliella salina* and the diatom *Thalassiosira weissflogii* (both from the AWI culture collection) were grown at 15° C and under a PFD of 100 µmol m^{-2} s^{-1}. Cultures were transferred weekly to fresh medium and were always in exponential growth when used in the experiments.

Experiment 1: Short-term effects of Alexandrium spp. on protistan targets

In the first experiments the "donor isolates", consisting of representatives of six different *Alexandrium* species, plus an isolate each of *Fragilidium subglobosum* and *Scrippsiella trochoidea* (as non-toxic control), were tested at a fixed cell concentration for their effects on different target species in an array of short-term mixed growth experiments (Table 2.1.2). The donor isolates were pre-cultured in 250 mL Erlenmeyer flasks at 15 °C with cool-white fluorescent light at a PFD of 100 µmol m^{-2} s^{-1} on a 16:8 h light-dark photocycle.

Table 2.1.2. Initial cell concentrations of donor and target species in Experiment 1.

Donor / Target	Species	Start-concentration (mL^{-1})
Donor species	*A. tamarense*	979
	A. ostenfeldii	1273
	A. lusitanicum	1602
	A. minutum	1471
	A. catenella	1004
	A. taylori	608
	F. subglobosum	778
	S. trochoidea	1539
Target species	*R. salina*	9300
	D. salina	7400
	T. weissflogii	3600
	O. marina	380
	A. crassum	256
	R. caudatum	20

Potential involvement of bacteria in the generation of lytic effects was determined by comparing effects of donor cultures treated with antibiotics (AB+) with those of untreated (AB–) cultures. One set of cultures were treated with the antibiotics penicillin (Sigma, final concentration 100 µg mL^{-1}) and streptomycin (Sigma, final concentration 25 µg mL^{-1}) whereas another set of cultures were untreated . All pre-cultures were grown for 14 days and than diluted to 500 cells mL^{-1} in normal K-medium (without antibiotics). After 1 week of growth, cells were counted (see below). Increase in cell density corresponded to expected growth rates for the species indicating that all cultures were in exponential growth phase. The culture of *A. taylori* treated with antibiotics was an exception; it grew poorly and cell numbers were too low to be included in the subsequent experiment. All other cultures (AB+ and AB–) were diluted in K-medium to yield a final cell concentrations of about 1000 mL^{-1} for *A. tamarense*, *A. ostenfeldii*, *A. catenella*, and *A. lusitanicum*, of 1500 mL^{-1} for the slightly smaller *A. minutum* and *S. trochoidea* , and 800 mL^{-1} for the larger *F. subglobosum*. From each dilution, three sub-samples of 10 mL were taken and either fixed with formalin (2 % final concentration) for subsequent estimations of bacterial abundance (see below), fixed with Lugol´s solution (final concentration 2 %) for algal cell counts (see below) or used for

pH measurements. For all AB+ cultures, lytic activity of whole cultures was also compared to culture filtrates from the same cultures. Therefore, two further sub-samples of 30 mL each were taken, one of which was filtered through 10 µm gauze (Nitex 03-10/2, SEFAR, Switzerland) and the other through syringe-mounted 0.2 µm pore-size cellulose acetate membrane filters (25 mm NALGENE®).

For each target isolate, cell concentration of stock cultures was determined by microscopic enumeration and dilutions were made to gain ten-fold the desired final concentration of target cells of each isolate in the experimental set up. For all target isolates, cell concentrations in these dilutions determined by triplicate cell counts was used to calculate final start concentration in the experimental 10 mL glass-vials.

For the short-term mixed growth experiment, 9 mL from each donor culture dilution as well as from both types of culture filtrate was pipetted into 20 mL glass vials (in triplicate) and spiked with 1 mL of a target species to start the experiment. *Rhodomonas salina* was selected as target species for all AB+ and AB– cultures and for both types of filtrate (<10 µm and <0.2 µm), whereas all other target isolates were subjected to AB+ cultures only. For each target, 9 mL of K-medium (triplicates) were used as control. Samples were incubated at 15 °C under cool-white fluorescent light at a PFD of 100 µmol m^{-2} s^{-1} on a 16:8 h light–dark photocycle. After 24 h, samples were fixed with 2 % Lugol´s solution and cell concentration of both donor and target species were determined. Final cell counts of target isolates were scaled to initial concentrations by calculating: N_{T24} x 100 / $N_{T0,}$ where N_{T24} = final target concentration and N_{T0} = initial target concentration.

Experiment 2: Cell-concentration dependent dose-responses

Based on results of the first experiment, a second experiment was set up in which dose-responses covering a wide range of donor cell concentrations were simultaneously determined for an array of three donor species (*A. ostenfeldii*, *A. catenella* and *A. minutum*) against three target species (*R. salina*, *D. salina* and *O. marina*). The whole experiment was done at the same time with the same *Alexandrium* cultures. Donor isolates were pre-cultured as described above (treated with antibiotics). Before the experiment, donor cell concentrations were determined by triplicate counts. Increase in cell density corresponded to expected growth rates for the species indicating that all cultures were in exponential growth phase. Dilutions were prepared to yield 7 to 11 final cell concentrations ranging from 50 to 8000 cells mL^{-1}. Target cell concentrations were diluted to yield 10 times the desired final target cell concentration. From each dilution of donor cells, 9 mL (in triplicate for each target species) were pipetted into 20 mL glass-vials; for each target species, 9 mL of K-medium (triplicates) were used as control. Vials were spiked with 1 mL of the respective target species. Samples were incubated as for Experiment 1. After 24 h, samples were fixed with 2 % Lugol´s solution and cell concentrations of both donor and target species were determined.

Experiment 3: detailed dose responses for A. catenella

The aim of the third experiment was threefold: (1) to further explore the small differences in lytic activity between AB+ and AB– cultures found for *A. catenella* in Exp.1; (2) to confirm the very low

EC_{50} values found in Exp. 2 for the donor/target combination of *A. catenella* and *D. salina*; and (3) to evaluate the variability in lytic potential between different batches of one strain pre-cultured independently.

Two sets of triplicate batch cultures of *A. catenella* were prepared in 250 mL Erlenmeyer flasks, half treated with antibiotics (AB+) and half without antibiotics (AB–). Cultures were grown as described for Experiment 2. Just prior to initiation of Exp. 3, cells were in exponential growth at concentrations ranging from 5.2 to 6.4 x 10^3 cells mL^{-1}. For each culture, nine dilutions (10 to 2000 cells mL^{-1}) were prepared. Sub-samples of all dilutions were taken to estimate initial *A. catenella* cell concentrations. Nine mL of each dilution were pipetted into 20 mL glass-vials (in triplicates), with 6 vials containing 9 mL K-medium as control. A culture of *Dunaliella salina* (2.7 x 10^5 cells mL^{-1}) was diluted to yield ten times the desired final concentration (10 x 10^3 cells mL^{-1}); 1 mL of that dilution was added to each glass-vial. From each control, 0.5 mL was taken immediately to estimate target cell start concentration. Samples were incubated, fixed, and counted as described for Experiment 2.

Determination of bacterial and algal cell concentrations

Bacteria were counted after staining with acridine orange according to a standard acridine orange staining method (Hobbie et al. 1977). Stained bacteria were counted under an epifluorescence microscope (Zeiss Axioskop 2 plus) with 100X oil immersion objective. At least 400 cells per filter (except for blank filters) were counted.

Concentrations of donor and target cells were determined with an inverted microscope (Zeiss Axiovert 40 C) from Lugol´s-fixed samples (final concentration 2 %). The volume set up for cell counts varied between different experiments (0.5 to 10 mL). Depending on the cell concentration, whole chambers or representative sub-areas were counted. For estimates of donor cell concentrations, total number of cells counted was always > 400 per sample, except for the two lowest *A. catenella* concentrations in Exp. 3, where 10 mL were counted resulting in 100 and 250 total counts per sample, respectively. For *R. salina*, *D. salina* and *T. weissflogii*, a volume corresponding to at least 800 cells in the control was counted. For *O. marina* and *A. crassum*, 1 mL sub-samples were counted (corresponding to 450 and 260 cells in the controls, respectively), whereas for *R. caudatum*, all ciliates in 10 mL were counted (corresponding to 200 cells in the control). In order to quantify lytic effects, only intact cells of the target species were scored.

Statistics

In Experiment 1, differences in final cell number of targets between each treatment and the control was tested using a Student´s *t*-test (n = 3). In dilution experiments (Experiment 2 and 3), final concentrations of target species followed a sigmoidal declining pattern when plotted against log-transformed *Alexandrium* cell concentrations. Estimates of $EC_{50,}$ i.e. the *Alexandrium* cell concentration yielding a 50 % decline in target cell concentration, were determined by fitting the data points to the following equation using the non-linear fit procedure of STATISTICA ™ (Statsoft, Germany) for Windows:

$$N_{final} = N_{control} / (1 + (x / EC_{50})^h)$$

where N_{final} is the experimental final target cell concentration, $N_{control}$ the final target cell concentration in controls, x is the log-transformed cell concentration of *Alexandrium* spp. and EC_{50} and h are fit-parameters. Results are expressed as EC_{50} (cells mL^{-1}) including 95 % confidence intervals.

2.1.4. Results

Experiment 1:

This experiment performed at one fixed donor cell concentration indicated qualitative and quantitative differences in effects of different *Alexandrium* species on various target species. In general, there was a high degree of variability for the different donor/target combinations. All *Alexandrium* species exhibited lytic effects on at least some target species (Fig. 2.1.1), reflected in the decrease in cell numbers of the target species relative to controls. Nevertheless, allelochemical activity was not restricted to *Alexandrium* because *Fragilidium subglobosum* was also highly potent, causing 100 % mortality of the ciliate *Rimostrombidium caudatum*, a reduction in *Oxyrrhis marina* cell numbers, and a lower growth rate in *Dunaliella salina*. Whereas the autotrophic flagellates *Rhodomonas salina* and *D. salina* were heavily affected by most *Alexandrium* species, the diatom *T. weissflogii* showed positive growth in all treatments except for the slight but significant inhibition of growth rate caused by *A. tamarense* and *A. ostenfeldii* ($p < 0.005$). Among *Alexandrium* species tested, *A. tamarense* and *A. ostenfeldii* were the most effective in lysing other

Table 2.1.3. Bacterial numbers (x 10^6 mL^{-1}) in algal cultures (Experiment 1) without (AB–) and with antibiotic (AB+) treatment. Mean ± SD of two filters per sample; n.d. = not determined.

Species	AB–	AB+	% reduction
A. tamarense	1.59 ± 0.02	0.10 ± 0.01	94.0
A. ostenfeldii	2.29 ± 0.15	0.14 ± 0.01	93.7
A. lusitanicum	1.25 ± 0.17	0.08 ± 0.02	93.6
A. minutum	0.91 ± 0.01	0.05 ± 0.01	94.8
A. catenella	1.28 ± 0.03	0.11 ± 0.01	91.7
A. taylori	5.60 ± 0.06	n.d.	
F. subglobosum	8.28 ± 0.18	1.91 ± 0.14	77.0
S. trochoidea	0.52 ± 0.02	0.03 ± 0.01	94.7

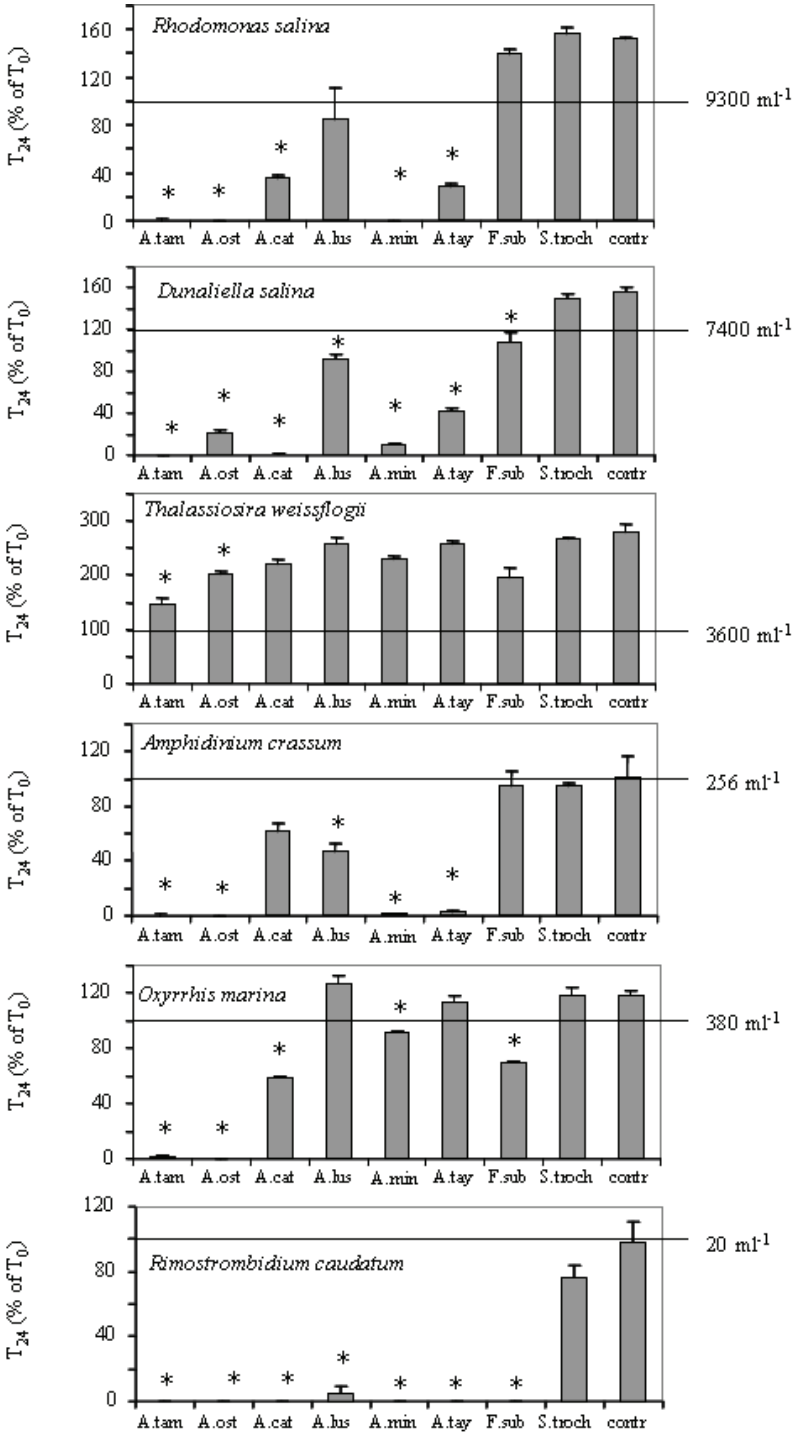

Fig. 2.1.1. Relative numbers of intact cells of different target organisms exposed to fresh culture medium (control) or to whole cell culture of a number of dinoflagellate strains. Target cell numbers after 24 h incubation were scaled to initial cell numbers, which are given on the right hand side. (A) *Rhodomonas salina*; (B) *Dunaliella salina*; (C) *Thalassiosira weissflogii*; (D) *Amphidinium crassum*; (E) *Oxyrrhis marina*; (F) *Rimostrombidium caudatum*. Results expressed as triplicate mean ± 1 SD. Asterisks above bars indicate values significantly ($p < 0.005$) different from control. (A tam = *Alexandrium tamarense*, A ost = *A. ostenfeldii*, A cat = *A. catenella*, A lus = *A. lusitanicum*, A tay = *A. taylori*, F sub = *Fragilidium subglobosum*, S troch = *Scrippsiella trochoidea*, contr = control).

species; almost 100 % mortality was observed for all target species except for *T. weissflogii*. *Alexandrium lusitanicum* caused marked reductions of only *R. caudatum* and *A. crassum* cells and therefore appears to be the least effective. Among the heterotrophic target species, *R. caudatum* was the most sensitive; almost no target ciliates were found in any treatments other than for *S. trochoidea* (non-toxic control). Figure 2.1.1 is based on cultures of donor species pre-treated with

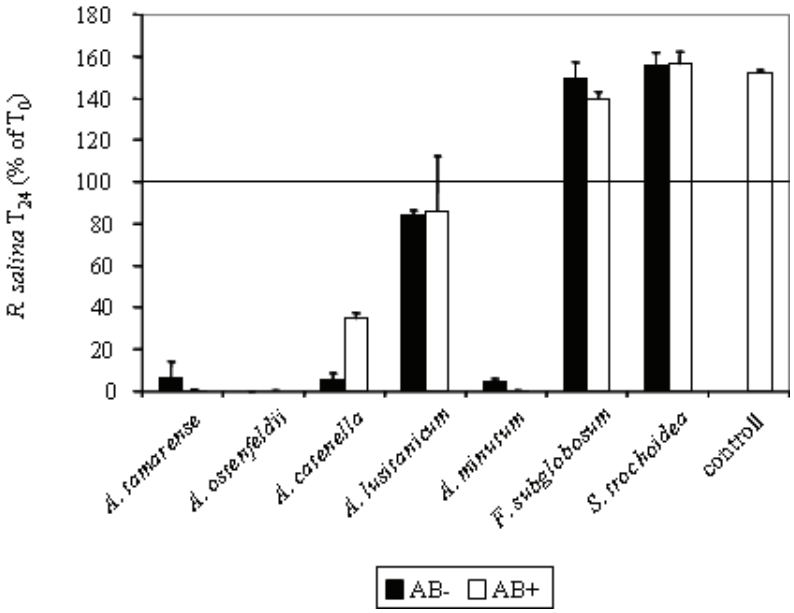

Fig. 2.1.2. Relative numbers of intact cells of *Rhodomonas salina* exposed to fresh culture medium (control) or to whole cell culture of a number of dinoflagellate strains. Target cell numbers after 24 h incubation were scaled to initial cell numbers. For each dinoflagellate, a comparison of cultures treated with antibiotics (AB+) and non-treated cultures (AB–) is shown. Results expressed as triplicate mean ± 1 SD.

antibiotics (AB+). Bacterial counts (Table 2.1.3) show that the treatment with penicillin and streptomycin does not yield axenic cultures. Bacterial numbers, however, were reduced by 94 % compared to untreated cultures (AB–).

A comparison of the lytic effect of AB+ and AB– cultures against *R. salina* (Fig. 2.1.2) shows that for each donor species, target cell numbers after 24 h incubation were in close agreement between AB+ and AB– cultures, except for *A. catenella* for which final target concentration was significantly lower in AB– treatment (*t*-test, $p < 0.001$).

Table 2.1.4. pH of donor-species test solution in Experiment 1. AB+ = cultures treated with antibiotics; AB– = cultures not treated with antibiotics. n.d. = not determined

Species	pH AB+	pH AB–
A. tamarense	8.20	8.24
A. ostenfeldii	8.08	8.27
A. lusitanicum	8.12	8.08
A. minutum	8.12	8.15
A. catenella	8.15	8.19
A. taylori	n.d.	8.50
F. subglobosum	8.20	9.04
S. trochoidea	8.16	8.24

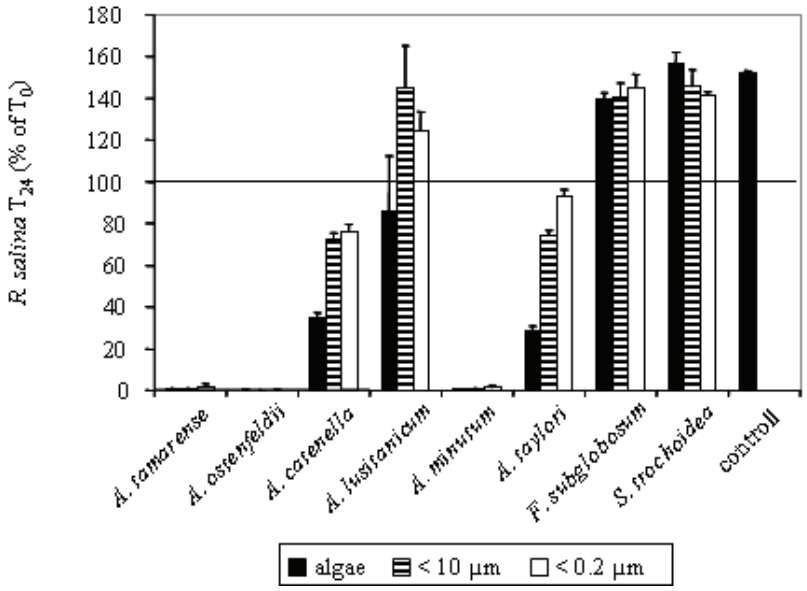

Fig. 2.1.3. Relative numbers of intact cells of *Rhodomonas salina* exposed to fresh culture medium (control) or to different fractions of a number of dinoflagellate strains. Target cell numbers after 24 h incubation were scaled to initial cell numbers. For each dinoflagellate, a comparison of whole cell culture and culture filtrate (both < 10 µm and < 0.2 µm) is shown. Results expressed as triplicate mean ± 1 SD.

The pH of all donor cultures (Table 2.1.4) was around 8.2, except for the *F. subglobosum* AB– culture (pH = 9.04).

For all *Alexandrium* species tested, lytic effects were observed with both whole cell samples and culture filtrate (<10 µm and <0.2 µm). For all donor isolates, no significant differences (*t*-test, $p > 0.05$) could be found between both types of filtrate. For *A. catenella* and *A. taylori* effects of culture filtrate were significantly (*t*-test, $p < 0.001$) lower than those of whole cell treatments (Fig. 2.1.3).

Experiment 2:

In Experiment 2, more detailed quantitative and qualitative differences in donor effectiveness and target susceptibility found from Experiment 1 were recorded as the dose-response curves for three *Alexandrium* and three target species (Fig. 2.1.4). For all donor-target combinations there was a sharp decline of target cell numbers with increasing *Alexandrium* cell concentrations. When plotted against log-transformed *Alexandrium* concentrations, the typical sigmoidal shape of the curves became evident (Fig. 2.1.4).

Table 2.1.5. EC_{50} concentrations (cells mL^{-1}) and 95 % confidence intervals for Experiment 2.

Donor/target species	***R. salina***		***D. salina***		***O. marina***	
A. ostenfeldii	230	(209–252)	170	(127–226)	70	(59–84)
A. catenella	649	(571–736)	22	(19–24)	1540	(1330–1782)
A. minutum	523	(472–579)	129	(112–148)	3136	(2414–4072)

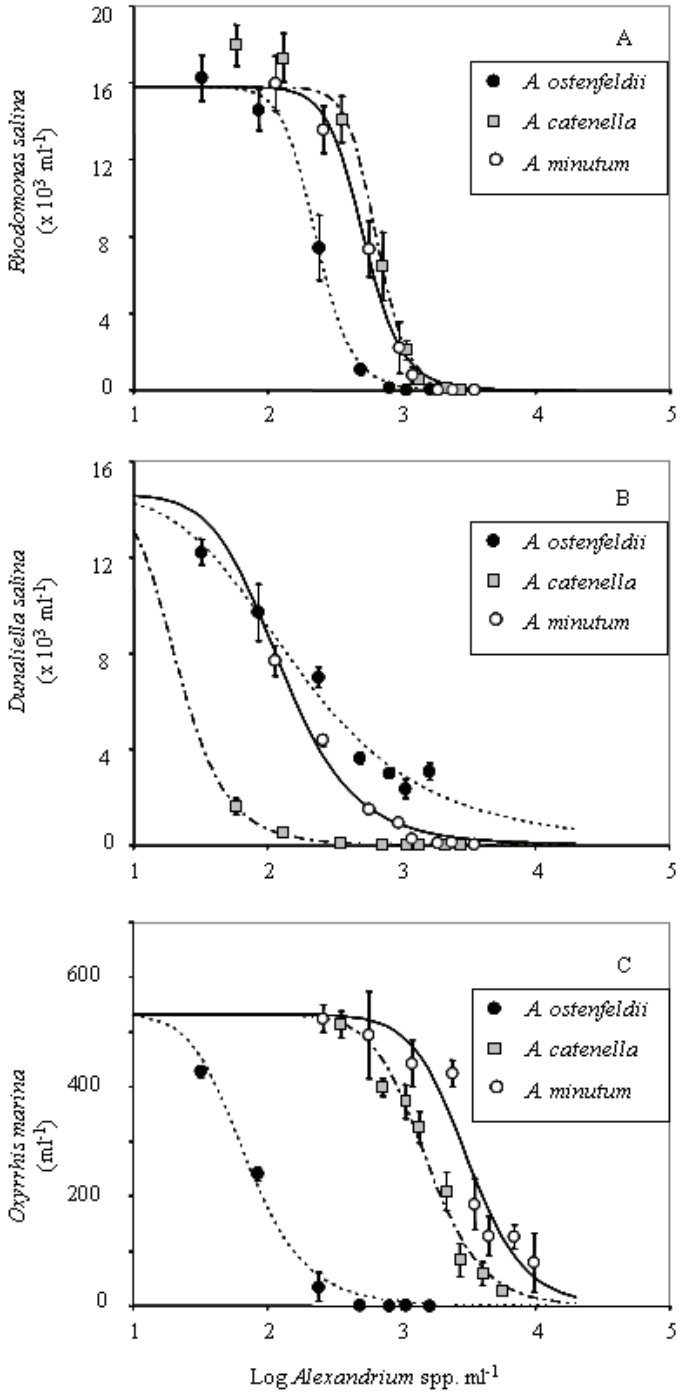

Fig. 2.1.4. Final cell numbers of (A) *Rhodomonas salina*, (B) *Dunaliella salina* and (C) *Oxyrrhis marina* after 24 h incubation with a dilution series of different *Alexandrium* concentrations, plotted against log-transformed *Alexandrium* concentration. For each target species, three different *Alexandrium* species were tested (*A. ostenfeldii*, *A. catenella* and *A. minutum*). Lines represent results of a non-linear fit procedure (see paragraph 2.1.3.). Results expressed as triplicate mean ± 1 SD.

Corresponding EC_{50} concentrations (Table 2.1.5) were significantly different for each combination (95 % confidence intervals do not overlap) and ranged from 3136 (*A. minutum*/*O. marina*) down to 22 cells mL^{-1} (*A. catenella*/*D. salina*). *Alexandrium catenella* was about 10 times more effective in lysing *D. salina*, but about 23 times less effective against *O. marina*, than *A. ostenfeldii*. A comparison between *A. minutum* and *A. ostenfeldii* showed that both species affected *D. salina* to the same extent, but *O. marina* was about forty times more affected by *A. ostenfeldii* than by *A. minutum*.

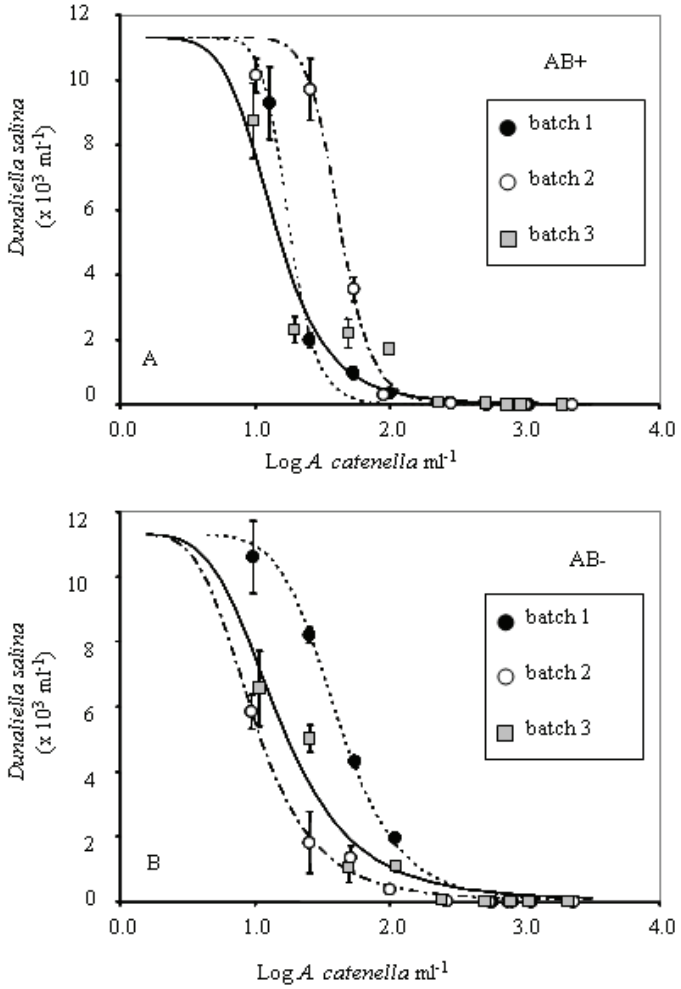

Fig. 2.1.5. Final cell numbers of *Dunaliella salina* after 24 h incubation with a dilution series of different *A. catenella* concentrations, plotted against log-transformed *Alexandrium* concentration. (A) Three batch cultures treated with antibiotics (AB+). (B) Three batch cultures untreated (AB–). Lines represent results of a non-linear fit procedure (see paragraph 2.1.3.). Results expressed as triplicate mean ± 1 SD.

Experiment 3:

The dose-responses curves show the final cell number of *Dunaliella salina* after 24 h incubation with a dilution series of different *A. catenella* concentrations treated with antibiotics (AB+) or untreated (AB–) (Fig. 2.1.5). Antibiotic treatment resulted in a 99.6 % reduction in bacterial numbers (AB+: 0.008 ± 0.001 x 10^6 mL^{-1}; AB–: 2.046 ± 0.228 x 10^6 mL^{-1}. Mean ± 1SD, n = 3). Final cell numbers of *D. salina* decreased sharply with increasing *A. catenella* cell concentrations; 100 % mortality was reached at about 250 *A. catenella* cells mL^{-1}.

Although there was some variability in final target density at the lowest *A. catenella* cell concentrations, and, consequently, in the corresponding EC_{50} values between replicate batches, EC_{50} values (AB+: 25 ± 12.5; AB–: 22 ± 13.6) revealed no significant differences between antibiotic-treated and untreated cultures (t-test, p = 0.847). The low EC_{50} concentrations of about 10–40 *A. catenella* cells mL^{-1} (Table 2.1.6) confirmed the high lytic potential of this dinoflagellate towards *D. salina* as found in Experiment 2 (Fig. 2.1.4B, Table 2.1.5).

Table 2.1.6. EC_{50} concentrations (cells mL^{-1}) and 95 % confidence intervals for *A. catenella* and *D. salina* (Experiment 3).

Batch	Treatment	EC_{50}	
1	AB+	18	(17–19)
2	AB+	42	(37–48)
3	AB+	14	(11–18)
1	AB–	41	(37–45)
2	AB–	10	(9–11)
3	AB–	15	(11–20)

2.1.5. Discussion

In the present study we showed the potential of *Alexandrium* strains from several different species (or species complexes), as well as the related gonyaulacoid species *Fragilidium subglobosum*, to produce extracellular compounds capable of immobilising and lysing different protistan species. The exact chemical nature of the compounds involved remains to be determined. In any case, they may be generally classified as allelochemicals – secondary metabolites that are directly targeted (e.g., against predators or competitors). These allelochemicals are distinct from the known phycotoxins that are vectored through the food web, and thus can cause broad-based trophodynamic effects.

Extracellular lytic activity is widespread among members of the genus *Alexandrium*, as evidenced by the previously described negative effects on other photosynthetic (Blanco and Campos 1988; Arzul et al. 1999) and heterotrophic protists (Hansen 1989; Hansen et al. 1992; Matsuoka et al. 2000; Tillmann and John 2002). The extracellular lytic effects of *Alexandrium* have been shown to be unrelated to the presence of PSP toxins (Tillmann and John 2002) and, in the case of *A. ostenfeldii*, to spirolides (Tillmann et al. 2007). Here we also report for the first time the presence of extracellular lytic compounds in *F. subglobosum*, which was not previously known to be allelopathic and from which no known phycotoxins have yet been detected.

The hypothetical role of such lytic allelochemicals, e.g., in mixotrophy and food acquisition, warrants further scrutiny. A number of species of *Alexandrium* have recently been shown to be mixotrophic (Jeong et al. 2005), but a high dependence on fixed organic and particulate nutrition is not universal in this genus. *Fragilidium subglobosum* is an avid mixotroph, apparently feeding on *Ceratium* species much larger than itself by direct engulfment (Skovgaard 1996). For the mixotrophic haptophyte *Prymnesium parvum* there is good evidence that lytic compounds are involved in prey capture, in that motile prey, which otherwise would be inaccessible, are immobilized or even killed before ingestion (Skovgaard and Hansen 2003; Tillmann 2003). For gonyaulacoid dinoflagellates, it would be interesting to know whether the lytic extracellular

compounds, shown here to negatively affect certain protistan species, are likewise involved in food acquisition and feeding.

Potential bias and misinterpretation of "toxicity" caused by the effects of high pH, even in short-term (e.g., 24 h) mixed algal culture experiments such as we employed, has been previously recognized (Schmidt and Hansen 2001). In our experiments, it is unlikely that elevated pH in *Alexandrium* cultures contributed to the observed short-term negative effects because the pH (8.2) of the donor cultures in Experiment 1 (Table 2.1.4) was typical of that of control seawater medium, and well below limits causing deleterious effects on marine protists (Schmidt and Hansen 2001; Pedersen and Hansen 2003).

The potential involvement of free-living or attached bacteria in provoking the observed lysis and immobilization effects was also carefully considered. There remains an ongoing debate as to whether or not bacteria are either directly or indirectly associated with phycotoxin production (Doucette et al. 1998; Kodama et al. 2006). In spite of several investigations on the potential role of bacteria on PSP-toxin production in *Alexandrium* (Gallacher et al. 1997; Gallacher and Smith 1999; Baker et al. 2003), there is no conclusive evidence that eubacteria are capable of independent biosynthesis of PSP toxins (and not just expressing Na-channel or other toxic activity) when isolated from dinoflagellate cultures. Nevertheless, the question of lytic activity and other allelochemical effects of free-living bacteria in xenic cultures of *Alexandrium* must still be addressed.

Obtaining axenic cultures of *Alexandrium*, at least free of associated bacteria in the medium, is difficult. In our culture experiments, antibiotic treatment (streptomycin and penicillin) to eliminate or reduce the presence of bacteria drastically reduced bacterial numbers, but cultures were not axenic. More complex antibiotic cocktails, alternative exposure regimes or higher dosages might be more successful (Martins et al. 2004; Ho et al. 2006), but other investigators using different antibiotics also failed to achieve axenic status for *Alexandrium* (Stolte et al. 2002; Ho et al. 2006) or did not provide adequate evidence of bacteria-free cultures. Caution must be exercised in the application of antibiotics because they can directly influence cell physiology in the short term (Martins et al. 2004; Wang et al. 2004; Ho et al. 2006) and could act as a genetic selection factor, either of which may affect allelochemical production. The potential role of putative intracellular bacteria also cannot be excluded by our approach. In any case, our antibiotic-treated cultures with highly reduced bacterial numbers did not show any general loss of lytic capacity. Though *A. catenella*, when tested at one fixed cell concentration, seem to show less lytic activity when treated with antibiotics (Fig. 2.1.2), full dose–response curves revealed no significant differences between antibiotic-treated and untreated *A. catenella* cultures (Fig. 2.1.5). Therefore, active involvement of extracellular bacteria in allelochemical production of *Alexandrium* is unlikely. Furthermore, lytic effects of antibiotic-treated cultures were not enhanced relative to untreated cultures, indicating that *in situ* organic enrichment and bacterial degradation are not major factors in lytic activity, at least over the time scale of a few days. Finally, there is no evidence that these allelochemicals bind non-specifically to organic surfaces, as might be expected when bacterial cell load was high within the cultures. Such removal of allelochemical activity by binding to eukaryotic target cells has been shown for lytic toxins from both *Prymnesium parvum* (Tillmann 2003) and *Alexandrium ostenfeldii* (Tillmann et al. 2007) lytic toxins.

The variable intensity of lytic effects among specific donor/target combinations (Fig. 2.1.1) indicates that target organisms might be differentially sensitive to the allelochemical substances. Such observations could provide insights into the ecological role of these allelochemicals. For example, the strong observed effect on the predatory ciliate *Rimostrombidium caudatum* is in concordance with experimental findings from natural plankton sample exposed to a culture filtrate of an *A. tamarense* strain with documented lytic effects. The numerically abundant ciliates were all drastically reduced compared to the untreated control (Fistarol et al. 2004b). In the same study, three abundant diatom species only showed a slight reduction in cell numbers (Fistarol et al. 2004b). The exceptional insensitivity exhibited by the diatom *T. weissflogii* against all donor strains in our experiments provides support for the hypothesis that diatoms are more resistant to the allelochemical substances produced by toxic dinoflagellates than ciliates and flagellates.

The specific mode of action of these allelochemical compounds against target species remains speculative. The immobilization of flagellates and ciliates results directly from jettisoning of the flagellae and interference with ciliary function, respectively, whereas the lytic activity indicates related deleterious effects on the structure and function of the cell membrane or the cytoskeleton of the target cell.

A direct comparison of target sensitivity is difficult because the target species differed in cell concentration and size and thus in total surface area/volume. Quantitative differences in target cell response may also be attributable to variation in the production of lytic compounds by the donor species because of physiological factors linked to nutrient status or stage of the cell- and culture cycle. Further differences among *Alexandrium* strains may be related to the chemical diversity expressed in the lytic substances – perhaps a cocktail of various components rather than a single common analogue. Support for the latter hypothesis is provided from Experiment 2 within which *A. ostenfeldii* co-incubated with *R. salina* and *O. marina* caused 50 percent mortality of the target at lower concentrations than for *A. catenella* and *A. minutum*, whereas the opposite was the case when incubated with *D. salina*. Furthermore, although EC_{50} values of *A. catenella* and *A. minutum* affecting *R. salina* were comparably high, *D. salina* was much more, and *O. marina* was slightly more affected by *A. catenella* than by *A. minutum* (Table 2.1.5 and Fig. 2.1.4). The results of this array of nine donor/target combinations, which were tested simultaneously, cannot be explained by quantitative differences in allelochemicals, but rather indicate qualitative differences in the composition from different *Alexandrium* species and strains.

Variation in toxin production and composition for several different classes of phycotoxins is well known among closely related dinoflagellate species and even among strains of the same species (summarized by Cembella 1998; Wright and Cembella 1998). For example, for lipophilic polyether toxin groups, such as yessotoxins (YTXs) and spirolides (SPXs), the number of known analogues ranges from as many as 100 for YTX (Miles et al. 2005) to about 10 for SPX (MacKinnon et al. 2006). The paralytic shellfish poisoning (PSP) toxins produced by various strains of dinoflagellates, including of the genus *Alexandrium*, comprise about two dozen naturally occurring saxitoxin (STX) analogues. Among *Alexandrium* species and strains it is generally accepted that although the total cell quota of STX analogues may vary according to life cycle, cell cycle or stage in culture and in response to environmental variables, a clonal strain tends to produce a certain limited spectrum of toxin derivatives, referred to as the toxin profile (Boczar et al. 1988;

Anderson 1990). While there may be some shifts in the percentage composition of the different PSP toxin derivatives, caused by abiotic parameters or due to biochemical changes during the course of culture growth (e.g., extreme nutrient limitation), the general ability to produce a certain suite of toxins seems to be genetically fixed for each clonal strain (Sako et al. 1992; Sako et al. 1995). Although we have ruled out a central role for STX derivatives and spirolides as the cause of the lytic activity, it would not be surprising if the unknown lytic compounds also represent a variable suite of closely relative bioactive compounds.

Whatever the mechanism, chemical structures and mode of action, the allelochemical immobilization and lytic effects could be of significant importance for the structure and dynamics of plankton populations. Both competitors and potential protistan grazers are affected, which should result in a competitive advantage for *Alexandrium* spp. and therefore might contribute to bloom formation of the species. More diatom species must be tested before making such a generalisation, but our results suggest that the effects are more pronounced against potential ciliate and flagellate predators, than against diatoms, which are only potential competitors, primarily for inorganic nutrients. This does make ecological sense if the allelochemicals play a role in chemical defence or mixotrophic food acquisition. For chemical defence, this lytic mechanism would not have to be overwhelming (i.e. 100 % mortality) or an "all or nothing response" to provide a selective advantage; even a slight reduction in the predation rate may be critical in sustaining the formation of a dinoflagellate bloom.

In theory, differences in sensitivity of target species have the potential to yield temporal shifts in plankton community composition. However, to asses the potential impact of allelochemical of *Alexandrium* populations *in situ*, EC_{50} concentrations from the present laboratory study must be compared to nominal cell numbers of *Alexandrium* found in field studies. Although *Alexandrium* spp. are often considered to be „background" taxa, in that they often are outnumbered by co-occurring phytoplankton (Anderson 1998), high-biomass blooms that even discolour the water ("red tides") do occur, including those of *A. minutum* in south Australia (Hallegraeff et al. 1988), or dense blooms (even >1 x 10^6 L^{-1}) of *A. tamarense* (= *Gonyaulax excavata*; *Protogonyaulax tamarensis*; *Protogonyaulax excavatum*) in the Argentine Sea (Carreto et al. 1986), coastal Nova Scotia (Cembella et al. 2002) and the Lower St. Lawrence estuary (Fauchot et al. 2005). Especially in the Mediterranean, dense blooms of different *Alexandrium* species are repeatedly observed (Vila et al. 2001a, and references therein). In confined areas, such as harbours or embayments along the Mediterranean coast, *Alexandrium* may achieve cell concentration up to 6 x 10^6 L^{-1} (Vila et al. 2001b). This particular peak concentration of *A. catenella* from Barcelona harbour is, for example, more than three orders of magnitude higher than concentration of a Mediterranean strain of *A. catenella* causing 50 % mortality of some target species (Tables 2.1.5 and 2.1.6) in our laboratory experiments.

While it seems obvious that the almost complete dominance of *Alexandrium* during such dense blooms could be supported by negative effects of allelochemicals on competitors and grazers, potential effects of allelochemicals on early bloom development, when cell concentrations are much lower, remain to be investigated. In this respect, it is important to note that even at low depth-integrated cell concentrations (e.g., averaged over the euphotic zone or a fixed depth interval), spatial variation in cell concentration may be high on both the horizontal and vertical axes

("plankton patchiness"). In culture flasks grown under non-turbulent conditions, cells of *Alexandrium* spp. often form dense patches and thin horizontal layers, as well as vertical strands or smaller globular patches (U. Tillmann, pers. observations), which may create locally enhanced concentrations of extracellular lytic compounds even at relatively low "average" cell concentrations. The cues for such aggregation behaviour, which probably are the result of active swimming coupled with physical convection, are unclear, but mesocosm experiments with *A. tamarense* have demonstrated nutrient-dependent vertical migration through physical gradients (thermal stratification) (MacIntyre et al. 1997). In field populations, high relative cell concentrations of dinoflagellates in certain strata of the water column (e.g., above or below pycnoclines) have been frequently observed and vertical migrations of dinoflagellates are a common behaviour of some dinoflagellate species (Smayda 1997). The formation of localised chemospheres in patches and thin-layers, within which *Alexandrium* cells can thrive and deter potential grazers, may constitute a key factor for bloom initiation.

The ecological significance of allelochemical properties in *Alexandrium*, as well as the chemical structures of the immobilizing/lytic compounds remains to be elucidated. Nevertheless, it is now feasible to begin extrapolation of laboratory experiments on allelochemical interactions to field studies in natural bloom populations, in particular to explore the range of phenotypic expression of allelochemical properties within natural populations of *Alexandrium*. Studies focussing on the role of allelochemicals at different stages of bloom formation will further increase our knowledge and understanding of the mechanisms of bloom formation in toxic dinoflagellates.

2.2. SIX NEW MICROSATELLITE MARKERS FOR THE TOXIC MARINE DINOFLAGELLATE *ALEXANDRIUM TAMARENSE*

2.2.1. Abstract

We report the characterization of six new microsatellite loci for the toxic marine dinoflagellate *Alexandrium tamarense* (North American ribotype), using 56 isolates from a range of locations. The numbers of alleles per locus ranged from five to nine and gene diversities ranged from 0.041 to 0.722. We tested primers for these six loci on other *A. tamarense* ribotypes and on other *Alexandrium* species; the results suggest that the primers are specific to *A. tamarense* isolates belonging to the North American ribotype.

2.2.2. Introduction

Alexandrium is a marine, planktonic dinoflagellate genus, with haploid vegetative cells and diploid motile and non-motile zygotes. Paralytic shellfish poisoning (PSP) neurotoxins are produced by nine of the 29 species so far described (Balech 1995). Three of these toxic *Alexandrium* species (*A. tamarense*, *A. fundyense* and *A. catenella*) form part of a taxonomically unresolved group, the *A. tamarense* 'species complex'. Phylogenetic studies indicate that ribotypes cluster depending on geographical origin rather than morphological similarity (Scholin et al. 1994). So far, six different ribotypes have been identified (John et al. 2003). The North American (NA), temperate Asian and tropical Asian ribotypes are exclusively toxic, whereas the Western European, Tasmanian and Mediterranean ribotypes are exclusively nontoxic. The application of microsatellite markers in population studies will help to understand better the development and dynamics of *Alexandrium* blooms. Microsatellite markers have been developed for Japanese strains of *A. tamarense* (Nagai et al. 2004). Here we report the development of six new microsatellite markers for Scottish strains of *A. tamarense* (NA ribotype) and the results of cross-amplifiability tests in other ribotypes and species.

2.2.3. Materials and Methods

Genomic DNA was extracted from an exponentially growing culture of *A. tamarense* (NA ribotype) strain BAHME 182 using a modified cetyltrimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1990). The DNA was enriched for CA repeats following (Edwards et al. 1996). In brief, the DNA was digested with *Alu*I (New England Biolabs), ligated to a *Mlu*I adapter, amplified using one of the adapter oligonucleotides and hybridized to CA dinucleotide repeat oligonucleotides immobilized on Hybond N+ filter paper (Amersham Biosciences). The enriched microsatellites

Table 2.2.1 Attributes of *A. tamarense* strain BAHME 182 (NA ribotype) microsatellite loci: starting annealing temperature during touchdown PCR cycles (TD-T_a, °C), annealing temperature during PCR cycles with constant annealing temperature (T_a, °C), allele size range (bp), number of Alleles (N_A) and number of non-amplifying samples (N_0). The numbers of alleles exclusive to the North Sea and culture collection isolates are given as N_A^{NS} and N_A^{CC}, respectively. All loci were tested in a total number of 56 isolates.

Locus (GenBank Accession no.)	Core sequence	Primer sequence (5'–3')	TD-T_a	T_a	Primer conc. (final)	Size range (bp)	N_A	N_0	N_A^{NS}	N_A^{CC}	Gene diversity (North Sea population)
ATB1 (DQ396619)	$(GT)_4GC(GT)_7GC(GT)_3GC(GT)_4(GC)_2(GT)_4GC((GT)_2GC)_9CTG_3(TCT(G)_3)_{13}$	F: CGCCTGCTCGAGAAAAGA R: TTGGGGGACAGTTGAGTTTC	n/a	53	0.5µM	260–292	5	0	1	3	0.041
ATB8 (DQ396620)	$(GT)_4(CT(GT)_3)_{15}CT(GT)_2(CT)_2G(TA)_2T_2(GC)_2GTGC(GT)_4(GCGT)_4GT(GC(GT)_2)_4GT(GC(GT)_2)_3GCGT(GTGC)_3GC(GT)_3$	F: CAGGGTAGCCGATCAAACAC R: CTTCCATCGCCTTGCATACT	61	54	0.1µM	377–415	8	26	7	0	0.685
ATD8 (DQ396621)	$(CA)_5GA(CA)_2GA((CA)_3GA)_6(CAGG)_3CA_2GC(A)_3CAG_2(CAGA)_3(CA)_3$	F: CAACACTGGAAGCGTGCTAA R: CCCATGCGCTACCTCTTACA	61	54	0.1µM	263–278	6	2	2	2	0.669

Table 2.2.1 (continued)

Locus (GenBank Accession no.)	Core sequence	Primer sequence (5'–3')	TD-T_a	T_a	Primer conc. (final)	Size range (bp)	N_A	N_0	N_A^{NS}	N_A^{CC}	Gene diversity (North Sea population)
ATF1 (DQ396622)	$(GT)_{12}(GCGT)_5(GC)_2$	F: CATTTAGGTTG CGGTGCATA R: TGAGCGACCAA CATGCTTAC	61	54	0.1µM	163–197	9	0	3	2	0.722
ATF11 (DQ396623)	$((GT)_3GC)_7(GT)_3$	F: AGCAGCGCGGC GGGAGATT R: ACCTGCGGCTG CGACACGACT	68.5	61.5	0.1µM	258–315	7	5	3	2	0.248
ATG6 (DQ396624)	$(GT)_{22}$	F: GGTATGCATGT GTGCAGGTG R: CCGATCGCAAG TCCTCTTAG	62.7	55.7	0.1µM	168–204	7	12	3	2	0.383

Table 2.2.2. Strains of the '*A.. tamarense* species complex' and of other *Alexandrium* species used for amplification testing of the six new microsatellite loci. (NA = North American ribotype, TA = Temperate Asian ribotype, WE = Western European ribotype sensu (Scholin et al. 1994), n.i. = no information). Allele length is given in number of base pairs; non-successful amplification is indicated by "–".

Species	Strain	Ribotype	Geographic origin	Collector	ATB 1	ATB 8	ATD 8	ATF 1	ATF 11	ATG 6
A. tamarense	BAH ME 182	NA	Orkney Islands (UK)	M. Elbrächter	286	403	274	169	299	204
	GTL 121	NA	Long Island (USA)	D. Anderson	277	–	267	197	299	176
	OF 84423–D3	NA	Ofunato Bay (Japan)	M. Kodama	286	–	263	167	–	–
	AL18b	NA	St. Lawrence (Canada)	A. Cembella	292	–	274	163	299	–
	31/4	WE	Cork Harbour (Ireland)	W. Higman	–	–	–	–	–	–
	UW 53	WE	Belfast (Northern Ireland)	W. Higman	–	–	–	–	–	–
	UW 42	WE	Belfast (Northern Ireland)	W. Higman	–	–	–	–	–	–
A. fundyense	GT 7	NA	Bay of Fundy (Canada)	A. White	292	–	274	165	268	172
	GTCH 28	NA	n.i.	n.i.	292	–	267	165	276	–
	CCMP 1719	NA	Gulf of Maine (USA)	D. Anderson	292	–	267	165	276	–
	NCCP 407	NA	n.i.	n.i.	260	–	272	175	258	168
A. catenella	BAH ME 215	TA	Tarragona (Spain)	M. Delgado	–	–	–	–	–	–
	BAH ME 217	TA	Tarragona (Spain)	M. Delgado	–	–	–	–	–	–
	BAH ME 220	TA	Tarragona (Spain)	M. Delgado	–	–	–	–	–	–
	BAH ME 222	TA	Tarragona (Spain)	M. Delgado	–	–	–	–	–	–

Table 2.2.2. (continued)

Species	Strain	Ribotype	Geographic origin	Collector	ATB 1	ATB 8	ATD 8	ATF 1	ATF 11	ATG 6
	BAH ME 255	TA	Tarragona (Spain)	M. Delgado	–	–	–	–	–	–
A. insuetum	CCMP 2082		Uchiumi Bay (Japan)	S. Yoshimatsu	–	–	–	–	–	–
A. lusitanicum	BAH ME 91		Laguna Obidos (Portugal)	E. Silva e Sousa	–	–	–	–	–	–
A. ostenfeldii	BAH ME 136		Timaru (New Zealand)	N. Berkett	–	–	–	–	–	–
	AOSH 1		Nova Scotia (Canada)	A. Cembella	–	–	–	–	–	–
A. taylori	Ay1T		Lagoon of Marano (Italy)	A. Beran	–	–	–	–	–	–
	Ay2T		Lagoon of Marano (Italy)	A. Beran	–	–	–	–	–	–

were re-amplified as above and cloned into pJV1 vector (Edwards et al. 1996). Cloned fragments were amplified using M13 forward and reverse primers (MWG Biotech) and sequenced using the DYEnamic ET Dye Terminator Cycle Sequencing Kit and a Mega-BACE 1000 sequencer (Amersham Biosciences).

2.2.4. Results and Discussion

Ninety-five clones (47 %) contained microsatellites. PRIMER 3 (Rozen and Skaletsky 2000) was used to design primer pairs flanking 13 cloned CA-repeats. Their ability to amplify the corresponding microsatellite loci was tested using a Mastercycler gradient thermal cycler (Eppendorf). The 20-µL reactions contained 10–20 ng of DNA, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl 2 , 0.2 mM dNTPs, 0.1 or 0.5 µM of each primer and 1 U of HotMaster™ Taq DNA polymerase (Eppendorf). After 5 min at 94 °C, 40 or 45 cycles were performed: 20 s at 94 °C, 10 s at 51.5–68.5 °C, and 30 s at 70 °C. The annealing temperature was either constant for 40 cycles, or was reduced by 0.3 °C per cycle for 25 cycles, followed by 20 cycles at a constant annealing temperature (Table 2.2.1).

Six microsatellite primer pairs (Table 2.2.1) generated amplification products of the expected size; 5'-fluorescently labelled forward primers (6-FAM, HEX or NED; Applied Biosystems) were used in repeat reactions using 74 isolates belonging to different *A. tamarense* 'species complex' ribotypes and *Alexandrium* species (*A. minutum*, *A. lusitanicum*, *A. insuetum*, *A. ostenfeldii* and *A. taylori*; se Table 2.2.2). Fifty-six NA ribotype isolates were genotyped: 48 were collected in 2004 from one eastern Scottish North Sea water sample and eight came from culture collections. Amplification of glutamate synthase (GOGAT; (Röttgers 2002) confirmed the amplifiability of each DNA template. One microliter of the PCR generated amplicons in 15 µL of Hi-Di formamide (Applied Biosystems) and 0.5 µL of the size marker GENESCAN–500 [ROX] (Applied Biosystems) were sized using a 3130xl Genetic Analyzer (Applied Biosystems). Microsatellite alleles were scored using GENEMAPPER version 3.5 software (Applied Biosystems). The six primer-pairs yielded 42 scoreable microsatellite alleles with a minimum of five and a maximum of nine per locus (Table 2.2.1). ARLEQUIN version 2.000 (Schneider et al. 2000) was used to test pairs of loci for linkage disequilibrium (Slatkin 1994). Using the data derived from the 48 North Sea individuals, none of the pairs of loci was found to be in significant linkage disequilibrium ($p = 0.05$).

Only NA ribotype isolates of the *A. tamarense* 'species complex' yielded amplification products, indicating that cross-amplification of the microsatellite loci does not occur between ribotypes and species. The numbers of alleles and gene diversities (Nei 1987) in the North Sea isolates suggest that the loci are sufficiently polymorphic for use in population studies. Eleven and 19 alleles were unique to the culture collection strains and North Sea isolates, respectively, suggesting a high degree of molecular diversity within the NA ribotype.

2.3. SPECIES DISCRIMINATION IN THE GENUS *ALEXANDRIUM* BY AMPLIFIED FRAGMENT LENGTH POLYMORPHISM

2.3.1. Abstract

Amplified fragment length polymorphism (AFLP) is a molecular technique for genotypic characterization of individuals or clonal strains, genetic differentiation of natural populations and phylogenetic analyses. We applied the AFLP technique for species discrimination by phylogenetic analyses within *Alexandrium*, a marine dinoflagellate genus that contains several PSP toxin-producing species of uncertain taxonomic status. Clonal isolates from three *Alexandrium* spp. – *A. tamutum*, *A. tamarense* and *A. ostenfeldii* – were included in the phylogenetic analysis. Evolutionary genome divergence, expressed as the estimated ratio of nucleotide substitutions per site showed a clear phylogenetic separation of the three species, which is in concordance with previous sequence data analysis. In addition, AFLP showed a separation of several distinct groups within *A. tamutum* and *A. tamarense* and thereby allows a deeper insight into the genetic sub-structure within these species. Analysis of genotypic variability among clonal isolates by AFLP is a promising tool for investigating correlations between genotypic and phenotypic population markers as well as the genetic causes of phenotypic diversity in biogeographical studies of natural populations.

2.3.2. Introduction

The dinoflagellate genus *Alexandrium* forms recurrent blooms in coastal regions around the world, and is the main organism causing paralytic shellfish poisoning (PSP). The genus includes more than 28 described species (Balech 1995), of which nine are known to produce the potent neurotoxin saxitoxin and/or some of the >20 naturally occurring derivatives. The taxonomic status of some species within the genus is under continuing debate (Fraga et al. 2006).

In addition to classical morphological characteristics, such as thecal plate morphology and pattern, analysis of molecular sequence data has been useful in elucidating phylogenetic relationships among *Alexandrium* spp. (Scholin et al. 1994; Montresor et al. 2004). However, in some instances, such as in the '*Alexandrium tamarense* species complex', apparently distinct morphospecies (e.g., *A. tamarense*, *A. catenella* and *A. fundyense*) cannot be separated by phylogenetic analysis of molecular sequence data, but rather they fall into ribotypes of different geographical origin, each containing one or several distinct morphospecies (Scholin et al. 1994).

To resolve phylogenetic relationships with a fine resolution on the specific or sub-specific level, molecular markers other than the slowly evolving nuclear genes are needed to provide additional support for taxonomic separation of closely related taxa. The amplified fragment length polymorphism (AFLP) technique has been applied to several groups of organisms at the population level, as well as to apparently closely related species in inter-specific studies. This techniques

produces a suite of multiple markers by restriction and selective amplification of subsets of genomic DNA restriction fragments, and thereby allows for characterization of individual isolates by their genotypic signature (Vos et al. 1995). Among the lower eukaryotes, AFLP has been used for discrimination of isolates at the inter- and intra-specific level (e.g., (Mueller and Wolfenbarger 1999) and references therein). While AFLP can distinguish among different geographical populations of *A. tamarense* (John et al. 2004; Alpermann et al. 2006b), its discriminating power at the inter-specific level has not been previously tested for dinoflagellates.

To test the applicability of AFLP in phylogenetic studies, we adopted a mathematical method that estimates the rate of nucleotide substitutions over whole genomes. The underlying assumption is that all AFLP fragments correspond to specific nucleotide sequences of the genome of a common ancestor, namely, the two restriction sites plus the selective bases of the selective primers for PCR amplification of AFLP fragments.

In addition, we investigated the potential of AFLP to describe genetic sub-structuring of populations of different *Alexandrium* spp. by a comparative analysis of AFLP patterns of isolates of *A. tamarense* and *A. tamutum*.

2.3.3. Materials and Methods

Twenty-three clonal cultures of dinoflagellates exhibiting an *Alexandrium*-like habitus under a stereo-microscope were established by single cell micro-pipette isolation from a natural water sample, obtained from coastal waters of the Scottish east coast. DNA was extracted from exponentially growing cultures (4 to 8 x 10^4 cells mL^{-1}) that were treated with antibiotics (100 µg mL^{-1} penicillin; 25 µg mL^{-1} streptomycin; both final concentrations) to minimize bacterial contamination. In brief, 240 ng of DNA was digested in 50 µL with the restriction endonucleases EcoRI and MseI. Specific adaptors were ligated to one end of each restriction site. Primers for both the MseI and the EcoRI restriction site, containing the adaptor and restriction site motif and one additional selective nucleotide, were used in the pre-amplification PCR to amplify a subset of the ligated DNA fragments containing both restriction sites. In four separate PCR amplification reactions with different combinations of one MseI and one EcoRI primer, which contained two more selective nucleotides, a smaller subset of pre-amplified fragments was amplified. The AFLP alleles were identified after capillary electrophoresis on an ABI 3170 XL automated sequencer with the GENEMAPPER ® v3.7 software (Applied Biosystems, Darmstadt, Germany). Binning of AFLP markers was performed in a size range from 100 bp to 500 bp at a threshold level of 250 for all AFLP primer combinations and subsequent automatic scoring of AFLP fragments was carried out with a threshold of 30.

Evolutionary genome divergence, expressed as the number of nucleotide substitutions per site was calculated using the Dice similarity index with the programme DISTAFLP after Mougel et al. (2002), where the evolutionary distance between two individuals is corrected to account for unobserved substitutions according to the standard Jukes-Cantor model (Swofford et al. 1996). A phylogenetic tree was constructed with PHYLIP (Felsenstein 1993) by the neighbour-joining

Table 2.3.1. Comparative characteristics of AFLP data for *A. tamutum* (12 isolates) and *A. tamarense* (10 isolates) generated with 4 different pairs of PCR primers and a total of 481 alleles produced for isolates from these two species.

	A. tamutum	***A. tamarense***
# of loci*	417	300
# of unique loci*	181	64
Average # of loci*	104 (S.D. 23.0)	116 (S.D. 22.1)
Average $\hat{t}$ to *A. tamutum*	0.063	0.089
Average $\hat{t}$ to *A. tamarense*	0.089	0.041

Method (Felsenstein 1985); 1000 bootstrapped replicates were run to confirm tree topology.

Sequencing of the D1/D2 region of the LSU rDNA was performed after PCR amplification with primers DIRF and D2CR (Scholin et al. 1994) and subsequent clean-up of PCR products using the forward primer DIR-F in the sequencing reaction. Sequences were compared to the NCBI database by BLAST search to confirm membership of our isolates to one of the species *A. tamutum*, *A. ostenfeldii* or the *A. tamarense* North American ribotype.

2.3.4. Results and Discussion

According to their partial rDNA sequences, one of the clonal *Alexandrium* isolates was identified as *A. ostenfeldii*, ten as *A. tamarense* and twelve as *A. tamutum*. The isolates of all *Alexandrium* spp. gave similar numbers of AFLP fragments – an average of about 25 fragments per clonal isolate per combination of selective primers. Interestingly, the isolates of *A. tamutum* produced a considerably higher number of AFLP loci, of which a greater proportion of loci were unique for the species when compared to *A. tamarense* (Table 2.3.1). This was also expressed in a higher averaged value for the evolutionary genome divergence among isolates of *A. tamutum*, when compared to that among *A. tamarense* isolates. The average estimated rate of nucleotide substitutions per site (0.089) between isolates of *A. tamutum* and *A. tamarense* indicates that considerable nucleotide substitution must have occurred in the evolution of the two species. However, since AFLP is a molecular marker that produces alleles irrespective if the DNA region is a coding region or not, these nucleotide substitutions do not necessarily reflect the degree of functional or physiological divergence of the two species from their common ancestor.

Phylogenetic analysis of AFLP genotypes revealed not only the existence of separated branches for the three species, but also showed that separated groups of genotypes supported by high bootstrap values exist within *A. tamutum* and *A. tamarense* (Fig. 2.3.1). The higher genetic variability

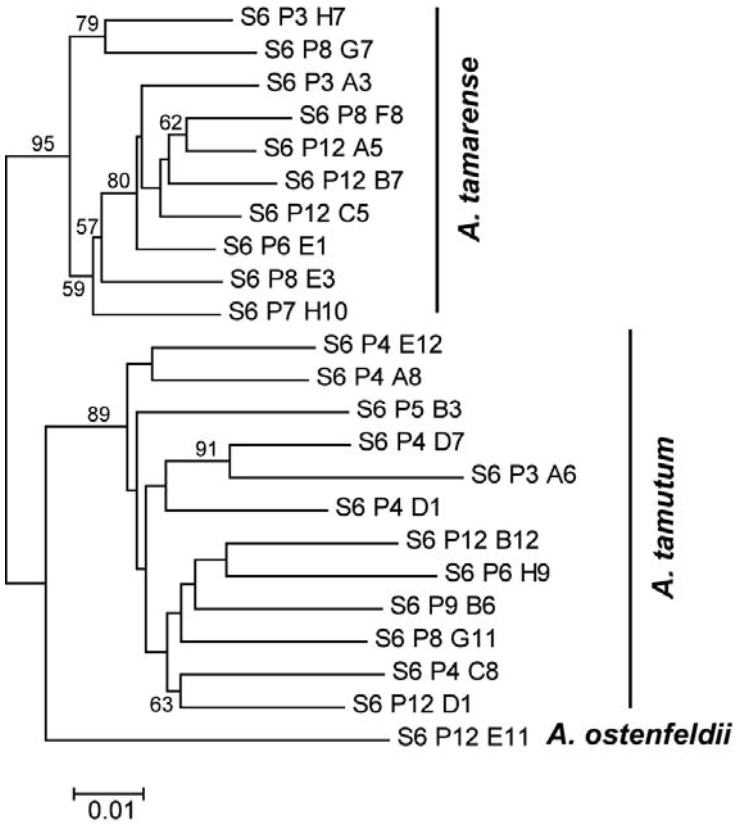

Fig. 2.3.1. Phylogenetic relationships of North Sea isolates of *Alexandrium* spp. inferred from evolutionary genome divergences determined by AFLP analysis. The dendrogram was generated by the neighbour-joining method with the estimated ratio of nucleotide substitution over whole genomes. Branching robustness is expressed as the percentage reliability after 1000 bootstrap resamplings corrected for fragment dependences (only bootstrap values above 55 % are indicated). The bar indicates the rate of base substitutions.

within *A. tamutum* is reflected in the comparatively deeper branching within the *A. tamutum* isolates included in this study. An evolutionary explanation for this observation is not readily at hand.

The general conclusion that populations of *A. tamutum* are genetically more diverse, or that separated groups of genotypes would persist if more isolates were included in the study, cannot be drawn with certainty. The number of isolates in this study is too small to represent the overall genetic diversity within natural populations of these two species. Additionally, not much is known about the variability and stability of the genotypic composition of natural populations of *Alexandrium* species during the different phases of bloom development and decline. Different physical and biotic factors may lead to the promotion of certain genotypes, which might be expressed in shifts of the genotypic composition of a population over a relatively short time. Whether or not neutral genetic markers such as AFLP can reflect such processes at all is crucial for these considerations.

In this study, we showed the usefulness of AFLP for species discrimination within the genus *Alexandrium*. Previous work using the same protocol, e.g., John et al. (2004), has shown the high reproducibility of AFLP band patterns in *Alexandrium*; we did not therefore specifically test this property in the inter-specific comparison. Each of the multilocus genotypes of *A. tamutum* and *A. tamarense* isolates represents a biological replicate in our analysis. Furthermore, the large number of markers generated with AFLP substantially advances the robustness of analyses based upon individual multilocus genotypes (Hollingsworth and Ennos 2004). At a time when the taxonomic and evolutionary significance of morphological characters for species discrimination among the lower eukaryotes must be reconciled with often discordant taxonomic and phylogenetic

inferences based on molecular markers of single genes (e.g., LSU and SSU rDNA, ITS, etc.), AFLP provides additional support for molecular phylogenies. In prokaryotes, the potential of AFLP to replace the obligate genetic marker for species definition (DNA–DNA hybridization) has already been shown (Mougel et al. 2002). For eukaryotic microalgae with unstable or indistinguishable morphologies, AFLP may prove to be a valid phylogenetic criterion, as well. Further applications of AFLP, e.g., for quantitative trait mapping or candidate gene identification with cDNA-AFLP, also seem to be auspicious for functional diversity studies.

2.3.5. Conclusions

This is the first published work showing the utility of AFLP for discrimination of members of the genus *Alexandrium*. Although the genetic differentiation of the three species was already shown by comparison of the LSU rDNA sequences, the novelty of the AFLP approach is supported by the possibility of gaining deeper insight into the inter-specific genetic differentiation at the species and population level at the same time. The universal application of AFLP to genetic diversity and species differentiation in dinoflagellates opens new avenues to study the evolution and functional ecology of marine microalgae.

2.4. PHENOTYPIC VARIATION AND GENOTYPIC DIVERSITY IN A PLANKTONIC POPULATION OF THE TOXIGENIC MARINE DINOFLAGELLATE *ALEXANDRIUM TAMARENSE* (DINOPHYCEAE)

2.4.1. Abstract

Multiple clonal isolates from a geographical population of *Alexandrium tamarense* (Lebour) Balech from the North Sea exhibited high genotypic and phenotypic variation. Genetic heterogeneity was such that no clonal lineage was repeatedly sampled according to genotypic markers specified by amplified fragment length polymorphism (AFLP) and microsatellites. Subsampling of genotypic data from both markers showed that ordination of individuals by pairwise genetic dissimilarity indices was more reliable by AFLP (482 biallelic loci) than by microsatellites (18 loci). However, resulting patterns of pairwise genetic similarities from both markers were significantly correlated (Mantel test $p < 0.005$). The composition of neurotoxins associated with paralytic shellfish poisoning (PSP) was also highly diverse among these isolates and allowed clustering of toxin phenotypes based upon prevalence of individual toxins. Correlation analysis of pairwise relatedness of individual clones according to PSP toxin profiles and both genotypic characters failed to yield close associations. The expression of allelochemical properties against the cryptophyte *Rhodomonas salina* (Wislouch) D. R. A. Hill et Wetherbee and the predatory dinoflagellate *Oxyrrhis marina* Dujard. manifested population-wide variation of responses in the target species, from no visible effect to complete lysis of target cells. Whereas the high genotypic variation indicates high potential for adaptability of the population, we interpret the wide phenotypic variation as evidence for lack of strong selective pressure on respective phenotypic traits at the time the population was sampled. Population markers as applied here may elucidate the ecological significance of respective traits when followed under variable environmental conditions, thereby revealing how variation is maintained within populations.

2.4.2. Introduction

Marine microalgae in coastal regions live in dynamic environments characterized by frequent and often sudden changes in ambient conditions. Abiotic parameters, such as turbidity, light intensity, salinity and nutrient availability, can vary dramatically within sub-meter spatial scales in the water column. Additionally, biotic interactions among microalgae and potential grazers or pathogens are major determinants that influence the population growth of particular microalgal species (Smayda 1997; Tillmann 2004). If changes in ambient conditions reduce growth, microalgal populations can secure their persistence or eventually restore net growth in two principally different ways. One mechanism is for all individuals of the respective population to express a common phenotypic plasticity, allowing them to physiologically acclimate to new conditions. The other mechanism to

cope with altered ambient conditions is by expression of population-wide phenotypic variation, enabling growth of those individuals that express the relatively better-adapted phenotypes for the respective environmental conditions. Physiological acclimation, with all individuals growing equally well under changing ambient conditions, would tend to result in more or less uniform populations for the respective phenotypes, whereas adaptation of populations would lead to phenotypic diversity due to balancing selection, especially if changes in determining environmental variables (forcing functions) occur frequently. The amount and patterns of intra-specific phenotypic variation are therefore crucial factors determining the ecological and micro-evolutionary fate of microalgal species and populations.

Underlying genetic variation, the basis for observed phenotypic variation, is assumed to reflect the ability of a population to adapt to changing environments (Fisher 1930; Barrett and Schluter 2008). Several studies of marine microalgae, particularly of diatoms (see Medlin et al. 2000) have revealed a high degree of cryptic variation, both phenotypic and genotypic, within microalgal species and even within single geographically defined populations. In some cases, microalgal species showing large variation were later attributed to species complexes (e.g., *Skeletonema costatum* (Gallagher 1980; Gallagher 1982; Sarno et al. 2005; Kooistra et al. 2008) and *Alexandrium tamarense* (Scholin et al. 1995)). Most studies of intra-specific variation within microalgal species or populations, however, have concentrated either on phenotypic or genotypic variation, or did not specifically compare variation at the population- or species-level. This is very unfortunate, since studies that integrate information on diversity and population-wide distribution of key phenotypic traits with that obtained by fine-scale genotypic markers are promising approaches to tackle questions on the evolutionary ecology of planktonic microalgae.

Such an integrative approach was chosen in this study to investigate population characteristics of the toxic marine dinoflagellate *Alexandrium tamarense*. This dinoflagellate can form harmful algal blooms (HABs), particularly along temperate and subtropical coasts. The '*Alexandrium tamarense* species complex' includes different morphotypes that were assigned species status before the advent of molecular genetic markers (Balech 1995 and references therein). According to LSU rDNA sequences, members of the '*A. tamarense* species complex' fall into ribotypes of geographically distinct origin and distribution, each containing one or more different morphotypes (Scholin et al. 1994; John et al. 2003). In addition to their genetic differentiation, ribotypes differ phenotypically by the presence or absence of neurotoxins associated with paralytic shellfish poisoning (PSP) (Scholin et al. 1994; John et al. 2003; Lilly et al. 2007). The PSP toxins comprise more than twenty naturally occurring derivatives of the tetrahydropurine saxitoxin that may be produced among toxigenic strains, although typically the toxin profile is dominated by few analogues within a given strain. The ecological and evolutionary significance of PSP toxins to the producing dinoflagellate is not well understood, but toxigenic clonal isolates of the same ribotypes may produce markedly different relative amounts of various saxitoxin derivatives (Cembella 2003). The resulting PSP toxin composition within a clonal isolate is rather stable under a range of environmental conditions (Hall 1982; Boyer et al. 1987; Cembella et al. 1987; Ogata et al. 1987a).

Variation in PSP toxins has been the focus of most previous studies (Hall 1982; Cembella et al. 1987; Anderson et al. 1994; Cembella and Destombe 1996; Ichimi et al. 2002) for comparison of regionally predominating toxin profiles or changes in toxin composition or cell content under

different ambient conditions in culture. Consequently, only single strains or a few isolates derived from local populations have been studied and these then were assumed to be characteristic of their geographic origin. However, an in-depth analysis of intra-population diversity of this intriguing phenotypic trait with a multitude of isolates is lacking. Such an analysis might provide important insights into the genetics of toxin production in *Alexandrium*.

Allelopathic interactions among microalgal species or allelochemical defence against grazing may have a major effect in determining which species attain bloom concentrations in a certain plankton community (Smayda 1997; Tillmann 2004). Almost all *Alexandrium* species tested contain strains known to express allelochemical properties, usually manifested as lytic or membrane-disruptive activity, against a wide spectrum of protistan target species (Tillmann et al. 2008a). The potency against protists is dependent upon susceptibility of the given target species, but capacity to elicit allelochemical activity also varies widely among strains of different *Alexandrium* species and within the same species among strains from different geographical origins (e.g., Tillmann and John 2002). In all cases, these allelochemical responses are apparently unrelated to the content of PSP toxins or other known biotoxins, such as the macrocyclic imine spirolides, which may be produced among *Alexandrium* strains (Tillmann and John 2002; Fistarol et al. 2004b; Tillmann et al. 2007).

Ribosomal DNA sequences have served well for grouping members of the *Alexandrium tamarense* species complex into regional ribotypes, but other genotypic markers with higher resolving power at the population level are needed to assess genotypic diversity within populations of established LSU ribotypes. Amplified fragment length polymorphism (AFLP) (Vos et al. 1995) and microsatellites or simple sequence repeats (SSR) (Tautz and Renz 1984) are two such presumably neutral genetic markers widely used for assessment of genotypic diversification within and among populations. Although these markers have been previously applied to *A. tamarense* in studies on gene diversity (Nagai et al. 2004; Alpermann et al. 2006a), population differentiation (Nagai et al. 2007a), regional genotypic differentiation (John et al. 2004) and genome divergence (Alpermann et al. 2008), the extent of population-wide genotypic diversity as a common feature in a natural population of *A. tamarense* has not been evaluated before.

Our study simultaneously addressed phenotypic variation and genotypic diversity among clonal isolates from a single *Alexandrium tamarense* population. Here our primary aim was to assess the degree to which different phenotypic and genotypic characters vary within one planktonic population from the Scottish east coast. Following from this, our second objective was to discover whether or not population-wide patterns that result from this variation by multivariate ordination are congruent for different characters or if certain AFLP loci are associated with phenotypic characteristics in the sampled population. The phenotypic markers we chose were profiles and cellular content of PSP toxins as well as allelochemical properties, which we assessed by a survey of a large number of clonal isolates derived from the same source population. For the characterization of the genotypic diversity in this natural population, we genotyped the isolates by multiallelic microsatellite (Nagai et al. 2004; Alpermann et al. 2006a) and biallelic AFLP markers (Vos et al. 1995; John et al. 2004). This study provides the first detailed multi-parameter evidence for high genotypic and phenotypic variation within a population of a marine dinoflagellate. The population-wide heterogeneities in phenotypic and genotypic traits apparent from this study provide

important indicators for the ecological significance of specific characters for development of *A. tamarense* populations and blooms.

2.4.3. Materials and Methods

Establishment and culture of clonal Alexandrium tamarense *isolates*

In May 2004 a natural population of *Alexandrium tamarense* (Lebour) Balech was sampled from the North Sea coast of Scotland at 56° 05' 47'' N and 1° 42' 35' 'W. Phytoplankton was collected by repeated vertical hauls of a 20 µm-mesh plankton net from 20 m depth to the surface. Utermöhl counts of *Alexandrium* spp. yielded an *in situ* concentration of about 90 cells L^{-1} based upon estimates from net tows from the water column. Single *A. tamarense* cells were isolated from this sample under a stereo-microscope by micropipette. The cells were transferred into individual wells of 96-well tissue culture plates (TPP, Trasadingen, Switzerland) containing 150 µL of K medium (Keller et al. 1987), supplemented with selenite (Dahl et al. 1989), prepared from 0.2 µm sterile-filtered natural Antarctic seawater diluted with seawater from the sampling location in a ratio of 1:10. Isolated cells were then incubated at 10 °C under artificial light at a photon flux density of 100 µmol photons m^{-2} s^{-1} on a 16:8 h light–dark photocycle. After three to four weeks, unialgal isolates were transferred to 24-well tissue culture plates, each well containing 1.5 mL of K medium diluted 1:5 with Antarctic seawater and incubated at 15 °C under the previous photo-regime. Exponentially growing isolates were finally used as inoculum for batch cultures in polystyrene cell culture flasks each containing 50 mL of K medium and were maintained thereafter under the same conditions as described. From a total of more than 100 clonal isolates of *A. tamarense*, 88 clones were arbitrarily chosen to study the genotypic and phenotypic diversity and genetic structure within this natural population.

DNA extraction and ribotyping of clonal isolates

Freshly inoculated batch cultures (800 mL) of 88 clonal isolates of *A. tamarense* were treated with the antibiotics (Sigma, Munich, Germany) penicillin (final concentration 100 µg mL^{-1}) and streptomycin (final concentration 25 µg mL^{-1}) and harvested about 3 weeks later when they reached mid- to late-exponential growth phase (3 to 8 x 10^3 cells mL^{-1}) by gravity filtration over a 10 µm polyethylene (Nitex) mesh. The cells were resuspended and washed twice in about 50 mL of filtered seawater and filtered again through the mesh. Finally, the cells were resuspended in 50 mL of filtered seawater and transferred to 50 mL conical centrifugation tubes and then centrifuged at 3,200 x g for 15 min. The supernatant was discarded and the pellet was resuspended in 1.5 mL of filtered seawater and transferred to a 2 mL reaction tube. After micro-centrifugation of the cell suspension at 16,000 x g for 5 min, the supernatant was removed and the cell pellet was shock-frozen in liquid nitrogen and stored at –20 °C until DNA extraction.

For cell disruption the frozen cell pellet was chilled in liquid nitrogen and homogenized twice at 20 Hz for 1 min in a mixer mill MM200 (Retsch, Haan, Germany). DNA was subsequently

extracted with a DNeasy Plant Mini Kit or a DNeasy 96 Plant Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The purity and quantity of the DNA was checked with a NanoDrop ND-1000 UV-spectro-photometer (Peqlab, Erlangen, Germany) and the integrity of DNA fragments of a molecular weight of about 20 kb was verified on a 0.8% agarose gel.

All isolates were confirmed to belong to Group I (Lilly et al. 2007) of the *A. tamarense* species complex (formerly North American ribotype) by sequencing of the D1-D2 LSU rDNA region (Scholin et al. 1994). Sequences were deposited in GenBank (accession numbers FJ404475 to FJ404562).

Amplified fragment length polymorphism (AFLP) genotyping

Assessment of polymorphic loci was carried out according to the AFLP protocol of John et al. (2004) (see also for sequences of adapters in the ligation and primers in the pre-amplification reactions). *Alexandrium* DNA (240 ng) was digested for 15 h at 37 °C in a 50 µL digestion reaction mix containing 1 µL EcoRI (20 U μL^{-1}), 1 µL MseI (10 U μL^{-1}), 0.5 µL BSA and 5 µL restriction buffer 2 (all reagents from New England BioLabs, Frankfurt am Main, Germany). Afterwards the enzymes were heat-inactivated at 65 °C for 15 min. The restriction mix (17 µL) was added to 8 µL of a ligation mix consisting of 2.5 µL water, 1.7 µL ATP (10 mM), 1 µL EcoRI adapter (10 pmol μL^{-1}), 1 µL MseI adapter (50 pmol μL^{-1}), 0.8 µL T4 ligase buffer and 1 µL T4 DNA ligase (1 U μL^{-1}, GE Health Care Life Sciences, Munich, Germany). The mix was incubated for 9 h at 16 °C and subsequently for 6 h at 21 °C. The ligation reaction was then stopped by heating for 10 min at 65 °C. The ligated DNA was diluted 1:5 in 0.1x Tris-EDTA buffer and 6.67 µL of it was added to a PCR mix consisting of 8.33 µL water 0.33 µL EcoRI+A primer (10 µM), 0.33 µL MseI+C primer (10 µM), 2 µL 10x Taq DNA polymerase buffer, 2 µL dNTP-Mix (1 mM each) and 0.33 µL HotMaster™ Taq DNA Polymerase (5 U μL^{-1}) (all PCR reagents from Eppendorf, Hamburg, Germany). This reaction mix was cycled 22 times for preamplification of the ligated DNA in a Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany) at 94°C for 30 s (denaturation), 56 °C for 30 s (annealing) and 72 °C for 1 min (extension). The PCR products were diluted 1:10 in 0.1x Tris-EDTA buffer. The final selective amplification was carried out with the diluted reaction product of the preamplification reaction and four different combinations of selective primers in separate reactions, where each selective primer contained three selective bases (see Table 2.4.1). The amplification reaction mix contained 3.33 µL of the diluted preamplification reaction product, 7.8 µL of water, 1.33 µL 10x Taq DNA polymerase buffer, 0.13 µL dNTP-Mix (10 mM each), 0.4 µL MseI amplification primer (10 pmol μL^{-1}), 0.07 µL of 6'-Fam labelled EcoRI amplification primer (10 pmol μL^{-1}) and 0.27 µL HotMasterTM Taq DNA Polymerase (5 U μL^{-1}) (all PCR reagents from Eppendorf, Hamburg, Germany). Amplification by touchdown PCR was performed with an initial denaturation at 94 °C for 30 s and a first cycle at 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. One µL of the product of the amplification reaction in 15 µL HiDi™ formamide and 0.5 µL of the size-marker GeneScan™–500[ROX]™ (both reagents from Applied Biosystems, Darmstadt, Germany) were sized by a capillary sequencer

Table 2.4.1. Combinations of primers and their characteristics in the AFLP amplification reaction.

Primer combination	EcoRI-Primer	MseI-Primer	Number of polymorphic loci	Mean number of present alleles (± SD)
1	EcoRI+AAG	MseI+CTA	109	26.5 (± 7.7)
2	EcoRI+AGG	MseI+CCG	117	23.2 (± 7.7)
3	EcoRI+AAG	MseI+CTT	133	41.5 (± 13.5)
4	EcoRI+AGG	MseI+CCT	123	34.5 (± 10.5)

ABI 3130XL (Applied Biosystems). Sizing and scoring of AFLP fragments in a range from 100 to 500 bp was carried out with the help of GENEMAPPER ® v4.0 (Applied Biosystems) with a signal intensity threshold of "250" for binning and a threshold of "50" for scoring of AFLP loci. Signal intensity was normalized over all samples for binning and scoring.

Microsatellite genotyping

Eighteen previously characterized microsatellite loci (Nagai et al. 2004; Alpermann et al. 2006a) were amplified from the same DNA templates of the 88 clonal *A. tamarense* isolates used for AFLP analysis, with specific primers developed for the *A. tamarense* North American ribotype (Table 2.4.2). The PCR conditions were as described in Alpermann et al. (2006). In brief, 20 µL PCR reactions were run for each pair of specific primers containing 10–20 ng of DNA, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.1 µM of each primer (except locus ATB1 with primer concentrations of 0.5 µM) and 1 unit of HotMaster™ Taq DNA Polymerase (Eppendorf, Hamburg, Germany). The PCR was performed either with a constant annealing temperature for 40 PCR cycles (only for loci ATB1) or as a touchdown PCR with 20 cycles, in which the annealing temperature was reduced by 0.3 °C every cycle followed by 25 cycles at constant annealing temperature (Table 2.4.2). One of the two primers in each PCR was 5' terminally labelled with either 6-FAM, Ned or Hex (Applied Biosystems, Darmstadt, Germany). Sizing and scoring of microsatellite alleles was carried out with the software GENEMAPPER ® v3.7 (Applied Biosystems) after capillary electrophoresis as performed for AFLP amplicons.

Analysis of AFLP and microsatellite genotypic data

From the 88 clonal isolates initially analyzed by AFLP and microsatellite genotyping, eleven isolates were excluded from the joint analysis of these markers. For two excluded isolates PCR amplification failed in the AFLP amplification reactions and nine isolates yielded scoreable amplification products for only half or fewer of the 18 microsatellite loci. The number of alleles per locus and of unique multilocus genotypes was assessed separately for the AFLP and microsatellite

Table 2.4.2. Specifications, experimental parameters and results of microsatellite loci for genotyping *A. tamarense* isolates. (TD-T_a, annealing temperature of first cycle of touch-down PCR; T_a, constant annealing temperature)

Locus	TD-T_a	T_a	Number of alleles	Gene diversity ($\hat{H}$) [a]
ATB1 [b]	–	53 °C	2	0.31
ATB8 [b]	61 °C	54 °C	9	0.76
ATD8 [b]	61 °C	54 °C	4	0.74
ATF1 [b]	61 °C	54 °C	8	0.70
ATF11 [b]	68.5 °C	61.5 °C	6	0.35
ATG6 [b]	62.7 °C	55.7 °C	5	0.68
Atama04 [c]	61 °C	54 °C	3	0.57
Atama06 [c]	67 °C	60 °C	3	0.53
Atama13 [c]	60 °C	53 °C	6	0.73
Atama15 [c]	63 °C	56 °C	8	0.68
Atama16 [c]	63 °C	56 °C	6	0.56
Atama17 [c]	59 °C	52 °C	5	0.73
Atama23 [c]	59 °C	52 °C	3	0.49
Atama26 [c]	56 °C	49 °C	4	0.50
Atama27 [c]	58 °C	51 °C	10	0.83
Atama32 [c]	60 °C	53 °C	7	0.83
Atama39 [c]	59 °C	52 °C	3	0.50
Atama42 [c]	59 °C	52 °C	7	0.70

[a] Nei 1987; [b] Alpermann et al. 2006; [c] Nagai et al. 2004

datasets of the remaining 77 isolates in the software ARLEQUIN Ver. 2.0 (Schneider et al. 2000). As a measure for the capacity of gene loci to detect genetically differentiated individuals gene diversity for each microsatellite and AFLP locus was estimated with the program LIAN (Haubold and Hudson 2000) after the formula for $\hat{H}$ (Nei 1987) through the web interface of Version 3.5 (http://adenine.biz.fh-weihenstephan.de/cgi-bin/lian/lian.cgi.pl). The value of $\hat{H}$ has a minimum of 0 (in the case of an uninformative locus with only one allele) and approximates 1 for the most

informative loci (i.e. loci with many alleles at equally low frequency). For biallelic AFLP loci the maximum value, however, is about 0.5.

The pairwise similarities of microsatellite and AFLP genotypes were calculated separately in BIONUMERICS v2.50 (Applied Maths, Sint-Martens-Latem, Belgium) statistical software according to the Dice and the Simple Match index of similarity, respectively (for a review on appropriate similarity indices for different genotypic markers see Kosman and Leonard 2005). The unweighted pair group method with arithmetic mean (UPGMA) dendrograms were constructed with the same software. Two different resampling approaches were adopted for each marker to obtain an independent measure of the reproducibility of the arrangement of individuals in the dendrograms. The dependence of the results on the number of loci was tested at the same time. To check the degree of consistency of pairwise dissimilarity matrices obtained with different markers and numbers of loci, two arbitrary, non-overlapping subsets of loci were sampled from all loci from the AFLP and microsatellite data with ten and three loci, respectively. Then pairwise Dice or Simple Match dissimilarities were calculated for each subset and a Mantel test (Mantel 1967) was performed on the resulting dissimilarity matrices by the Pearson product-momentum correlation. The procedure was repeated 50 times and the average correlation coefficients and significances were obtained. The number of markers included in the bootstrapped subsets was then stepwise raised by five loci for AFLP and one locus for microsatellites, until 240 and nine loci were reached, respectively. The second approach, which corresponds to that developed by King et al. (1993) and Tivang et al. (1994), also involved bootstrapping of subsets of different numbers of loci for each marker. Here 100 subsets of a certain number of AFLP or microsatellite loci were arbitrarily sampled from all loci and pairwise Simple Match or Dice dissimilarities were calculated for each subset of AFLP or microsatellite loci, respectively. The average coefficient of variation (CV) was calculated from all pairwise distances. The markers included in the bootstrapped sets of loci were increased stepwise for AFLP and microsatellite subsets by ten and one loci, starting with eleven and two loci per bootstrapped subset, respectively, until subsets were as large as the total number of AFLP and microsatellite loci. A linear regression was then calculated with log transformed numbers of loci and mean CV values for each number of loci used in the bootstrap procedure. The number of loci needed to reach CV values of 0.1 and 0.05 were estimated by inter- or extrapolation using the regression function. All bootstrap procedures and regression analyses were performed in the statistical software package R (R Development Core Team 2007); the R functions with example data and usage instructions are available for download (http://epic.awi.de/epic/Main?static=yes&page =abstract&entry_dn=Alp2010a) or may be obtained on request from the authors.

Qualitative and quantitative analysis of PSP toxins

Batch cultures of all 88 clonal *A. tamarense* isolates were grown in 50 mL cell culture flasks and harvested by centrifugation as for DNA extraction when cultures were in early to mid-exponential growth phase (at about 1.5 to 4 x 10^6 cells L^{-1}). One mL of the same cultures was fixed with Lugol's iodine solution and later cells were counted on an Axiovert (Zeiss, Jena, Germany) inverted microscope.

Toxin analysis was performed by automated reverse-phase high performance liquid chromatography (HPLC), applying ion-pair chromatographic separation, followed by post-column oxidation with periodic acid and fluorescence detection, based on the method of Thielert et al. (1991) modified with details by Hummert et al. (1997) and Yu et al. (1998). The sample preparation protocol of Hummert et al. (1997) was followed for extraction of PSP toxins from the frozen cell pellets by addition of 1 mL 0.03 N acetic acid to each pellet and sonication of cells. The 0.45µm PTFE-filtered acetic acid cell lysates were injected into a HPLC system consisting of a SIL-10A intelligent autosampler, a LC-10ATvp intelligent pump, a SCL-10Avp system controller, a 1 mL CRX400 post-column reaction unit (Pickering Laboratories, Mountain View, CA), two LC-9A pumps for delivery of post-column reaction solutions, and a RF-10Axl fluorescence detector (all Shimadzu, Duisburg, Germany). The *N*-sulfocarbamoyl toxins were indirectly determined after hydrochloric acid-mediated hydrolysis by determining the ratios of the difference in peak areas to those obtained directly from the original acetic acid extract. Toxin standards were obtained from the CRMP, Institute for Marine Biosciences, National Research Council, Halifax, Canada. A standard for dcSTX was provided by the European Commission (BCR, Community Bureau of Reference), Brussels, Belgium.

The total PSP toxin cell quota as well as the cellular content of the different derivatives was calculated from molar concentrations after estimation of cell numbers from the Lugol's fixed samples. Tandem mass-spectrometry was performed to directly confirm the presence of *N*-sulfocarbamoyl toxins in unhydrolysed samples as described in Krock et al. (2007). Molar quantities of the epimeric pairs C1/C2, GTX2/GTX3, GTX1/GTX4 were fused in subsequent analyses because of the known facile epimerization during storage and processing (Hall et al. 1990). A cluster analysis of toxin profiles was performed after calculation of the Euclidean distances among all pairs of isolates. To equally weight all toxin groups, the molar percentages of each toxin group in each isolate were expressed as a proportion of the highest molar percentage for the respective toxin group within all isolates (Cembella et al. 1987). Calculations of pairwise distances and construction of a UPGMA dendrogram was performed in BIONUMERICS v2.50 (Applied Maths, Sint-Martens-Latem, Belgium). Chauvenet`s criterion ($p < 0.05$) and the Shapiro-Wilk W test in STATISTICA ™ V.6 (StatSoft®, Hamburg, Germany) were used to determine if potential outliers in the data on PSP toxin content (fmol cell^{-1}) of *A. tamarense* isolates could be identified and to determine if the data was normally distributed, respectively.

Allelochemical activity of A. tamarense isolates

The allelochemical and lytic activity of 67 clonal *A. tamarense* isolates was tested in two series of experiments against different target organisms, the cryptophyte *Rhodomonas salina* and the dinoflagellate *Oxyrrhis marina*. Clonal isolates of *A. tamarense* were grown in batch cultures as described herein until they reached mid-exponential growth phase. After estimation of cell concentrations of *A. tamarense* by counting Lugol's iodine fixed cells within a subsample that contained at least 400 cells, the batch cultures were diluted with K medium to a final cell concentration of 1,500 cells mL^{-1}. Then 15 mL of diluted cultures were dispensed into triplicate 20 mL scintillation glass vials. Each replicate was spiked with a dense culture of *R. salina* to a final

concentration of 10 x 10^4 cells mL^{-1}. The test vials were then incubated under the same culture conditions as described.

Two negative and one positive control were performed in the same way as the experimental assays. The first negative control contained only 15 mL of K medium whereas the second negative control was spiked with 15 mL of a batch culture of the non-toxic dinoflagellate *Scrippsiella trochoidea* (Stein) Balech ex Loeblich III (final cell concentration 1.5 x 10^3 cells mL^{-1}). The positive control was produced by adding 15 mL of a culture of the allelochemically active *A. tamarense* strain SZNB01 (Tillmann and John 2002), established by M. Montresor from the Gulf of Naples (final cell concentration of 1.5 x 10^3 cells mL^{-1}). After 24h (T_{24}) cells of *A. tamarense* and *R. salina* were fixed with 0.3 mL Lugol's iodine solution and concentrations of intact cells of both species were determined under an inverse microscope.

For the second experimental series, 200 µL of diluted *A. tamarense* cultures were dispensed in triplicate to wells of a 96-well cell culture plate (TPP, Trasadingen, Switzerland). Each replicate was spiked with 10 µL *O. marina* culture (at *ca.* 1 x 10^4 cells mL^{-1}) to obtain a final density of *O. marina* of about 500 cells mL^{-1}. Two negative and one positive control were performed in the same way as for the experiments with *R. salina* After 24h, cells of *A. tamarense* and *O. marina* cells were fixed by addition of 5 µL Lugol's iodine solution and the concentration of intact cells was enumerated under an inverse microscope.

The magnitude of the lytic effects of each *A. tamarense* isolate was expressed by calculating the fraction of intact cells versus the average cell number of the respective controls after 24h. The Shapiro-Wilk W test in STATISTICA ™ V.6 (StatSoft®, Hamburg, Germany) was used to determine if the data on allelochemical properties of *A. tamarense* isolates against *O. marina* (%T_{24}) were normally distributed.

Statistical analyses for correlations among different markers

Matrices of pairwise similarities among individuals used for the construction of UPGMA dendrograms with the data sets from AFLP, microsatellite and PSP analyses were subjected to Mantel tests (Mantel 1967) with 9,999 permutations in the statistical software package BRODGAR Ver. 2.5.0 (Highland Statistics Ltd., Newsburgh, UK). Pearson product-momentum correlation was analyzed to determine the degree of correlation between different marker data sets.

The association of certain AFLP markers with total cellular PSP toxin content and the allelochemical potency was analyzed by multiple Student's *t*-tests in EXCEL software (Microsoft, Redmond, USA). Data sets included phenotypic and genotypic data from 86 and 67 isolates in the case of PSP toxin content and allelochemical properties towards *O. marina*, respectively. Clonal isolates were grouped for each AFLP locus according to the presence or absence of an amplified band for this specific locus. Student's *t*-tests between the resulting presence and absence groups were performed for all AFLP loci for which the number of isolates assigned to either group was at least 10, yielding 301 and 265 tests of association of AFLP loci with PSP toxin content and allelochemical activity, respectively. *P*-values were adjusted by Bonferroni correction to account for the number of tests.

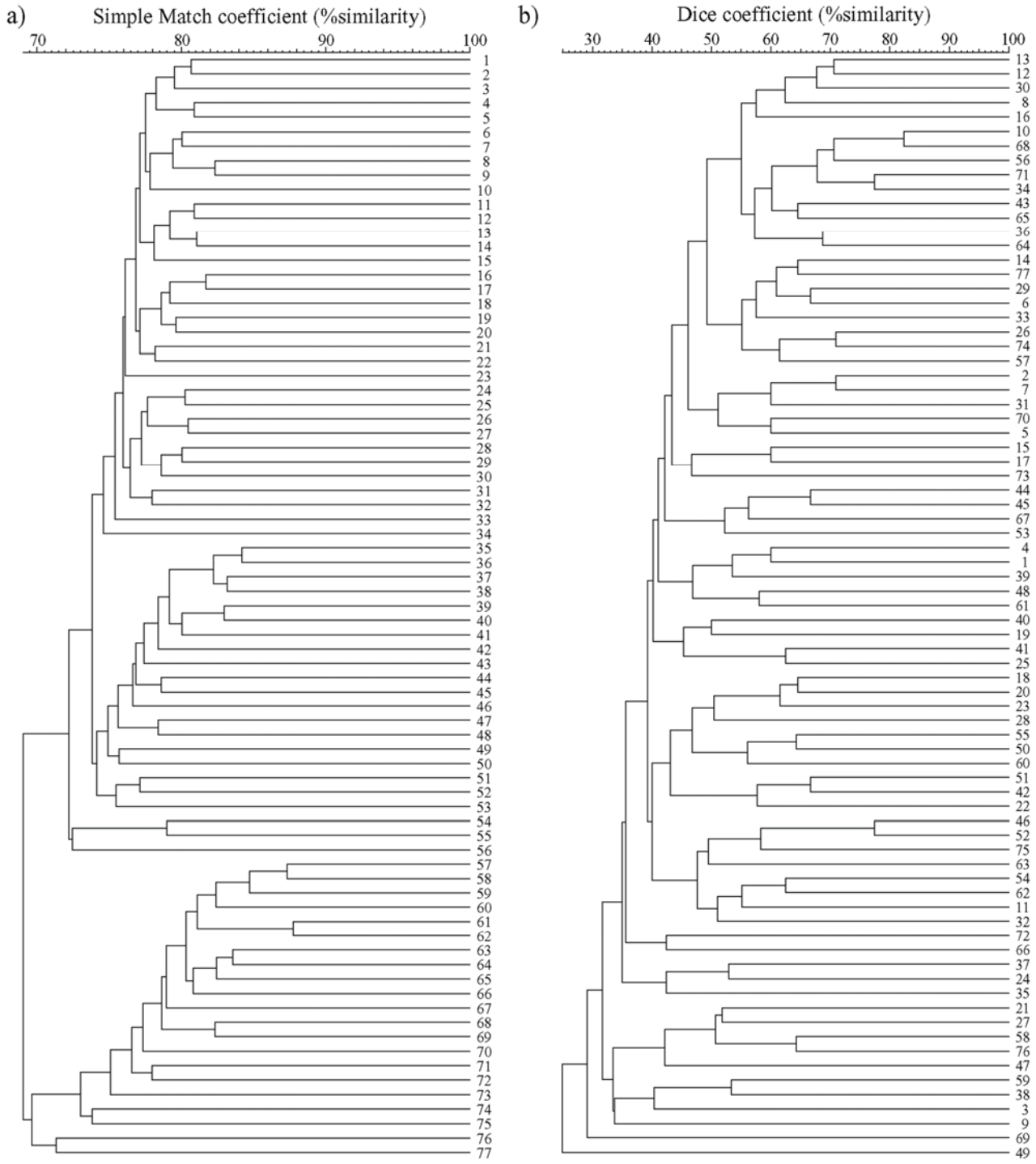

Fig. 2.4.1. UPGMA dendrograms of multi-locus genotypes based on the Simple Match coefficient of similarity for AFLP data (a) and on the Dice coefficient of similarity for microsatellite data (b). *A. tamarense* isolates from the North Sea are numbered from 1 to 77 according to their order in the dendrogram obtained by AFLP data.

2.4.4. Results

AFLP genotypic data and analysis

The four combinations of primers in the AFLP amplification reactions produced a total of 482 well-scoreable PCR products of distinct size (i.e. presence alleles) in the 77 clonal isolates of the combined AFLP-microsatellite data set. The number of AFLP loci per primer pair ranged from 109 to 133 with an average number of 120.5 (SD ± 8.8) (Table 2.4.1). Gene diversity ($\hat{H}$) at AFLP loci ranged from 0.03 to 0.51, while the mean gene diversity was estimated as $\hat{H}$ = 0.27 (SE ± 0.007). Comparison of binary AFLP profiles showed no identical multilocus AFLP genotype within the group of 77 isolates. The UPGMA dendrogram based on Simple Match similarities of all pairs of isolates (Fig. 2.4.1a) showed that average branch length ranged from 65 to 70%, but clearly separated clusters were not formed.

Correlations of similarity matrices derived from subsets of AFLP loci as tested by Mantel tests were always highly significant ($p < 0.001$) when subsets included 45 or more arbitrarily selected AFLP loci. The correlation coefficients increased rapidly until subsets included about 225 AFLP loci, but then tended to increase only slightly as more AFLP loci were included (Fig. 2.4.2).

The highest correlation coefficient was observed for subsets that included the maximum number of AFLP loci. When only up to 9 AFLP loci were included in the subsets, correlation coefficients were always low and rarely significant at $p < 0.01$ (Fig. 2.4.3a). Analysis of coefficients of variation (CV) of pairwise genetic similarities by bootstrapped subsets with increasing number of AFLP loci resulted in a steep decline of CV values for the smaller subsets with each additional locus included. The threshold value of a CV of 0.1 was already reached with about 270 loci and the largest AFLP dataset analyzed including 481 of the 482 sampled loci resulted in a CV of 0.075 (Fig. 2.4.4). Extrapolating the linear regression function beyond the range of AFLP loci assessed in this study provided an estimate that about 1080 loci would yield a CV of 0.05.

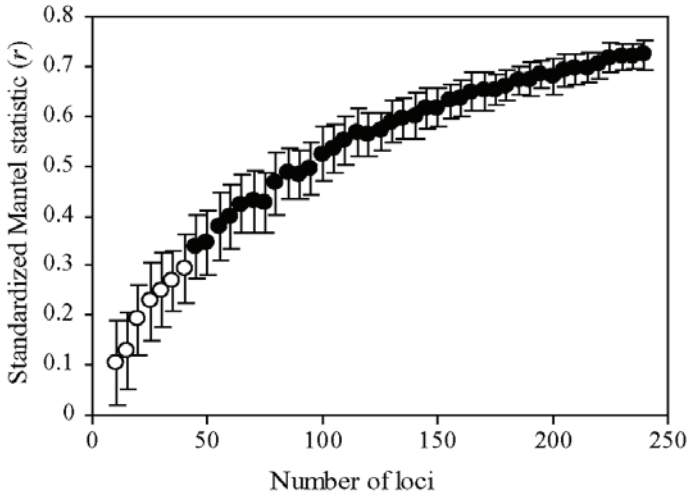

Fig. 2.4.2. Standardised statistic of Mantel tests (r) versus number of loci that were contained in each bootstrapped subset (± SD) for AFLP data. Black circle symbolize significant values at $p < 0.01$.

Microsatellites

The genotypes obtained by microsatellite analysis were very diverse. No identical multilocus genotype was found among the 77 clonal isolates of *A. tamarense*. The number of alleles for a specific microsatellite marker varied considerably and ranged from two to ten (Table 2.4.2) with an average of 5.5 (SD ± 2.3). Gene diversity among microsatellite loci also varied and ranged from 0.31 to 0.83 (Table 2.4.2). The mean gene diversity over all microsatellite loci was $\hat{H} = 0.62$ (SE ± 0.036). The cluster analysis of clonal *A. tamarense* isolates based on the pairwise Dice similarity index of microsatellite genotypes did not allow identification of separated subgroups within the population (Fig. 2.4.1b).

The correlation among the subgroups as tested by Mantel tests performed on the similarity matrices obtained for the subgroups was never significant (Fig. 2.4.3b), when arbitrarily composed subsets of nine microsatellite markers were each taken to evaluate the consistency of genetic similarities among isolates. The mean correlation coefficient obtained for nine microsatellite loci was 0.09, comparable to the degree of correlation obtained with about the same number of AFLP loci in this study (Fig. 2.4.3a).

The analysis of CV of pairwise genetic similarities by bootstrapped subsets with an increasing number of microsatellite loci yielded a steep decline of mean CV values for the larger subsets with each additional locus included. The lowest mean CV (about 0.2) was reached with the largest bootstrapped subset (18 loci). Analysis of the AFLP data set yielded a comparatively low mean CV only when at least 70 loci were included in the bootstrapped subsets. However, the threshold CV value of 0.1 was not reached with 18 microsatellite loci (Fig. 2.4.4). Extrapolating the linear regression function beyond the range of microsatellite loci assessed in this study allowed an estimate that about 58 and 224 microsatellite loci would yield CV values of 0.1 and 0.05, respectively.

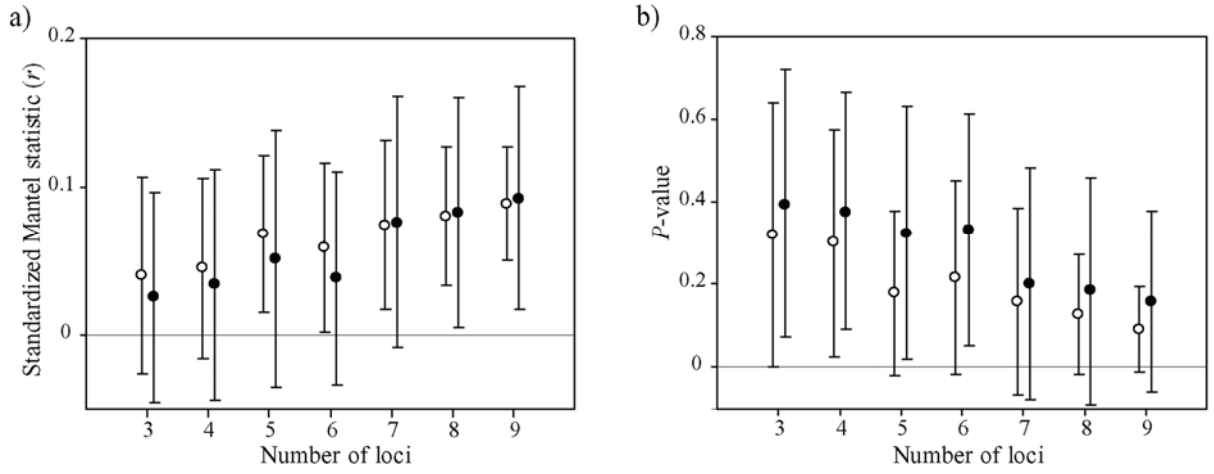

Fig. 2.4.3. (a) Standardised statistic of Mantel tests (r) versus number of loci that were contained in bootstrapped subsets (mean ± SD) for microsatellite data (open circles) and AFLP data (filled circles) and (b) the corresponding p-values of 50 replicates (mean ± SD).

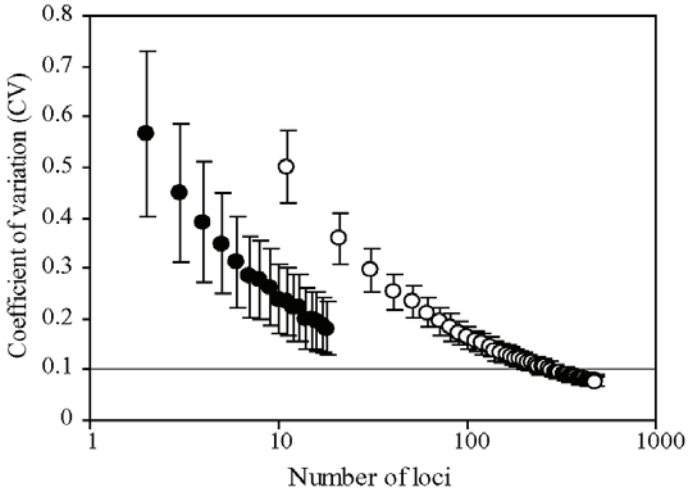

Fig. 2.4.4. Average coefficient of variation (CV) versus number of loci in bootstrapped subsets (± SD) for microsatellite data (filled circles) and AFLP data (open circles). X-axis is scaled logarithmically.

Variation in PSP toxins

The clonal isolates showed a remarkable variation with respect to both toxin content per cell and the relative amount of the different toxin derivatives, expressed as the toxin profile characteristic for individual isolates (Fig. 2.4.5b). Data on cellular toxin content were normally distributed when the two extreme values (471.1 and 428.9 fmol cell^{-1}) were excluded from the test for normal distribution according to Chauvenet's criterion (Taylor 1997) and ranged from 25.3 to 367.7 fmol cell^{-1}. The mean PSP toxin cell quota for the *A. tamarense* isolates was 165.2 fmol cell^{-1} (SD ± 85.4; n = 88). Plotting the data showed that values were continuously distributed with a distinct maximum, but also displayed skewness towards high toxin content (data not shown). Neosaxitoxin (NEO) was on average the most abundant of all PSP toxins and reached highest molar percentages. The epimers C1/C2 were the second most prominent toxin group, followed by saxitoxin (STX). The residual toxins, GTX1/GTX4, GTX2/GTX3, B1 and B2, contributed on average <10 mol% to the PSP toxin composition, but in some isolates, these toxins contributed relatively much more, e.g., maximum values for GTX2/GTX3 almost reached those of NEO (Table 2.4.3).

Table 2.4.3. Range and mean (± SD) of the cellular content of PSP toxins (in fmol cell^{-1}) and the relative contribution of analogues to the toxin composition of 88 clonal isolates of *A. tamarense*.

Toxin	Cellular content	Mean	Mole%	Mean
STX	0.2–67.0	18.5 (±14.3)	0.3–39.6	11.1 (±6.9)
NEO	6.8–359.1	89.5 (±61.6)	7.2–80.8	52.5 (±15.9)
GTX1/GTX4	0.0–99.5	9.3 (±16.3)	0.0–45.7	5.4 (±7.9)
GTX2/GTX3	0.0–73.6	8.6 (±10.6)	0.0–77.8	6.0 (±9.2)
B1	0.0–13.4	1.8 (±3.0)	0.0–11.1	1.2 (±2.0)
B2	0.0–93.2	4.9 (±11.9)	0.0–25.3	2.4 (±4.3)
C1/C2	0.0–89.8	32.6 (±18.3)	0.0–49.4	21.4 (±9.6)

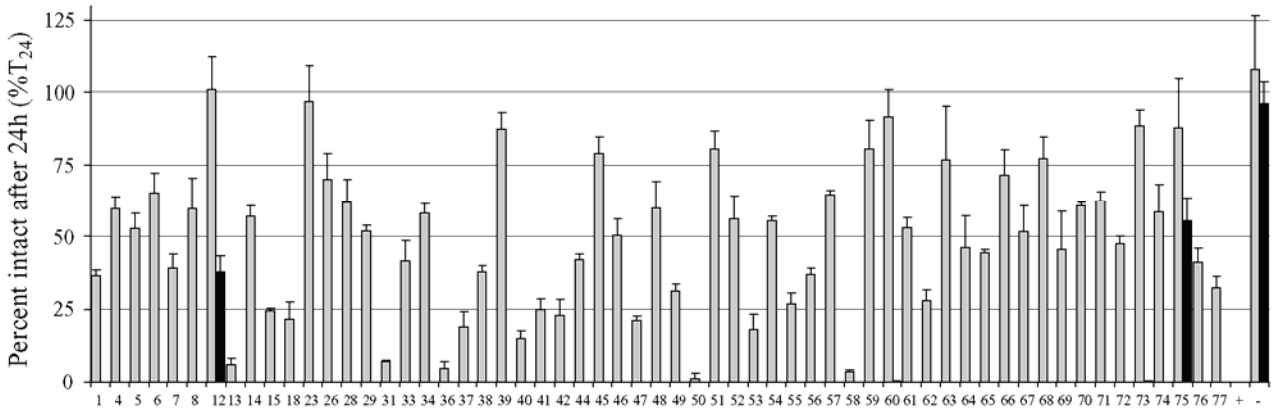

Fig. 2.4.6. Allelochemical effects of *A. tamarense* isolates towards *Oxyrrhis marina* (grey bars) and *Rhodomonas* sp. (black bars) as percentage of intact target cells after 24h co-incubation of intact cells in the negative control with K medium after 24h co-incubation ($\%T_{24}$). Identifiers of *A. tamarense* isolates correspond to those used in Fig. 2.4.1 and Fig. 2.4.5; + : positive control for lytic effect by addition of *A. tamarense* strain SZNB01; − : negative control by addition of non-lytic *Scrippsiella trochoidea.*

Neither decarbamoyl NEO (dc-NEO) nor any decarbamoyl gonyautoxins (dc-GTX1/dc-GTX4 and dc-GTX2/dc-GTX3) were detected, but traces of dc-STX were present in some isolates to a maximum of 0.6 mol% of total toxin and an average of 0.12 mol% (SD ±0.13). Small amounts of dc-STX found in *Alexandrium* may be due either to its presence as a transient biosynthetic intermediate or as an artefact resulting from decarbamoylisation of either STX or B1. This component was therefore excluded from further analyses in a conservative approach because further analyses were based on toxin profiles equally weighted to the different toxin groups.

Variation in allelochemical activity

The allelochemical potency of *A. tamarense* isolates against *Rhodomonas salina*, expressed as percent of target cells in treatment related to the number of target cells in the negative control without dinoflagellate cells ($\%T_{24}$), ranged from 0 to 55.5. Interestingly, only two isolates of *A. tamarense* did not cause severe damage to *R. salina* target cells (Fig. 2.4.6). In the control treatments, the positive control with *A. tamarense* strain SZNB01 caused complete lysis of *R. salina* cells, whereas the negative control treatment with *Scrippsiella trochoidea* did not cause a significant reduction in *R. salina* cell numbers (Fig. 2.4.6).

Against *Oxyrrhis marina* allelochemical activity of *A. tamarense* ($\%T_{24}$) ranged from 1.2 to 100.9 with a mean value of 48.3 (SD ± 24.6). The allelochemical properties against *O. marina* ($\%T_{24}$) among the 67 *A. tamarense* isolates were confirmed to be normally distributed.

Statistical analyses for correlations among different markers

The Mantel tests on the similarity matrices obtained by the genotypic markers (AFLP and microsatellites) and PSP toxin profiles as a phenotypic character did not show significant ($p < 0.05$) correlation. The two genotypic markers, however, exhibited weakly ($r = 0.12$) but significantly ($p < 0.005$) correlated similarity matrices.

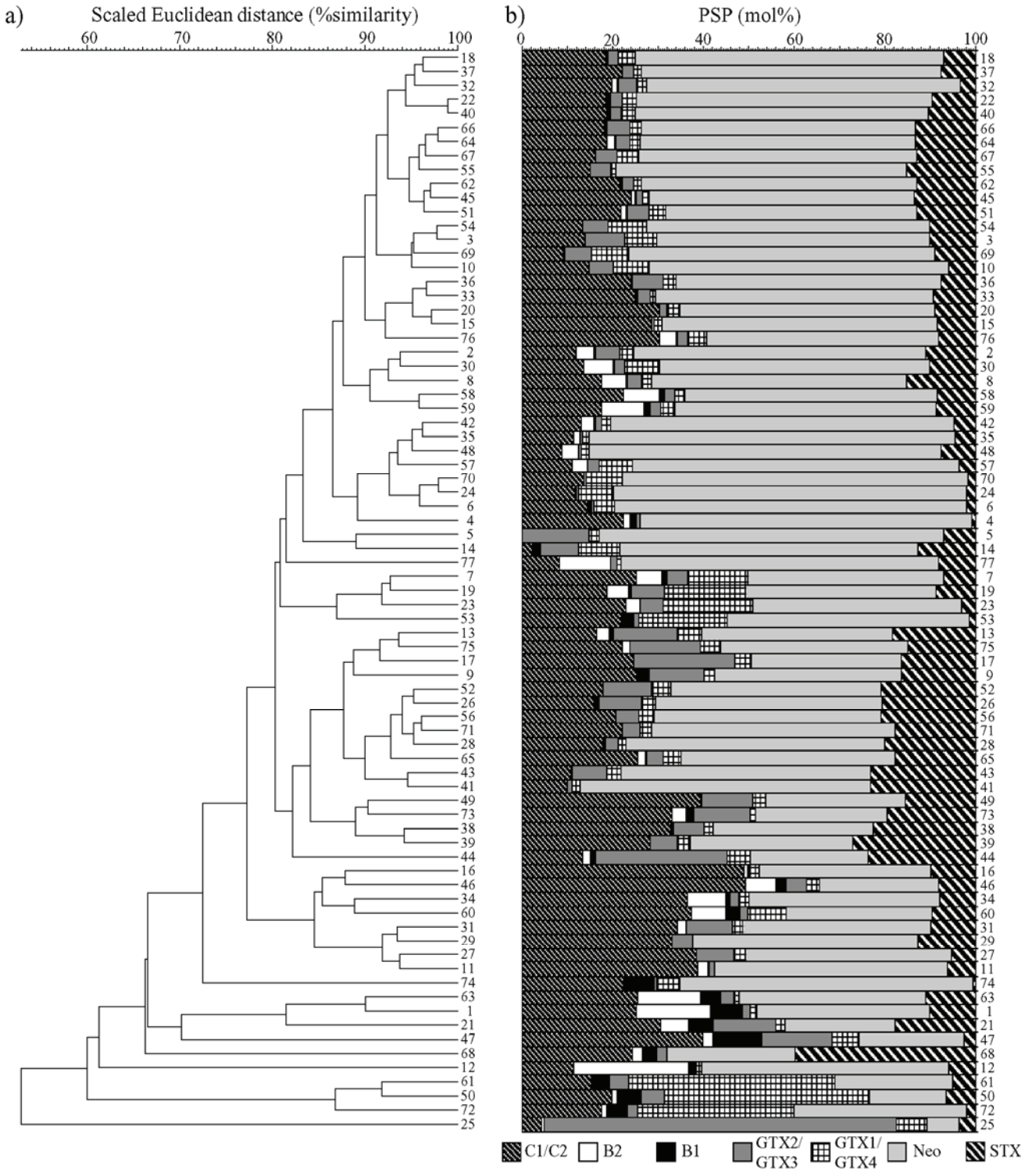

Fig. 2.4.5. UPGMA dendrogram of PSP toxin profiles based on the Euclidean distance calculated for the relative molar composition of toxins (a) and respective toxin profiles in mol% (b) of all genotyped isolates from the North Sea population. *A. tamarense* isolates from the North Sea are numbered as in Fig. 2.4.1 and Fig. 2.4.6. Distance is scaled in percent of the maximum distance among characters in the data set and converted to percent similarity (100 – scaled Euclidean distance = % similarity).

The multiple *t*-tests performed to test the association of alleles at certain AFLP loci with either the cellular PSP toxin content of clonal *A. tamarense* isolates or their allelochemical activity against *O. marina* did not show any significant association ($p < 0.05$) between any of the polymorphic AFLP loci and either of the two phenotypic characters. The lowest *P*-value ($p = 0.00018$) for the pairwise *t*-tests for association of cellular PSP toxin content with any of the AFLP loci, however, almost met the Bonferroni corrected significance limit of 5% ($p = 0.00017$; $n = 301$).

2.4.5. Discussion

The nature of genotypic population markers

In our study, the two markers (AFLP and microsatellites) did not give concordant patterns of genetic relatedness among the individual clones from the North Sea population, although matrices of pairwise similarities obtained for both markers were significantly correlated (Mantel test) at a very low level. A plausible explanation for this apparent mismatch of pairwise genetic relatedness derives from testing the congruence of bootstrapped subsets of each genotypic marker with independent subsets of the same marker. Whereas pairwise similarity matrices of subsets of AFLP loci always reached significant correlations for subsets with 45 or more loci, the missing significance and low correlation indices of the largest subsets of microsatellite markers (nine loci in each subset) indicated that the representation of genetic relatedness of isolates by this marker with the number of loci in this study is not stable. The high number of available AFLP markers and the high degree of correlation for those subsets that contained only half the AFLP loci analyzed in this study (see Fig. 2.4.2), indicates that the pattern will stabilize further, as more markers are included. The set of AFLP loci are therefore better suited for the characterization of genotypic similarities of *A. tamarense* isolates than the microsatellites applied in the study. Bootstrap simulations of the CV showed that values for both genotypic markers tended to decrease rapidly, when only a few more marker loci were added to the subsets, but the CV for microsatellites did not reach the threshold of 0.1. This value is generally expected to be the minimum for the generation of reliable patterns of pairwise genetic relatedness among individuals (see Garcia et al. 2004 and references therein). However, the CV values for pairwise genotypic dissimilarities obtained for bootstrapped microsatellite subsets decreased much faster with additional loci than for AFLP, generally interpreted as an indication of the higher resolving power of microsatellite loci. The higher resolution is most likely due to the predominantly multiallelic nature of microsatellites, when compared to biallelic AFLP markers, which also leads to higher average gene diversity in comparison to AFLP (Nybom 2004). The technical demands in obtaining a sufficiently high number of polymorphic microsatellites, however, hinder its usefulness for studies of intra-population differentiation, including apparently the present study of *Alexandrium*.

Significance of genotypic diversity within natural populations

In contrast to the present study, in which we did not find repeated multilocus genotypes, other extensive surveys on genotypic composition of planktonic dinoflagellate populations (e.g., Shankle

et al. 2004 and Nagai et al. 2007) and other phytoplankton species such as diatoms (Rynearson and Armbrust 2000; Evans et al. 2005; Godhe et al. 2006) reported repeated sampling of genotypes. Theoretically, there is a high likelihood of eventually finding isolates of identical clonal origin in any such study on genotypic richness in predominantly clonally reproducing organisms − if only sample size were big enough (Halkett et al. 2005). The appearance of identical genotypes, however, does not necessarily reflect true clonal identity, since genotyping in previous studies relied on different types of genetic markers and/or numbers of loci. The chances of detecting multilocus genotypic differences in the North Sea population of *A. tamarense*, for which as many as 18 microsatellites and 482 polymorphic AFLP loci were used, were consequently higher than in previous studies that made use of less polymorphic loci. The repeated detection of certain genotypes in other studies might be the result of the lower differentiating capacity of the respective set of genotypic markers employed. Interestingly, the two most similar microsatellite genotypes from the North Sea population share the same alleles in 14 out of 18 microsatellite loci, but carry different alleles at the residual four loci. This illustrates that a large set of polymorphic genotypic markers is needed to yield good resolution of genotypic richness in phytoplankton populations, if the aim of such a study is to gain insight into the genotypic diversity or the frequency of occurrence of clonal individuals.

With respect to the supposition that the genetic diversity in a population reflects its potential to adapt to changing environments (Fisher 1930; Barrett and Schluter 2008), the discovered high genetic diversity in the population of *A. tamarense* indicates that it is well prepared to withstand frequent changes in environmental conditions. The number of possible combinations of microsatellite and AFLP alleles due to recombination should be enormously high. The theoretically possible number of AFLP genotypes (1.25×10^{145} with 482 binary loci) exceeds by far the number of *A. tamarense* cells that could be present within a given spatial-temporal frame in any natural planktonic population − even in blooms of high cell concentrations ($>1 \times 10^{6}$ cells L^{-1}). Taking the estimates of gene diversity as the probability of sampling different alleles at a specific locus, one minus the gene diversity is the probability of sampling the same allele at a locus. Hence, the product of the latter estimates from all loci yields the probability of sampling the same multilocus genotype at the given allele frequencies, namely 8.9×10^{-9} for microsatellites and 5.2×10^{-73} for AFLP. The number of both molecular markers, applied in this study, therefore, would suffice to reveal clonal identity with high certainty, if identical multilocus genotypes were found in several isolates.

Both molecular markers employed in this study of *A. tamarense* are assumed to be neutral with respect to phenotypic variation among the clonal isolates. Yet the high genotypic and clonal diversity that they reveal raises speculations that this diversity also reflects broad phenotypic variation caused by variation in genes involved in the expression of these phenotypic traits.

PSP toxin content and profile variation

The diverse set of toxin profiles from this North Sea population is − with a few exceptions − characterized by very small transitions in the proportions of PSP toxins. Consequently, the toxin profiles among subclusters vary rather little within the next higher hierarchical cluster (Fig. 2.4.5a). The small differences between cluster hierarchies suggest no clear separation of toxin profiles into

discrete groups. The observation that certain derivatives or groups of toxins, such as NEO and C1/C2 in our samples, dominate the toxin profiles of most isolates from a certain geographical region has been made in previous studies (e.g., Cembella et al. 1987; Anderson et al. 1994; Cembella and Destombe 1996).

Cellular toxin content, however, is a less stable phenotypic character of a clonal isolate than the relative composition of PSP toxins, which changes significantly only under rather extreme differences in growth conditions in batch and semi-continuous cultures (Boczar et al. 1988; Anderson et al. 1990a). Direct comparison of the toxin content of *A. tamarense* isolates analyzed in this study with that of clonal isolates from other studies is therefore not advisable because of differences in culture conditions, sampling point along the growth curve and toxin analytical methodologies. Time-series differences in cellular toxin content within nutrient-replete batch cultures of *Alexandrium* are typically less than 2-fold (e.g., Boczar et al. 1988; Kim et al. 2005)), but much larger differences are expressed under stress conditions such as nutrient limitation . The clonal isolates in our study were cultured simultaneously under standardized nutrient-replete conditions and harvested in exponential growth phase. The drastic differences in cellular toxin content of more than one order of magnitude found among the clonal isolates from the North Sea therefore likely represent real clonal differences among isolates, and are not simply time- or condition dependent culture artefacts.

The within-population variation in cellular PSP toxin content found among the isolates from the North Sea corresponds well to values reported for clonal isolates from populations of *A. tamarense/fundyense* (North American ribotype/Group I) from the western North Atlantic (Cembella and Destombe 1996) as well as the eastern (Cembella et al. 1987) and the western North Pacific (Yoshida et al. 2001). Our conclusion that high variation in cellular PSP toxin content is a general feature of natural *A. tamarense* (Group 1) populations, therefore, is in good agreement with other studies (Cembella et al. 1987; Kim et al. 1993; Anderson et al. 1994; Cembella and Destombe 1996; Yoshida et al. 2001).

Variation in allelochemical activity

The unexpectedly high level of phenotypic variation in allelochemical properties of *A. tamarense* is similar to the high diversity in PSP toxin profiles and content among *Alexandrium* isolates. Allelochemical activity in *Alexandrium* spp. has previously been shown to be independent of PSP toxins (Tillmann and John 2002). Furthermore, the potency of allelochemical effects does not seem to be influenced by associated bacteria (Tillmann et al. 2008a) nor is it altered detectably in clonal isolates after maintenance in culture for years (U. Tillmann, unpublished data). We therefore suggest that the differences in allelopathic characteristics are genetically fixed.

The results support the conclusion that *R. salina* is in general far more susceptible to the deleterious effects of *A. tamarense* allelochemicals than *O. marina*. This is in congruence with observations made with single strains from several species of the genus *Alexandrium*, for which the effect on *R. salina* was always higher than that on *O. marina* (Tillmann et al. 2008a). As in other toxicity assays, percent lysis of target cells is a function of the dose (*A. tamarense* cell concentration), typically following a sigmoid dose-response curve (Tillmann et al. 2008a). In the

present experiments, recording full dose-response curves was not feasible because of the large number of clones tested. In the case of *R. salina*, the one fixed *A. tamarense* cell concentration applied in the bioassay (1500 cells mL^{-1}) was obviously too high to resolve quantitative differences among the strains. However, due to the lower sensitivity of *O. marina*, the identical *A. tamarense* cell concentration was within the optimal range for this target to resolve quantitative differences among clones. It is important to note that among these 67 clones, only two isolates did not cause severe damage to *R. salina* target cells. This indicates that although there are large quantitative differences in the amount of lytic compounds produced among isolates, the absence of measurable lytic activity within *A. tamarense* clones is a rare exception.

Correlations between genotypic and phenotypic characteristics

Agreement of the similarity matrices for data on microsatellites and molar PSP toxin composition was not expected, since the similarity matrix obtained for the microsatellite data did not stabilize in our test. In contrast, since AFLP provided reliable patterns of genotypic differentiation among individuals (clones) within the North Sea population, we assumed that if there was congruence between population-wide diversification based on molar toxin composition and either of the genotypic markers, AFLP would more likely reveal such a correlation. Nevertheless, the lack of congruence of population-wide similarity matrices for molar toxin composition and either genotypic marker (Mantel test) indicates that no global genotypic-phenotypic relationships are resolved for the North Sea population of *A. tamarense*. Since both molecular markers are generally assumed to be neutral with respect to phenotypic expression, a global correlation of this kind can be expected only under certain conditions. One such condition would be simultaneous differentiation of neutral markers and evolution of phenotypic differences according to an evolution by distance model.

Association of AFLP loci with phenotype expression

In this study, both phenotypic traits tested for association with individual AFLP loci − cellular PSP toxin content and allelochemical activity − were normally distributed. Accordingly, they resemble quantitative traits, implying that several genes are involved in the expression of these phenotypes. Studies on the relation of phenotypic characteristics and genomic markers, e.g., by linkage analysis or association studies, can be expected to contribute substantially to the elucidation of the genetic basis of the development and maintenance of this phenotypic variation.

Our approach of testing for association of individual AFLP loci with the PSP toxin or allelochemical phenotype, failed to show significant association between any of the genetic markers and either phenotype. Recombination of genes responsible for phenotypic expression is expected to occur frequently in outbreeding populations of *Alexandrium*. Few examples are reported where correlation of phenotypic traits and genetic markers have been detected in natural populations of closely related individuals (Erickson et al. 2004 and references therein). From a probabilistic perspective, the chances of detecting such associations are determined by several factors, such as genome size, the rate of genetic recombination around the phenotype-effective genes, the mutation rate at AFLP loci, as well as the number of individuals and AFLP loci included in the analysis. The probability of detecting phenotype-genotype associations might be very low in *A. tamarense*

because the genome size is estimated at around 200,000 Mb in 143 chromosomes (D.L. Erdner and D.M. Anderson, unpublished data, cited in Hackett et al. (2005)). However, evidence that the rate of recombination is similar to that in other eukaryotes is lacking, as the degree to which the genetic material is involved in meiotic recombination is unknown. In general, the number of AFLP loci required for screening to detect such functional linkage within natural, outbreeding populations is considerably higher than the number that allows for association analysis in experimental populations. Experimental crosses between allelochemically active and non-active strains or between 'high' and 'low' PSP toxin-phenotypes, therefore could facilitate the detection of functionally linked loci (Erickson et al. 2004).

The near significance of association of PSP toxin content with one AFLP locus (*t*-test, $p = 0.053$ after Bonferroni correction) prompts the question of whether or not significant results may have been obtained by testing a moderately higher number of clones.

Implications of phenotypic variation on evolutionary ecology

The specific cause of the enormous phenotypic variation in allelochemical properties and PSP toxin composition cannot be determined without secured knowledge on the ecological significance of the respective trait. The functional role of PSP toxins in the ecology and evolution of *Alexandrium* populations remains unknown (Cembella 2003), but if the expression of a high-toxicity phenotype was of evolutionary benefit to a certain clonal lineage, the question arises as to how lineages with low toxicity persist in the long run within natural populations. Directional selection would be expected to reduce the frequency of low-PSP toxicity phenotypes. The production of a suite of secondary metabolites must also incur metabolic costs. In the absence of a selective advantage of a PSP toxin phenotype, these costs, if high enough to diminish growth performance or other critical reproductive factors, must discriminate against the respective individual. This is the classic dilemma in the chemical ecology of secondary metabolites: balancing metabolic costs of production against evolutionary advantage.

The ecological significance of an allelopathic phenotype is believed to be reflected in its specific effects on certain target taxa (Tillmann and John 2002; Fistarol et al. 2004b). Thus, by mediating a group-selective advantage, the presence of a number of allelochemically active *Alexandrium* clones could benefit the population as a whole. A reasonable explanation for the wide phenotypic variation in expression of this trait in the studied population is that allelochemical interactions among *Alexandrium* and its competitors and grazers only play an important role at certain stages of population development. Under this scenario, in phases of population development/growth when the selective pressure on weakly allelochemically active lineages is low due to the absence of sensitive grazers or competitors, expression of other phenotypic traits may be more critical. If certain phenotypic traits are of selective importance only for certain periods during population growth, balancing selection on other − at times more relevant − traits may lead to the maintenance of broad phenotypic diversity over time. The population-wide distribution of allelochemical activity suggests an important ecological role for this phenotype, though under the constraints at the time of sampling a strong selective pressure on respective phenotypic trait seems to be lacking. For the population under study the stage of population development is not known, but

future studies on the ecological significance of the allelopathic phenotype might benefit from directing a focus on allelochemically mediated species interaction during the cause of population and HAB development and maintenance.

2.4.6. Conclusions

We provide a comprehensive scenario of variation and diversity of phenotypic and genotypic characters of a large contemporaneous collection of clonal isolates representing a geographically defined population. The genotypic and phenotypic analysis tools applied here can clarify the ecological significance of these expressed characters in the context of population and bloom development, as well as to discover environmentally driven directional selection on the phenotype. In order to answer questions on the evolutionary benefit of certain phenotypic characters, such as biologically active secondary metabolites, detailed analyses of natural populations over longer time periods and larger-scale experiments (e.g., in mesocosms) in which environmental variables can be semi-controlled must be performed. Such studies will shed further light on the evolution of these traits in the marine dinoflagellate *Alexandrium*, as well as in other toxigenic eukaryotic microalgae responsible for harmful algal blooms.

2.5. INTRA-POPULATION CLONAL VARIABILITY IN ALLELOCHEMICAL POTENCY OF THE TOXIGENIC DINOFLAGELLATE *ALEXANDRIUM TAMARENSE*

2.5.1. Abstract

Clonal variability in exponential growth rate and production of secondary metabolites was determined from clonal isolates of *Alexandrium tamarense* originating from a single geographical population from the east coast of Scotland. To assess variability in the selected phenotypic characteristics over a wide spectrum, 10 clones were chosen for experimentation from 67 clonal isolates pre-screened for their lytic capacity in a standardized bioassay with the cryptophyte *Rhodomonas salina*. Specific growth rates (μ) of the 10 clonal isolates ranged from 0.28 to 0.46 d^{-1} and were significantly different among clones. Cell content (fmol $cell^{-1}$) and composition (mol%) of paralytic shellfish toxins (PSTs), analyzed by liquid chromatography with fluorescence detection (LC-FD), varied widely among these isolates, with total PST quotas ranging from 20 to 89 fmol $cell^{-1}$. Except for strain 3, the toxins C1/C2, neosaxitoxin (NEO), saxitoxin (STX), and gonyautoxins-1 and -4 (GTX1/GTX4), were consistently the most relatively abundant, with lesser amounts of GTX2/GTX3 evident among all isolates. Only clone 3 contained >20 mol% of toxin B1, with C1/C2, GTX2/GTX3 and NEO in almost equimolar ratios.

Eight of the 10 clones caused cell lysis of both *R. salina* and the heterotrophic dinoflagellate *Oxyrrhis marina*, as quantified from the dose-response curves from short-term (24 h) co-incubation bioassays. For two clones, no significant mortality even at high *Alexandrium* cell concentrations (ca. 10^4 mL^{-1}) was observed. Allelochemical activity expressed as EC_{50} values, defined as the *Alexandrium* cell concentration causing lysis of 50% of target cells, varied by about an order of magnitude and was significantly different among clones. No correlation was observed between growth rate und allelochemical potency (as EC_{50}) indicating that at least under non-limiting growth conditions no obvious growth reducing costs are associated with the production of allelochemically active secondary metabolites.

2.5.2. Introduction

In phytoplankton research the majority of field data is based on measurements of bulk parameters like chlorophyll-*a* or C^{14} uptake. Without question, however, these community level measurements mask the variability within the biological community which typically is composed of a large number of different species. Consequently, and complementary to bulk measurements, the extrapolation of experimental work conducted on cultured plankton species in the laboratory is often used to interpret and predict the activity of microorganisms in nature. In this approach, the concept of species plays a central role because organisms, which are genetically so closely related

as to form a species, are expected to have similar ecological requirements. With this assumption of little intra-specific genetic variation, phytoplankton investigators (and especially modellers) frequently extrapolate rates and parameters from a single clonal culture to the entire species and then often claim to identify significant inter-specific differences without estimating the magnitude of intra-specific variation. In fact, since Braarud´s discussion on phytoplankton ecotypes more than 50 years ago (Braarud 1951), there is increasing evidence that significant intra-specific variability in ecologically important traits in phytoplankton is widespread (reviewed by Brand 1988/1989; Wood and Leatham 1992). Genotypic and phenotypic variability has been detected among clones or isolates separated either geographically (Maranda et al. 1985) or temporally (Shankle et al. 2004) among clones from a single population (Brand 1981) or even clones from a single bloom (Rynearson and Armbrust 2005). Physiological and biochemical traits which have been proven to vary among clones include growth rate, nutrient and vitamin requirements, luminescence and toxicity (Wood and Leatham 1992). It is often assumed that toxin production is a biological process intrinsic to some toxin-producing dinoflagellates and its genetic basis is encoded by the algal genome (Ishida et al. 1993; Ishida et al. 1998). Nevertheless for many harmful algal bloom (HAB) species, tremendous intra-specific variability in toxicity has been shown (reviewed by Burkholder and Glibert 2006).

Among HAB flagellates, dinoflagellates of the genus *Alexandrium* are among the most ecologically important, as they have been involved in massive fish kills (Mortensen 1985; Cembella et al. 2002) and marine mammal morbidity and mortalities (Durbin et al. 2002; Doucette et al. 2006). Furthermore, *Alexandrium* blooms are the proximal source of toxins associated with paralytic shellfish poisoning (PSP), a neurological affliction that has caused human illness for centuries via consumption of contaminated shellfish (Prakash et al. 1971). In addition, there is increasing evidence that within the genus *Alexandrium*, lytic activity of extracellular metabolites and other negative effects upon other microalgae (Blanco and Campos 1988; Arzul et al. 1999; Fistarol et al. 2004b; Tillmann et al. 2007; Tillmann et al. 2008a) and towards heterotrophic protists (Hansen 1989; Hansen et al. 1992; Matsuoka et al. 2000; Tillmann and John 2002) is rather widespread. The allelochemical mechanism against other protists, typically immobilization followed by cell lysis or temporary cyst formation in the target species, was shown to be due to unknown extracellular substances – independent of both groups of known phycotoxins, paralytic shellfish toxins (PSTs) (Tillmann and John 2002) and spirolides (Tillmann et al. 2007), produced among various *Alexandrium* strains. Whereas intra-specific variability of PST content and composition are well known (Maranda et al. 1985; Cembella et al. 1987; Ogata et al. 1987b), there are very few quantitative data on the inter- and intra-specific variability in lytic capacity. Variability in lytic capacity was obvious when comparing one clone each of seven different *Alexandrium* species (Tillmann et al. 2008a). However, these results can not be used as evidence for significant inter-specific differences as long as the range of intra-specific variability is unknown. Therefore, a detailed study was performed to simultaneously investigate genotypic diversity and phenotypic variation, with respect to both PSTs and lytic potency, among clonal isolates from a single *Alexandrium tamarense* population (Alpermann et al. in press). In this previous study, a high genotypic richness among 67 clonal cultures was in congruence with very diverse PST profiles and with a very variable expression of lytic properties. Lytic activity was estimated by determining

percent cell lysis of two different target organisms at one fixed *A. tamarense* cell density. However, detailed quantitative comparisons of this kind of toxicological data require full dose-response curves over a wide range of different donor concentrations, from which comparable parameters (e.g., EC_{50} values, toxin concentration where 50 % of target cells are affected) can be derived. In the present study, we therefore aimed to fully quantitatively determine the lytic capacity of a subset of 10 selected clones of *A. tamarense*, taken from a single population, by recording complete dose-response curves of two different planktonic target species.

2.5.3. Materials and Methods

Origin and maintenance of cultures

Ten clonal strains of *Alexandrium tamarense* (Alex1–Alex10) were selected for the experiments from a larger collection of >60 clones of the North American ribotype (Type 1) of this species isolated simultaneously in May, 2004 at the same geographical location (56° 05' 47'' N and 1° 42' 35' 'W) from the Scottish east coast of the North Sea. The clones were isolated by micro-capillary pipette from a single net haul (20 µm mesh, from 20 m depth) and maintained as unialgal stock cultures under standardized growth conditions. To assure that true clonal cultures were established and maintained all isolates were genotyped with microsatellites (Alpermann et al., 2006). The absence of more than one allele at six microsatellite loci for all cultures implies that only a single haplotype was present in each culture (Santos and Coffroth 2003), as distinguished from the multiple haplotypes that could have arisen from isolation of diploid planomeiocytes. Stock cultures were grown non-axenically in K-,medium (Keller et al. 1987), supplemented with selenite (Dahl et al. 1989), prepared from 0.2 µm sterile-filtered (VacuCap, Pall Life Sciences) seawater from the North Sea (salinity: 32 psu, pH adjusted to 8.0) in 100 mL Erlenmeyer flasks under controlled conditions at 15 °C under fluorescent light at a photon flux density (PFD, measured with a LICOR spherical light sensor) of 100 µmol m^{-2} s^{-1} on a 16:8 h light–dark cycle. Stock cultures were diluted regularly and were in exponential growth for the experiments, all of which were conducted about 1 year after isolation of the clones.

In order to cover a wide spectrum of lytic potency, selection for the experimental isolates was based on the results of prior screening experiments to test their lytic capacity against whole cell cultures of the cryptophyte *Rhodomonas salina* and the heterotrophic dinoflagellate *Oxyrrhis marina* at one fixed *Alexandrium* concentration in a standardized bioassay (Tillmann et al. 2008a) In particular, we selected the only two (out of 67) clones which had caused no severe damage to *Rhodomonas*, together with 8 other clones covering the whole range of percent target mortality.

To quantify lytic capacity of the *A. tamarense* clones, two different target protistan species were cultured for short-term exposure experiments. The heterotrophic dinoflagellate Oxyrrhis *marina* (Göttingen culture collection, strain B21.89, isolated from the North Sea at Helgoland, Germany) was grown at 15° C and at a PFD of 20 µmol m^{-2} s^{-1} with the chlorophyte *Dunaliella salina* as food. Cultures were transferred once or twice per week to fresh medium containing food organisms. Cultures of *O. marina* for the experiments were grown to high cell concentrations until

they became almost deprived of food, as checked by microscopic examination. The phototrophic test alga *Rhodomonas salina* (Kalmar culture collection, KAC 30) was grown in K-medium at 15° C and under a PFD of 100 µmol m^{-2} s^{-1}. Cultures were transferred weekly to fresh medium and maintained as stock cultures prior to experimentation.

Growth rate estimates

Stock cultures of the ten selected *A. tamarense* clones were simultaneously set up under standard culture conditions as described. Before starting the growth experiments, stock cultures were verified to be in exponential growth phase according to three consecutive microscopic cell counts performed every other day. For the experiments, triplicate cultures of 80 mL of each *A. tamarense* clonal isolate were initially prepared in 100-mL Erlenmeyer flasks by dilution of the stock cultures (density: 2 – 4 x 10^3 cells mL^{-1}) to reach a starting concentration of about 150 cells mL^{-1}. Immediately after mixing, 2-mL samples were taken to estimate the initial cell concentration. Flasks were placed haphazardly on an illuminated bench under a PFD of 100 µmol m^{-2} s^{-1} on a 16:8 h light–dark cycle in a temperature-controlled culture room at 15° C. Although irradiance measurements showed that the irradiance field was homogenous, positions of the flask were changed haphazardly every second day. Samples for cell counts (2 mL) were taken the following day and then at two-day intervals for about 4 weeks.

Cell counts and growth rate calculation

Cell counts were performed in sedimentation chambers with an inverted microscope (Zeiss Axiovert 40C) on samples fixed with Lugol´s solution (10 g potassium iodide; 5 g iodine in 100 mL distilled water) at a final concentration of 2 %. Depending on the cell concentration, the volume set up for cell counting varied from 0.1 to 2 mL. Total number of cells counted was always >400 per sample, except for the initial few days when cell concentrations were <200 mL^{-1}. Exponential growth rate µ (d^{-1}) was calculated separately for each replicate by linear regression of ln-transformed cell number versus time for a defined period (see below) during exponential increase. For replicate cultures, co-variance analysis of regression lines showed that exponential growth in replicate cultures did not differ significantly ($p > 0.2$, $n = 3$); therefore, growth curves for each clone were plotted from mean cell numbers of the three replicates (see Fig. 2.5.1). Growth of *A. tamarense* exhibited a lag-phase for most clones and a more or less gradual shift from exponential to a more linear increase at higher cell concentrations before stationary phase. In order to account for and to objectively define the lag phase, cell counts from Day 0 were not included and cell counts from Day 1 were omitted (because of a initial lag phase) when $(\ln(N_{t3})–\ln(N_{t1}))/2$ (i.e. the rate of increase from Day 1 to 3) was <50 % of $(\ln(N_{t5})–\ln(N_{t3}))/2$ (i.e. the rate of increase from Day 3 to 5). From the newly estimated starting time, a stepwise regression of ln-transformed cell numbers versus time was calculated. The last time point to include in the final regression estimate was defined when the next time point reflected a >5% reduction in the growth rate. Analysis of variance (ANOVA) and Tukey's HSD post-hoc tests were used to determine significant differences in exponential growth between clones as assessed by µ calculated for each replicate. All statistical analyses were carried out with STATISTICA ™ software (StatSoft inc., USA).

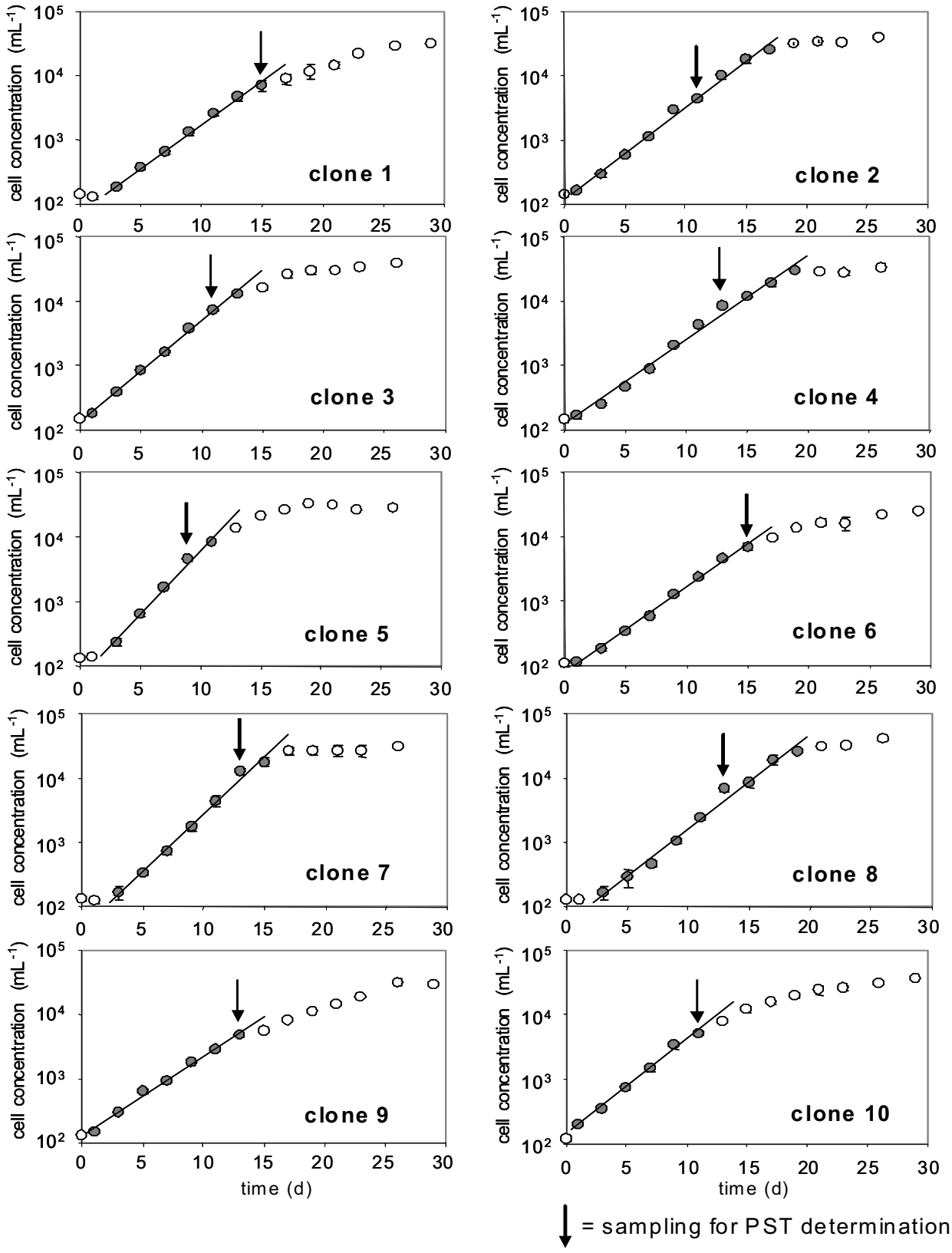

Fig. 2.5.1. Growth curves of 10 *A. tamarense* clonal cultures. Data points represent mean cell counts of three replicate cultures (± 1SD). Grey dots were used to calculate exponential growth rate (see paragraph 2.5.3.). Arrows indicate sampling points for PSP toxin determination.

PSP toxin determination

As cell toxin quota of *Alexandrium* spp. can vary widely over various growth stages in batch culture (Cembella 1998), we collected all samples for PSP toxin analysis only within the same growth stage. All clones were harvested during late exponential phase of the growth rate experiment (see Fig. 2.5.1) when cell concentrations were >4 x 10^3 mL^{-1}. A sample (40 mL) of each replicate culture was centrifuged (3,200 x g, 10 min at 10° C) and the cell pellets were re-suspended in 1.0 mL of 0.03 M acetic acid, and subsequently transferred into a FastPrep tube containing 0.9 g of lysing matrix D (Thermo Savant, Illkirch, France). The samples were homogenized by reciprocal shaking at maximum speed (6.5 m s^{-1}) for 45 s in a Bio101 FastPrep instrument (Thermo Savant, Illkirch, France). After homogenization, samples were centrifuged (Eppendorf 5415 R, Hamburg, Germany) at 16,000 x *g* at 4 °C for 15 min and 400 µL of the supernatant were transferred to a spin-filter (pore-size 0.45 µm, Millipore Ultrafree, Eschborn, Germany) and centrifuged for 30 s at 800 x *g*. The filtrate was transferred into an LC vial and analyzed by liquid chromatography with fluorescence detection (LC-FD) with post-column derivatisation (Krock et al. 2007).

Water was deionised and purified (Millie-Q, Millipore GmbH, Eschborn, Germany) to 18 $M\Omega cm^{-1}$ quality or better. Formic acid (90 %, p.a.), acetic acid (p.a.) and ammonium formate (p.a.) were purchased from Merck (Darmstadt, Germany), nitric acid (p.a.) and phosphoric acid (p.a.) were from AppliChem (Darmstadt, Germany), periodic acid, 1-heptanesulphonic acid, 1-octanesulphonic acid and diammonium hydrogen phosphate were from Sigma (Deisenhofen, Germany). The solvents, methanol, tetrahydrofurane (THF) and acetonitrile, were HPLC grade (Merck, Darmstadt, Germany).

Standard solutions of PSP toxins: saxitoxin (STX), neosaxitoxin (NEO), decarbamoyl saxitoxin (dcSTX), gonyautoxins 1&4 (GTX1/GTX4), gonyautoxins 2&3 (GTX2/GTX3), decarbamoyl gonyautoxins 2&3 (dcGTX2/dcGTX3), and B1, were purchased from the Certified Reference Material Programme of the Institute for Marine Biosciences, National Research Council, Halifax, NS, Canada.

The LC-FD analysis was carried out on a LC1100 series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) coupled to a PCX 2500 post-column derivatisation system (Pickering Laboratories, Mountain View, CA, USA). The LC-system consisted of a G1379A degasser, a G1311A quaternary pump, a G1229A autosampler, a G1330B autosampler thermostat, a G1316A column thermostat and a G1321A fluorescence detector. Chromatographic conditions were slightly modified from the method described by Hummert et al. (1997) as follows: mobile phase A: 6 mM 1-octanesulphonic acid and 6 mM 1-heptanesulfonic acid in 40 mM ammonium phosphate, adjusted to pH 7.0 with dilute phosphoric acid and 0.75 % tetrahydrofurane (THF) for the B-toxins and the gonyautoxin group; mobile phase B: 13 mM 1-octanesulphonic acid in 50 mM phosphoric acid adjusted to pH 6.9 with ammonium hydroxide and 15 % (v/v) of acetonitrile and 1.5 % of THF for the saxitoxin group. The flow rate was 1 mL min^{-1} with the following gradient: 0 min, 100 % A isocratic to 15 min, switch to 100 % B until 16 min, isocratic B until 35 min, switch to 100 % A until 36 min, isocratic 100 % A until 45 min (= total run time). The autosampler was cooled to 4°C and the injection volume was 20 µL.

The separation of analytes was performed on a 250 × 4.6 mm i.d., 5 µm, Luna C18 reversed-phase column (Phenomenex, Aschaffenburg, Germany) equipped with a Phenomenex SecuriGuard pre-column. The eluate from the column was continuously oxidized with 10 mM periodic acid in 550 mM ammonium hydroxide at a flow rate of 0.4 mL min^{-1} in a reaction coil set at 50 °C. Subsequently, the eluate was continuously acidified with 0.75 N nitric acid at a flow rate of 0.4 mL min^{-1} and the derivatised toxins were detected by a dual monochromator fluorescence detector (λ_{ex} 333 nm; λ_{em} 395 nm). Data acquisition and processing were performed with the HP ChemStation software (Agilent,Waldbronn, Germany). The C-toxins were identified indirectly by desulfonation to the respective gonyautoxins with 1 M hydrochloric acid for 15 min at 90°C. Hydrolyzed samples were neutralized by the addition of 150 µL 1 M sodium acetate and re-injected after filtration (30 s, 800 x *g* spin-filter, pore-size 0.45 µm, Millipore Ultrafree, Eschborn, Germany). Gonyautoxin concentrations of the non-hydrolyzed samples were subtracted from the respective hydrolyzed samples. The obtained gonyautoxin concentration is equivalent to content of the respective C-toxin. PSP toxin concentrations were determined by external calibration (Krock et al. 2007)

Quantification of lytic effects

Allelochemical potency of *A. tamarense* clones was quantified with two short-term (24 h) bioassays with the cryptophyte *R. salina* or the heterotrophic dinoflagellate *O. marina* as target organisms. After exposure to *A. tamarense*, these target protists first slightly increased in size due to swelling, then formed blisters and finally lysed. Counting the number of intact target cells (normal cell shape still visible) after 24 h incubation period enabled quantification of the lytic effect. In order to quantitatively compare the lytic potency of the clonal strains, dose-response curves over a wide range of *A. tamarense* cell concentrations were recorded. For each clonal strain, preliminary dose-response curves (both *R. salina* and *O. marina*) were estimated to define the *A. tamarense* cell concentrations for the lytic test.

Three replicate cultures (100 mL each) for each *A. tamarense* clonal strain were grown under standard culture conditions as described. Before starting the experiment, the *Alexandrium* cell concentrations of each replicate was estimated by cell counts (which ranged from 3 to 7 x 10^3 mL^{-1} for the eight "lytic" strains and were about 17 x 10^3 mL^{-1} for the two "non-lytic" strains). Appropriate dilutions (2.9 mL each) were prepared to yield a series of 6–7 final cell concentrations ranging from 50 to 1.6 x 10^4 cells mL^{-1}, depending on the *A. tamarense* clone and target species. For each replicate culture, each dilution was set up in duplicate in 5 mL glass-vials; three vials with only K-medium served as the control.

The bioassay was performed as follows. Cell concentrations of stock cultures of *R. salina* and *O. marina* were determined by microscope cell counts and subsequently diluted to a final concentration of about 3.3 x 10^5 mL^{-1} (*R. salina*) or 1.8 x 10^4 mL^{-1} (*O. marina*). Each set of experimental vials was spiked with 0.1 mL of either *R. salina* (final cell concentration: 10 x 10^3 mL^{-1}) or *O. marina* (final concentration: 0.6 x 10^3 mL^{-1}) to start the experiment. Immediately after adding the target species, 0.5 mL of each control vial was fixed with 2 % Lugol´s solution to estimate the actual initial target concentration. Samples were incubated at 15 °C under low photon

flux density (about 5 µmol m^{-2} s^{-1}) on a 16:8 h light–dark cycle. After 24 h, samples were fixed with 2 % Lugol´s solution and cell concentrations of both *A. tamarense* and the intact target species were determined by inverted microscope counts of subsamples taking after gentle mixing. The volume for cell counts was 0.5 mL and 1 mL for the *Rhodomonas* and *Oxyrrhis* bioassay, respectively. For *O. marina*, whole chambers were counted, whereas for *R. salina*, a sub-area corresponding to at least 800 cells in the control was counted. In order to quantify lytic effects, only intact cells of the target species were scored. Depending on the cell concentration, *A. tamarense* was counted in the whole chamber or in representative sub-areas.

In these dilution experiments, final concentrations of target species followed a sigmoidal declining pattern when plotted against log-transformed *A. tamarense* cell concentrations. In order to estimate EC_{50} concentrations (i.e. the *Alexandrium* cell concentration yielding a 50 % decline in target cell concentration), data points were fit to the following equation from the non-linear fit procedure in STATISTICA ™:

$$N_{final} = N_{control} / (1 + (x / EC_{50})^{h})$$

where N_{final} is the final target cell concentration, $N_{control}$ is the final target cell concentration in control samples, x is the log-transformed *A. tamarense* concentration and EC_{50} and h are fit-parameters. Results are expressed as EC_{50} (cells mL^{-1}) including 95 % confidence intervals. In order to visually compare all curves (which had slightly different target control concentrations), plots were normalized by setting the control as 100 %. Statistical comparisons of the lytic effects between strains were compared by ANOVA and post-hoc Tukey's HSD tests.

2.5.4. Results

Growth rate

Growth curves of all ten clones are plotted in Fig. 2.5.1. For a number of clones, a lag phase of 1–3 days was evident. Whereas some clones (2, 4, 7, 8) grew at a maximum (and constant) exponential rate until stationary phase, others showed a more or less gradual shift from exponential to a linear increase at higher cell concentrations. Maximum cell concentration in stationary phase was similar for all clones, ranging from 25 to 40 x 10^3 cells mL^{-1}. Only the maximum cell concentration of clone 6 was significantly lower than that of clones 2, 3, and 8 (ANOVA, Tukey's HSD test, $p < 0.05$). Specific growth rate µ ranged from 0.28 to 0.46 d^{-1} (Fig. 2.5.2) and was significantly different among clones (ANOVA, $F = 61.4$, $p < 0.0001$). Post-hoc tests revealed that growth rates of clone 7 and 5 were significantly different from all others and that among the other clones, three homogenous subgroups can be clustered (Tukey's HSD, $p < 0.05$).

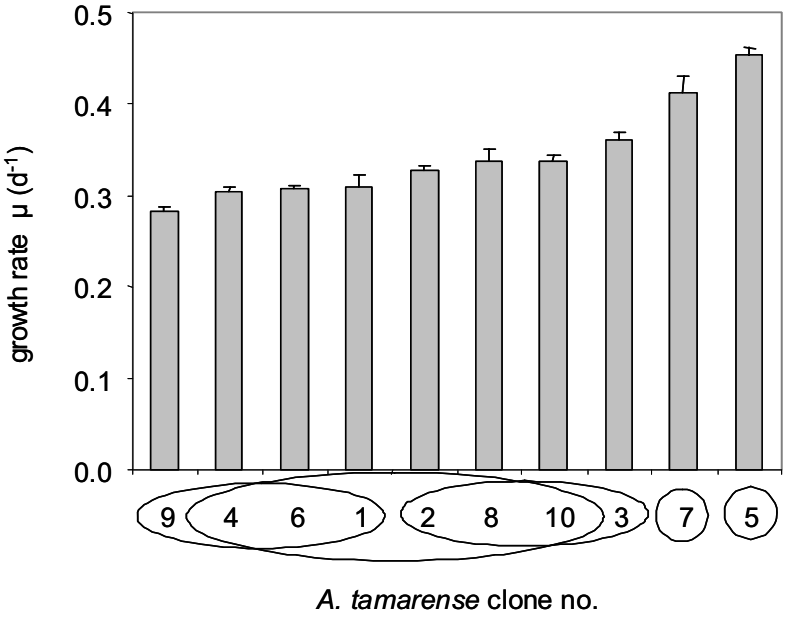

Fig. 2.5.2. Mean growth rate (n = 3, ± 1 SD) of 10 *A. tamarense* clonal cultures. Circles indicate classification of clones forming homogenous groups (ANOVA, Tukey's HSD test, $p < 0.05$).

PSP toxins

The qualitative PSP toxin composition (Table 2.5.1) was fairly consistent among the 10 *A. tamarense* clones sampled during late exponential growth phase (see arrows in Fig. 2.5.1). All clones contained C1/C2, GTX1 and 4, GTX 2 and 3, NEO and STX, with C1/C2, NEO and STX present in the highest relative abundance in all isolates except clone 3. Decarbamoyl toxins and B2 were never >0.5 % of total toxin content, and were often below detection limit. Clone 3 differed in PSP toxin composition from all other clones, because of its relatively high amount of B1 (26 mol%) and GTX1/GTX4 (23 mol%), as well as a very low STX (3.3 mol%) content.

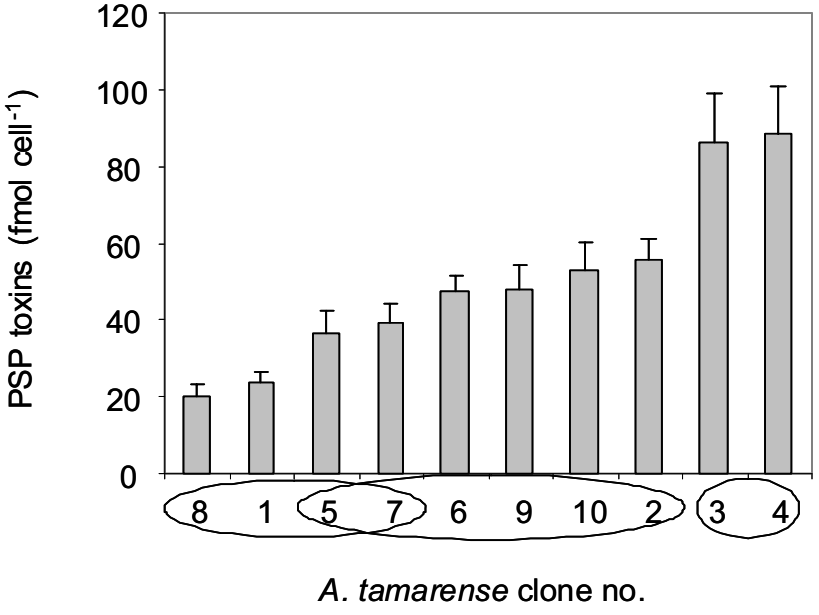

Fig. 2.5.3. Mean PSP toxins **c**ell quota (n = 3) ± 1 SD of 10 *A. tamarense* clones. Single PST compounds are summed up on a molar basis. Circles are used to classify clones forming homogenous groups (ANOVA, Tukey HSD test, $p < 0.05$).

Table 2.5.1. PST profiles of 10 *A. tamarense* clones. Values are mol% of total PST content (± SD). nd = not detected.

Clone	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
STX	14.8 ±0.5	43.1 ±1.1	3.3 ±0.3	31.3 ±0.8	27.4 ±0.6	25.6 ±1.5	22.7 ±2.9	11.3 ±0.1	26.4 ±0.6	23.7 ±1,0
NEO	39.1 ±0.6	26.4 ±0.6	18.1 ±0.6	18.2 ±0.8	31.6 ±0.6	29.5 ±2.8	19.5 ±1.3	14.5 ±0.9	25.7 ±.05	18.7 ±0.7
GTX1/ GTX4	5.4 ±0.3	3.6 ±0.2	23.2 ±0.9	8.6 ±0.5	6.4 ±0.1	9.2 ±0.1	11.1 ±2.0	5.7 ±0.2	6.2 ±0.3	6.6 ±0.3
GTX2/ GTX3	3.2 ±0.3	3.8 ±0.4	1.2 ±0.1	9.5 ±0.3	2.2 ±0.1	8.8 ±0.5	9.8 ±0.5	6.5 ±0.1	4.1 ±0.1	10.5 ±0.3
dcSTX	nd	0.3 ±0.01	0.3 ±0.01	0.1 ±0.01	0.4 ±0.01	nd	0.1 ±0.02	nd	0.1 ±0.00	nd
dcGTX2/ dcGTX3	0.5 ±0.03	0.3 ±0.01	0.3 ±0.01	nd	nd	nd	nd	0.1 ±0.01	nd	nd
B1	2.5 ±0.1	nd	26.4 ±0.8	nd	nd	nd	0.4 ±0.04	1.2 ±0.1	1.9 ±0.3	0.5 ±0.06
B2	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
C1/C2	33.3 ±1.2	21.5 ±1.4	24.2 ±2.0	32.3 ±2.2	32.0 ±0.2	26.7 ±1.6	36.3 ±0.6	60.8 ±1.2	35.5 ±1.2	40.1 ±0.7

Total toxin content ranged from 21 to 89 fmol cell^{-1} (Fig. 2.5.3) and was significantly different among clones (ANOVA, $F = 30.1$, $p < 0.0001$). Post-hoc tests (Tukey's HSD, $p < 0.05$) indicated that three homogenous subgroups of clones could be clustered (Fig. 2.5.3). Clones 8 and 1 had a low toxin content (ca. 20 fmol cell^{-1}), clones 2, 5, 6, 7, 9 and 10 had intermediate toxin content (38 - 58 fmol cell^{-1}) and clones 3 and 4 had high total toxin amount (> 80 fmol cell^{-1}).

Lytic activity

In the bioassay 8 of 10 clones caused cell lysis of the cryptophyte *R. salina* and the heterotrophic dinoflagellate *O. marina* (Fig. 2.5.4 and 2.5.5). In the 24h bioassay dose response experiments, there was a sharp decline in target cell number with increasing *A. tamarense* concentrations, but no significant mortality of target cells even at high *Alexandrium* cell concentrations was observed for clones 5 and 9 (Fig. 2.5.4 and 2.5.5).

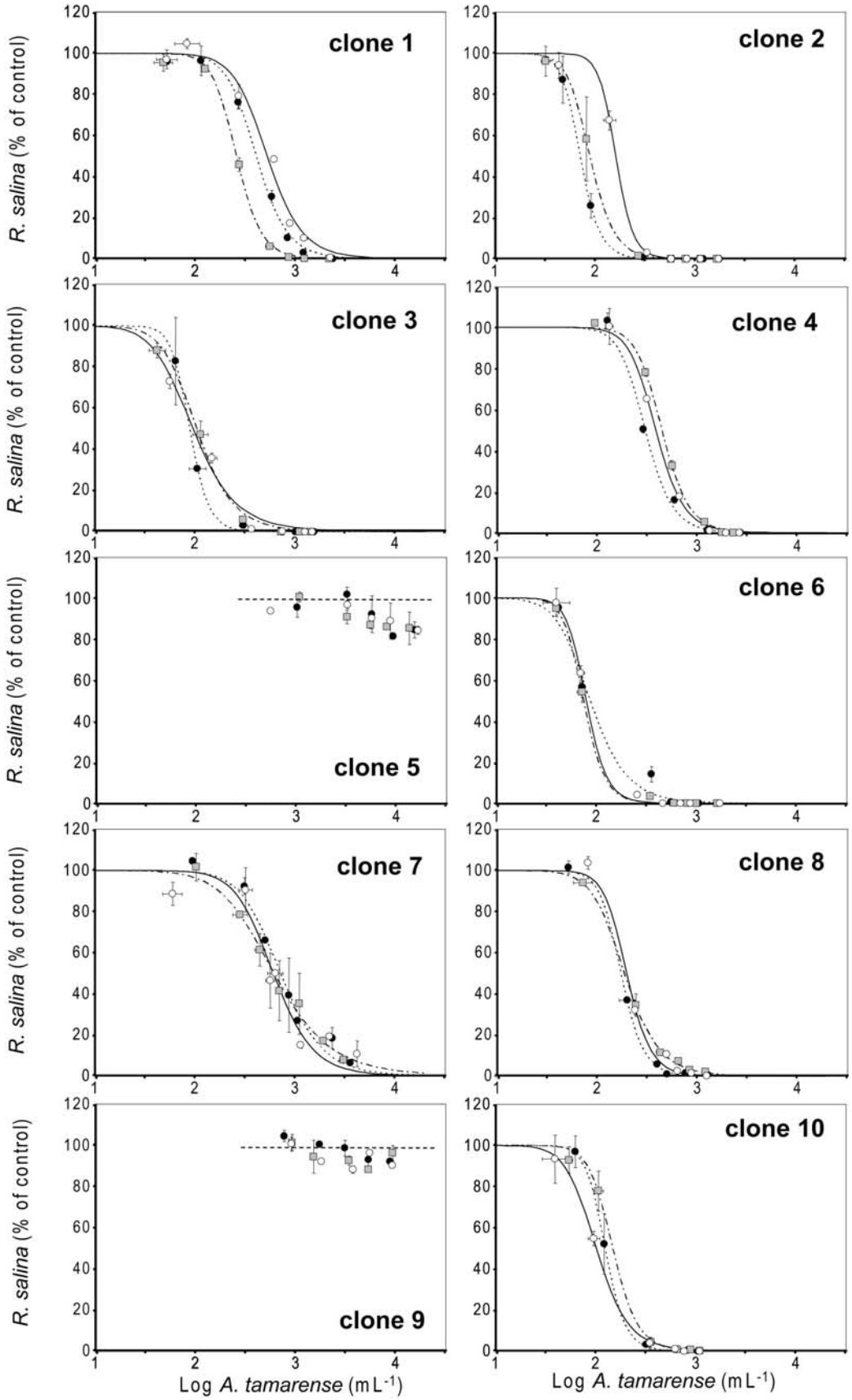

Fig. 2.5.4. Dose–response curve describing lytic capacity of 10 *A. tamarense* clones as quantified with the *Rhodomonas* bioassay. Each graph shows the concentration of *R. salina* after 24 h incubation (as % of control) as a function of log-transformed *A. tamarense* concentration of 3 replicate cultures (white circles, black circles and grey squares). Each data point represent mean (n = 2) ± 1SD. Lines represent a non-linear, sigmoidal curve fit (see paragraph 2.5.3.).

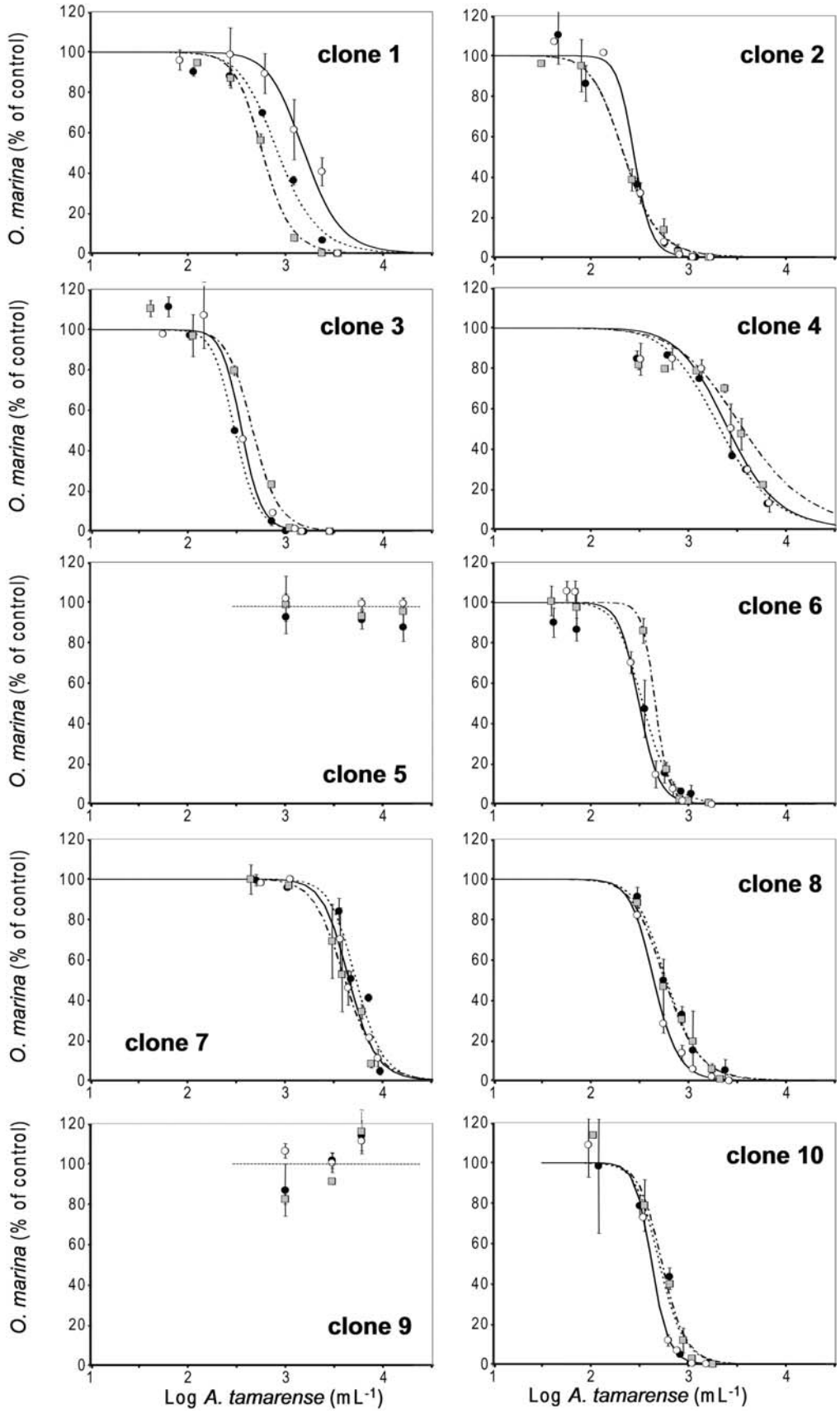

Fig. 2.5.5. Dose–response curve describing lytic capacity of 10 *A. tamarense* clones as quantified with the *Oxyrrhis* bioassay. Each graph shows the concentration of *O. marina* after 24 h incubation (as % of control) as a function of log-transformed *A. tamarense* concentration of 3 replicate cultures (white circles, black circles and grey squares). Each data point represent mean (n = 2) ± 1SD. Lines represent a non-linear, sigmoidal curve fit (see paragraph 2.5.3.).

EC_{50} values for the lytic clones ranged from 80 to 640 mL^{-1} (*Rhodomonas* bioassay) and from 240 to 4500 mL^{-1} for the *Oxyrrhis* bioassay (Fig. 2.5.6). For both bioassays, EC_{50} values were significantly different among clones (ANOVA, $F = 42.8$ for *Rhodomonas*, $F = 47.2$ for *Oxyrrhis*; $p < 0.0001$ for both) with three homogenous subgroups defined as clusters according to Tukey's HSD test ($p < 0.05$, Fig. 2.5.6). The EC_{50} values could not be calculated for clones 5 and 9 because unlike the other clones they had no apparent lytic capacity. For both bioassays, Tukey´s HSD test merged almost the same clones to homogenous subgroups; the only exception was clone 1, which was included in the largest subgroup for EC_{50}-*Rhodomonas*, but formed a subgroup together with clone 4 for EC_{50}-*Oxyrrhis*. EC_{50} values for both targets were significantly correlated ($r^2 = 0.868$)(Fig. 2.5.7).

Rank correlation analysis of lytic effect (as EC_{50} values for both *Rhodomonas* and *Oxyrrhis*) compared with PSP toxin content (both separately for each compound/group and as total toxin content) did not show any significant relationship (complete data set not shown, but exemplified in Fig. 2.5.8 for EC_{50}-*Rhodomonas* and total PSP-toxins). Likewise, rank correlation analysis of lytic effect (as EC_{50} values of both *Rhodomonas* and *Oxyrrhis*) and *A. tamarense* growth showed that lytic activity was not related to growth rate (exemplified in Fig. 2.5.9 for EC_{50}-*Rhodomonas* and growth rate μ).

2.5.5. Discussion

The nature and extent of intra-population clonal variation is an important concept in the autecological interpretation of species interactions (e.g., grazing, allelopathy, competition) and population dynamics (e.g., net growth rate) of phytoplankton. Maintenance of a multitude of co-existing genotypes in a geographical population may be particularly critical for the dinoflagellates, which are nominally haploid and therefore must present phenotypes for selection and fitness testing without the potential risk mitigation of recessive mutation. In one of the few studies on intra-population genotypic and phenotypic diversity in dinoflagellates (Alpermann et al. 2009; Alpermann et al. in press), the authors demonstrated both high intrinsic variation in molecular markers (microsatellites and amplified fragment length polymorphism [AFLP]) and lack of correlation of defined genotypes with the high variability observed in phenotypic characteristics – specifically lytic activity and PSP toxin content and composition – in a single population of *Alexandrium tamarense* from the Scottish coast. The current work presented here focused more intensively on the expression of phenotypic characters from a limited subset (10 isolates) selected from the bank of >60 isolates from this geographical population. The subset selection comprised the known wide spectrum of PSP toxin composition and allelochemical activity among these isolates, including two apparently non-lytic isolates (defined as causing no severe damage to *Rhodomonas* at a concentration of 1500 cells mL^{-1}(Alpermann et al. in press). By recording full dose–response curves, supplemented by PSP-toxin measurements the subset isolates yielded detailed data on growth rate and quantitative estimates of lytic capacity not available from the previous study.

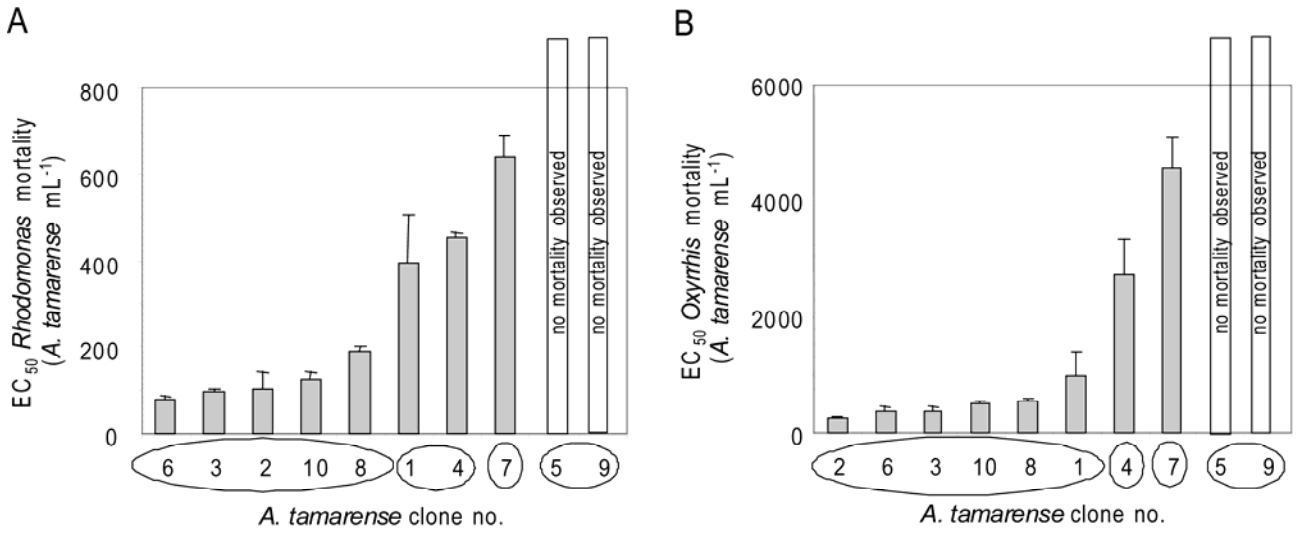

Fig. 2.5.6. EC_{50} values for 10 *A. tamarense* clones for (A) the *Rhodomonas* bioassay and B) the *Oxyrrhis* bioassay. Circles are used to classify clones forming homogenous groups (ANOVA, Tukey HSD test, $p < 0.05$).

Growth rate

Growth rate is an extremely significant parameter in population dynamics and phytoplankton ecology as it integrates numerous biochemical processes to yield a single resultant "output", usually defined in terms of changes in cell number or biomass. Ideally, in determining intra-specific or intra-population growth variability, cells of each clonal lineage should be acclimated under identical conditions in a constant environment and growth rates determined only after acclimation has been achieved. This "acclimated growth rate" determination from cultures is usually accomplished by measuring growth over a number of generations, and even over successive transfer cycles from the original stock cultures. The few studies that have examined temporal stability in growth rates among dinoflagellates have generally reported constant rates after complete acclimation for clonal cultures (Brand 1981; Shankle 2001, cited in: Shankle et al. 2004). In the present study, the temporal stability of growth rate for a given clone was not estimated by strict adherence to the principle of "acclimated growth rate"; however, growth rate was determined over several cell division cycles in exponential growth phase under standardized "optimal" conditions. Absolute values of μ depend on the interplay between intrinsic (genetic) factors and environmental conditions (light, temperature, nutrient status, etc.) and are thus hard to compare among different studies even with the same species. In any case, the range of growth rates among the 10 clones in our experiments ($\mu = 0.28 - 0.45\ d^{-1}$; C.V. 14.5 %) is generally in line with that from an extensive study on genetic variability in acclimated growth rates ($n = 75$; $0.19 - 0.66\ d^{-1}$; C.V. 10.4 %) among contemporaneous clones of *A. tamarense* (cited as *Gonyaulax tamarensis*) (Brand 1981). Significantly, in this previous study, growth rates tended to vary within a rather restricted range (for $n = 72$; $\mu \geq 0.5 \leq 0.65\ d^{-1}$), with very low growth rates only recorded for the remaining 3 isolates. Nevertheless, due to the exponential function of the growth rate (μ), even seemingly small

differences will cause large differences in cell abundance of clonal lineages within a population after a certain time. For example, hypothetical growth of our fastest growing clone ($\mu = 0.45\ d^{-1}$) in "competition" with the slowest growing one ($\mu = 0.28\ d^{-1}$) in a simple numerical model with no differential loss parameters would change an initial proportion of 10 % to about 95 % after one month. On the other hand, "constant" growth conditions, as are assumed to exist during optimal balanced growth in exponential phase in batch culture experiments, are unlikely to prevail over longer periods in the plankton. Shifts in growth rate of clonal lineages under different environmental pressures may also contribute to re-equilibration of clonal abundances in natural populations.

Toxin cell content and composition

Intra-specific variability of toxin levels and composition among different isolates of *A. tamarense* is well known (Alam et al. 1979; Schmidt and Loeblich III 1979b; Schmidt and Loeblich III 1979a) and has been clearly documented in many cases (Maranda et al. 1985; Orlova et al. 2007). Within the *A. tamarense* species complex, there are even clones for which PSP toxin levels are beneath the analytical detection limit (Cembella et al. 1987). For *A. tamarense* from the western Pacific, Ogata et al. (1987b) reported a 100-fold variation in total toxicity among clones from a given area and even 20-fold differences among subclones originating from a single clonal culture. This led Ogata et al. (1987b) to suggest that quantitative aspects of toxin production may not be a hereditary characteristic, and may arise from stochastic variation and epi-genetic factors.

In contrast, the "profile" or toxin composition (%mol) has repeatedly been shown to be a relatively stable characteristic of a given *A. tamarense* clone (Cembella 1998), subject to modification only under extreme condition (e.g., long-term *N*-starvation (Boczar et al. 1988)). Stable variability in toxin profile has been shown among isolates of different geographical origin (Cembella et al. 1987), but also among clones from a single population (this paper, Ichimi et al. 2002; Alpermann et al. in press). The variation in toxin composition among clonal strains ($n = 10$) of *A. tamarense* determined in our current study was consistent with measurements on the complete set of clones (Alpermann et al. in press). Furthermore, in spite of the differences in experimental design, growth conditions and time in culture, the clonal toxin profiles have not changed significantly between the two sets of experiments.

Lytic activity

Studies on allelochemical potency and attendant clonal variability represent a relatively new field of dinoflagellate research, compared with the investigations of acclimated growth rates and PSP toxin profiles which have been carried out for several decades. Our results here (e.g., Fig. 2.5.8) are consistent with the well established idea that PSP toxins are not evidently involved in the negative effects observed on other protistan species (Tillmann and John 2002; Fistarol et al. 2004b; Alpermann et al. in press). In fact, in spite of a few speculations in the literature (Hansen 1989; Cho and Matsuoka 2000; Tillmann and John 2002), there are no validated reports on direct effects

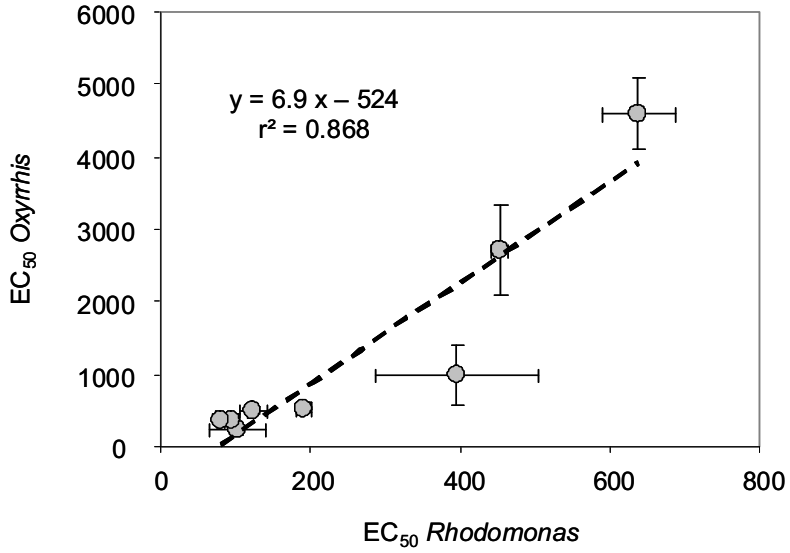

Fig. 2.5.7. A comparison of EC_{50} values obtained with the *Rhodomonas* bioassay (X-axis) and the *Oxyrrhis* bioassay (Y-axis). Each data point represents mean (n = 3) ± 1 SD values for one of the 8 *A. tamarense* clones for which EC_{50} values could be calculated. Dotted line represents a linear fit.

of PSP toxins on marine protists. Harmful effects of *Alexandrium* spp. against a wide range of marine protists have been shown to be due to extracellular lytic compounds, and not to secondary effects such as pH shifts or bacteria present in the cultures (Tillmann and John 2002; Tillmann et al. 2008a). The extracellular lytic compounds produced by *Alexandrium* spp. remain poorly characterized and the chemical structures are unknown. In any case, through the direct destructive effect on phytoplankton competing for limiting resources and/or against protistan grazers, these compounds are likely to be of great importance for *Alexandrium* bloom development and persistence. Within the genus *Alexandrium*, lytic activity has been detected in all species tested so far, but the strength of lytic activity varied considerably among different species and strains of the same species of different geographical origin (Tillmann et al. 2007; Tillmann et al. 2008a).

In the present detailed study, eight out of 10 selected clones of *A. tamarense* caused cell lysis of both photosynthetic (*R. salina*) and heterotrophic (*O. marina*) protistan targets, whereas for two clones, no significant mortality was observed, even at high *Alexandrium* cell concentrations. These were the only two non-lytic isolates found among the much larger set of clones (n = 67) previously screened with respect to lytic potency (Alpermann et al., in press). At least within this Scottish North Sea population, the absence of measurable lytic activity is thus a rare phenotypic trait. Among the subset *A. tamarense* isolates, EC_{50} concentration for *Rhodomonas* as target ranged widely from 80 to 640 cells mL^{-1}. Interestingly, a comparison of this range of values to the EC_{50} range (based on the same bioassay) estimated for single isolates of *A. ostenfeldii*, *A. catenella* and *A. minutum* (230 to 520 cells mL^{-1}) (Tillmann et al. 2008a) indicate that the intra-specific and intra-population clonal variability in lytic potency within *A. tamarense* may be even higher than variability among strains of different *Alexandrium* species.

Little is known about long-term stability of the lytic phenotype. There are some indications that EC_{50} values of a given strain may vary somewhat when tested at different times (Tillmann et al. 2007). On the other hand, EC_{50} values obtained for *O. marina* in the present study compared with

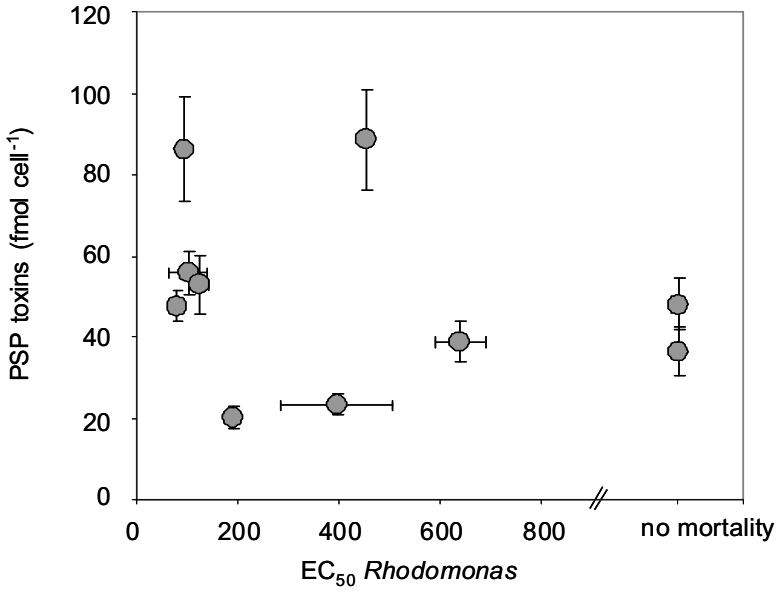

Fig. 2.5.8. A comparison of EC_{50} values (*Rhodomonas* bioassay) and PSP-toxin content (fmol $cell^{-1}$) for 10 *A. tamarense* clones. Each data point represent mean (n = 3) ± 1 SD values.

the % mortality of *O. marina* cells previously presented (Alpermann et al., in press) for the same 10 clones showed that they were significantly correlated (Spearman's $R = 0.80$, $p < 0.01$). The other study was performed about one year before the measurements reported here, indicating that at least over an annual time frame the quantitative lytic activity is a relatively stable trait of a given clone. Moreover, at least two of the strains tested (lytic clone 2 and non-lytic clone 5), which have been extensively tested and experimented upon, have retained their quantitative expression of the lytic trait for over 4 years (U. Tillmann, unpublished).

We address the question: what are the implications and consequences of this observed high variability in lytic capacity within single populations? High clonal variability of a phenotypic trait could be interpreted as an indication that the lytic phenotype is under low or at least inconstant evolutionary selective pressure. In the case of extracellular lytic compounds, however, it is reasonable to suggest that this "low evolutionary pressure" applies mainly to quantitative aspects, as local aggregations of cells temporarily yield a "chemical cloud" of benefit to all clones in this patch regardless of their actual quantitative contribution to the exuded substances. In other words, based upon group selection, extracellular allelochemicals might be selectively favoured on the population level as a whole rather than on individual cells that produce high amounts of allelochemicals. This explanation is consistent with the finding that only two out of 67 clones of *A. tamarense* completely lack the lytic activity expressed by the large majority of individual clones, albeit in variable quantities.

Unfortunately we do not know the growth history and the state of development of the *A. tamarense* population from which these clones were sampled. The low *A. tamarense* concentrations at the time of sampling (ca. 90 cells L^{-1}) are not indicative of a "bloom" population but it is not possible to clearly discriminate among the alternative explanations for the low cell numbers – a newly initiated *in situ* population, advective dispersal, extensive grazing, post-senescent decline, etc. At an early stage of bloom development, the population tends to high phenotypic diversity, because its individuals originate mainly from a large number of freshly hatched cysts. Composed

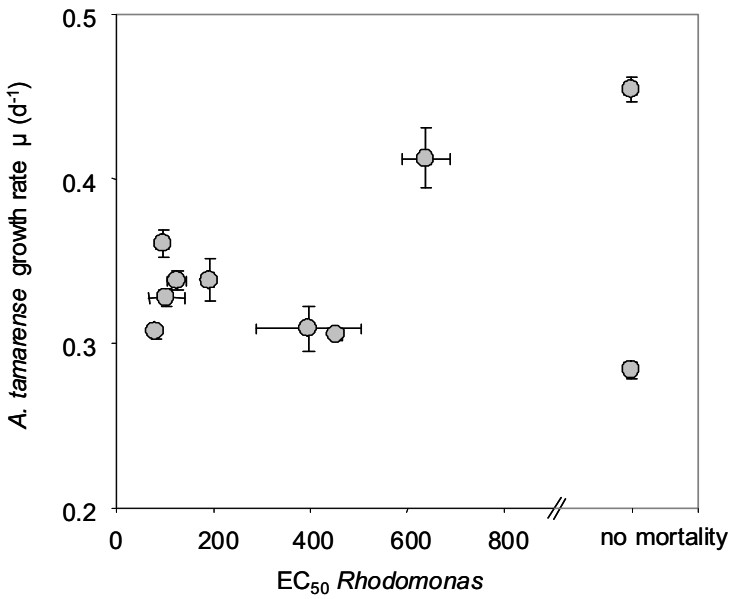

Fig. 2.5.9. A comparison of EC_{50} values (*Rhodomonas* bioassay) and growth rate for 10 *A. tamarense* clones. Each data point represent mean (n = 3) ± 1 SD values.

mainly of post-meiosis products, such a population would also be expected to be genetically more diverse than a more mature one that already grew substantially by vegetative cell division, at least if certain clonal lineages became relatively more abundant. In the course of population development (bloom formation), certain genotypes (e.g., those with high lytic capacity) would be selected for and gain at least a particular advantage in a bloom population. Indeed, findings on population genetic parameters such as population-wide multilocus linkage disequilibrium together with the phenotypic differentiation of genetic population subgroups that may represent offspring cohorts from different years indicate that such processes do take place in natural *Alexandrium* populations (Alpermann et al. 2009).

Our results showing no correlation between growth rate and lytic activity (Fig. 2.5.9) indicate that at least under non-limiting growth conditions, no obvious growth reducing costs seem to be associated with the production of allelochemically active secondary metabolites, such as the lytic substances. However, as the chemical composition of these compounds remains unknown, significant growth reducing "costs" for the production, might be expected under nutrient-limited conditions, especially when the molecules are rich in the particular limiting element, or under sub-optimal energetic regimes (e.g., light limitation).

Allelopathy is generally considered to be especially effective in stress situations (Reigosa et al. 1999), such as under nutrient limitation. Target organisms can be more susceptible to allelochemicals under stress (Fistarol et al. 2005), and/or producer organisms might induce or augment the production of allelochemically active compounds under such conditions (Gross 2003; Legrand et al. 2003) In any case, quantitative differences of lytic compounds need to be investigated under "stress" and/or limiting growth conditions to completely address the question of metabolic costs and effectiveness associated with the production of these secondary metabolites.

In addition to quantitative differences in the amount of lytic compounds produced, qualitative differences may also be expressed. In a detailed characterization of lytic effects of different *Alexandrium* strains on a number of different protists (Tillmann et al. 2008a), variable ratios in EC_{50}

values between donor/target combination could not be explained by quantitative differences in allelochemical production, but rather indicated qualitative differences in the composition of lytic substances produced by *Alexandrium* strains belonging to different species (Tillmann et al. 2008a). For the data presented here, however, there is no evidence for such qualitative differences occurring among clonal strains of *A. tamarense* because EC_{50} values for both targets were significantly correlated (Fig. 2.5.7). The slope (α) of the linear regression line ($\alpha = 7$) nicely fits to a calculated ratio (β) of surface area of the two targets ($\beta = 6$), assuming a sphere of 8 and 20 µm diameter for *R. salina* and *O. marina*, respectively. This is further evidence for the notion that these lytic compounds act on the cell surface, presumably on outer membranes. Nevertheless, this finding should not be generalized to imply that "small" target species are "susceptible"; in fact, without any relation to size, a few target species were found to be relatively refractory or even unaffected by extracellular compounds of *A. ostenfeldii* (Tillmann et al. 2007) indicating that structural modification might be involved in susceptibility towards extracellular allelochemicals.

Conclusion

All parameters tested showed high intra-specific and intra-population variability within a geographical site for the planktonic dinoflagellate *A. tamarense*. We interpret this to indicate that there exists a large genetic reservoir allowing the population to flexibly respond to short- and long-term changing abiotic and biotic conditions. Nevertheless, knowledge of the amount of genetic variability expressed under one set of environmental conditions does not necessarily allow inferences about the genetic variability expressed under another set of environmental conditions (Brand 1985). With respect to lytic capacity, the production of lytic compounds by *A. tamarense* seems to be common but quantitative variability was also shown to be high. Despite the complications generated by the existence of phenotypic and genotypic variability within populations, such variation can be used in a comparative approach to investigate the ecological advantages (or disadvantages) of particular traits. For example, the obviously non-lytic clonal *A. tamarense* strains identified in this study can be compared with lytic strains to explore various aspects of the ecological effects, consequences and metabolic costs of expressing the lytic trait. The genotypic uniqueness of these clones allows the analysis of specific microsatellite loci (Nagai et al. 2004; Alpermann et al. 2006a) for the enumeration of single, pre-characterized clones in micro- and mesocosm experiments to follow the success of certain phenotypic traits in mixed assemblages in the course of population and bloom development.

2.6. IMPLICATIONS OF LIFE HISTORY TRANSITIONS ON THE POPULATION GENETIC STRUCTURE OF THE TOXIGENIC MARINE DINOFLAGELLATE *ALEXANDRIUM TAMARENSE*

2.6.1. Abstract

Genotypic or phenotypic markers for characterization of natural populations of marine microalgae have typically addressed questions regarding differentiation among populations, usually with reference to a single or few clonal isolates. Based upon a large number of contemporaneous isolates from the same geographical population of the toxigenic species *Alexandrium tamarense* from the North Sea, we uncovered significant genetic substructure and low but significant multilocus linkage disequilibrium (LD) within the planktonic population. Between the alternative molecular genotyping approaches, only amplified fragment length polymorphism (AFLP) revealed cryptic genetic population substructure by Bayesian clustering, whereas microsatellite markers failed to yield concordant patterns. Both markers, however, gave evidence for genetic differentiation of population subgroups as defined by AFLP. A considerable portion of multilocus LD could be attributed to population subdivision. The remaining LD within population subgroups is interpreted as an indicator of frequency shifts of clonal lineages during vegetative growth of planktonic populations. Phenotypic characters such as cellular content and composition of neurotoxins associated with paralytic shellfish poisoning (PSP) and allelochemical properties may contribute to intra- or inter-annual differentiation of planktonic populations, if clonal lineages that express these characters are selectively favoured. Nevertheless, significant phenotypic differentiation for these characters among the genetically differentiated subgroups was only detected for PSP toxin content in two of the four population subgroups. By integrating the analysis of phenotypic and genotypic characteristics, we developed a conceptual model of population genetics to explain the importance of life cycle dynamics and transitions in the evolutionary ecology of these dinoflagellates.

2.6.2. Introduction

Eukaryotic microalgae of diverse phylogenetic origin often share alternations among phases of vegetative (asexual) reproduction and events of sexual reproduction, including the formation and fusion of gametes, as a common feature in their life cycle. Whereas the former mode of reproduction generates multiple individuals from the existing clonal lineages, the latter mode leads to the formation of new clonal lineages following meiotic division. Natural populations of extant microalgae are the ecological entities within which evolutionary processes and selection occur, raising questions regarding the consequences of life cycle events on the genetic structure of populations. Analysis of planktonic populations of microalgae by genotypic and phenotypic population markers is therefore appropriate to address these questions.

Within prokaryotic species, populations are alternatively organized in a panmictic, clonal or epidemic structure, with population entities dominated by a single or few clonal lineages in the two latter cases (Maynard Smith et al. 1993). These alternatives have been shown in studies on the marine cyanobacteria ("blue-green algae") *Microcoleus chthonoplastes* (Lodders et al. 2005) and *Nodularia* sp. (Barker et al. 2000), which display panmictic or epidemic population structures, respectively.

In contrast to the prokaryotic microalgae, comparatively little is known about the consequences of life cycle peculiarities and transitions on genetic structure within populations of eukaryotic microalgae. In any case, the occurrence and prevalence of mitotic versus meiotic reproduction modes within eukaryotic microalgal lineages, as well as the expression of species-specific ecological traits, certainly have genetic implications in natural populations. The few population genetic studies that have been conducted on marine eukaryotic microalgae, e.g. those on the diatoms *Ditylum brightwellii* (Rynearson and Armbrust 2005) and *Pseudo-nitzschia pungens* (Evans et al. 2005), or those on the prymnesiophyte *Emiliania huxleyi* (Iglesias-Rodríguez et al. 2006) and the dinoflagellate *Prorocentrum micans* (Shankle et al. 2004), have revealed a high level of genetic and genotypic diversity. Yet these studies presented little or no evidence for genetic differentiation within a "geographical population" over time. Whether or not standard models of population structure apply to eukaryotic microalgae remains therefore an open question.

Complete descriptions of life cycles are available for only about 1 % of the approximately 2500 extant dinoflagellate taxa (Elbrächter 2003). Nevertheless, at least the free-living forms can be subdivided into species that form benthic resting stages (hypnozygotic cysts) following sexual reproduction and those that do not. The capacity for sexual cyst production is a critical life history transition for certain planktonic dinoflagellates capable of bloom formation and is thus a well studied phenomenon (if only in a few dinoflagellate species), but the attendant implications for the population structure are poorly understood.

The free-living planktonic marine dinoflagellate *Alexandrium tamarense* (Lebour) Balech provides an interesting model organism for an integrative study on population characteristics. First, this species is found over a wide geographical range from polar to tropical latitudes, albeit with a distributional bias towards mid-latitudes and temperate coastal and shelf seas. Second, the physiology, growth characteristics and environmental tolerance have been well studied (e.g., Prakash 1967; Brand 1981; Watras et al. 1982; Glibert et al. 1988; Parkhill and Cembella 1999; Yamamoto and Tarutani 1999). Third, many strains isolated from natural populations produce potent neurotoxins associated with paralytic shellfish poisoning (PSP) (e.g., Ghazarossian et al. 1974; Shimizu et al. 1975b; Oshima et al. 1977; Alam et al. 1979; Hall 1982; Cembella et al. 1987; Anderson et al. 1994; Ichimi et al. 2002) and/or allelochemical substances causing lysis of co-occurring protists (e.g., Arzul et al. 1999; Tillmann and John 2002; Fistarol et al. 2004b; Tillmann et al. 2008a) − these can serve as additional phenotypic markers. Fourth, this species has high ecological relevance because of its role in outbreaks of PSP due to the accumulation of these toxins in the food chain. Fifth, the life cycle characteristics of different ecotypes of *A. tamarense* have been described in detail (see Wyatt and Jenkinson (1997) and Anderson (1998) and references therein), which represents an exceptionally sound base of knowledge. Finally, and perhaps most

importantly, the requisite genotypic and phenotypic population markers have been developed with specific reference to this species (John et al. 2004; Nagai et al. 2004; Alpermann et al. 2006a).

Planktonic populations of *A. tamarense* from coastal marine waters are initiated by hatching of diploid planomeiocytes from resting hypnozygotes from sea floor cyst beds at the beginning of the annual growth cycle (Wyatt and Jenkinson 1997; Anderson 1998). The lack of observations of overwintering populations of vegetative cells in the plankton indicates that this is not an important annual re-seeding mechanism. Although the exact temporal sequence of meiosis has not been determined with certainty, it is generally assumed that meiotic divisions of the planomeiocyte then lead to the formation of haploid cells that reproduce asexually by vegetative cell division and form part of the marine phytoplankton (Wyatt and Jenkinson 1997; Anderson 1998). The formation of gametes is essential to complete the annual life cycle at the end of the planktonic population growth since durable hypnozygotes that overwinter in sea-floor cyst beds are only formed after fusion of gametes and maturation of the resulting quadriflagellate planozygote (Wyatt and Jenkinson 1997). From this general life cycle, deviations such as the formation of temporary pellicular cysts may occur (Anderson and Wall 1978), but their adaptive significance (if any) in natural populations is unclear. Furthermore, direct transition from the planozygote to the planomeiocyte stage could possibly occur in the plankton without prior formation of hypnozygotes, as it has been described in several other species from the same genus (Figueroa et al. 2006; Figueroa et al. 2007). However, such direct sexual reproduction in planktonic populations of *Alexandrium* probably does not significantly contribute to numerical population growth.

Considering that sexual reproduction occurs cyclically in *A. tamarense* populations, genetic recombination is assumed and consequently clonal diversity is expected. Formation of gametes and consecutive events of sexual reproduction are restricted in time and presumed to be triggered by certain changes in environmental conditions and/or internal mechanisms (e.g. “endogenous clock”) (Anderson 1998), predominantly occurring towards the end of the planktonic bloom phase, when growth conditions are no longer optimal. Sexual reproduction and thus the potential for formation of new genotypes therefore occurs far less often than asexual reproduction by vegetative cell division, which constitutes the basis for population growth in planktonic dinoflagellates (Anderson 1998).

Whereas repeated sampling of genotypes can be a first step in the detection of clonal or partially clonal reproduction within natural populations, other indicators such as heterozygote deficiency and linkage disequilibrium might assist in the detection of shifts in the genetic population structure caused by clonal reproduction (Halkett et al. 2005). Repeated sampling of genotypes, however, has proved to be unfeasible in natural populations of *A. tamarense* due to the high genotypic diversity found within a single planktonic population (Nagai et al. 2007a). Heterozygote deficiency may be a useful potential indicator of clonal reproduction in populations of diplontic microalgae such as diatoms (Rynearson and Armbrust 2004; Evans et al. 2005; Rynearson and Armbrust 2005) or among diploid life stages of prymnesiophytes (Iglesias-Rodríguez et al. 2006) but it is not an applicable concept in dinoflagellates. With the probable exception of the genus *Noctiluca*, the free-living marine dinoflagellates are characterized by a nominally haploid genome within vegetatively dividing planktonic cells (Elbrächter 2003).

In contrast to heterozygote deficiency, linkage disequilibrium (LD), i.e. non-random associations of alleles at two or more gene loci, might help in the detection of clonal or partially clonal reproduction modes, even in cases where sampling of repeated genotypes is impractical, because clonal dominance may leave a signature with respect to linkage of loci (Halkett et al. 2005). Molecular markers not only allow the identification of clonal lineages and the extent of LD as a consequence of clonality, but also open up further possibilities to address questions regarding evolutionary ecology. For example, inference of genetic population structure by Bayesian clustering methods that make use of multilocus genotype data has become a widely applied approach in evolutionary ecology (e.g., Pritchard et al. 2000a; Dawson and Belkhir 2001; Corander et al. 2003; Guillot et al. 2005; François et al. 2006; Chen et al. 2007). Population genetic processes such as the admixture of genetically differentiated subpopulations of the same microalgal species can be potentially identified by such or similar approaches.

Our intention for this study was to investigate how the two reproduction modes, vegetative and sexual reproduction, may influence the genetic structure of a planktonic population of *A. tamarense*. The primary objective was to determine whether or not the planktonic population under study displays genetic features that can be linked to certain life cycle characteristics. A secondary objective was to establish the importance of multilocus linkage disequilibrium in the planktonic population and evaluate its validity as a measure for the impact of clonality on the genetic population structure in *A. tamarense*. Finally, by analysing the distribution of phenotypic characters within the population, we intended to provide evidence that the population genetic characteristics are linked to selection processes. This is the first study to comprise an in-depth analysis of within-population genetic parameters such as multilocus linkage equilibrium and population substructure in a planktonic dinoflagellate. Our results suggest that the complex life history of this species leaves a signature detectable in genetic variation of even a single local population. This underlines the need for an integrative framework combining evolutionary genetic and life-history perspectives for understanding intra-specific variation within dinoflagellates. Combining our results on the population genetic characteristics, we propose a novel conceptual model of the effects of life cycle characteristics on the genetic population structure of *A. tamarense*.

2.6.3. Materials and Methods

Origin and culture of clonal isolates

Clonal isolates of *Alexandrium tamarense* were produced by micropipette isolation of single cells from a near-surface phytoplankton sample from North Sea waters off the Scottish east coast (56° 5.47’ N latitude, 1° 42.35’ W longitude). Clonal cultures derived from vegetatively dividing single cells were maintained in K medium (Keller et al. 1987), supplemented with selenite (Dahl et al. 1989), prepared from 0.2 µm sterile-filtered natural Antarctic seawater at 10 °C under fluorescent light at a photon flux density of 100 µmol photons m^{-2} s^{-1} on a 16:8 h light–dark photocycle. Cultures for DNA extraction and toxin analysis were grown under the same conditions but with supplement of the antibiotics penicillin (final concentration 100 µg mL^{-1}) and streptomycin (final

concentration 25 µg mL^{-1}). Dinoflagellate cells were collected in exponential growth phase by filtration over a 10 µm polyethylene (Nitex) mesh to prevent bacterial contamination of DNA.

Genotypic and phenotypic analysis

Of the more than 100 isolates 86 were genotyped by amplified fragment length polymorphism (AFLP) (Vos et al. 1995) according to the protocol of John *et al.* (2004). In addition to AFLP analysis, 77 of the 86 isolates were genotyped at 18 simple sequence repeat or microsatellite loci (Tautz and Renz 1984) by the methods given in Nagai *et al.* (2004) and Alpermann *et al.* (2006) for 12 and 6 loci, respectively. Sizing and scoring of AFLP and microsatellite loci were carried out with GENEMAPPER ® V4.0 software (Applied Biosystems, Darmstadt, Germany) after capillary electrophoresis.

Different phenotypic markers were examined to obtain information on the distribution of phenotypic characters among the sampled isolates. Paralytic shellfish poisoning (PSP) toxins were quantified from exponential phase batch cultures of all genotyped isolates by automated reverse-phase high-performance liquid chromatography with fluorescence detection (LC-FD), applying ion-pair chromatographic separation, followed by post-column oxidation with periodic acid and fluorescence detection, based on the method of Thielert *et al.* (1991), as described in detail in Tillmann and John (2002). Toxin concentrations were converted to molar quantities using the known molecular weight of each toxin and total PSP toxin cell quota as well as the cellular content of the different derivatives was calculated. Molar quantities of the epimeric pairs of saxitoxin derivatives (C1/C2, GTX2/GTX3, and GTX1/GTX4) were fused in subsequent analyses, since interconversions among epimeric pairs of toxins are known to occur during storage and processing of samples (Hall et al. 1990).

As an additional phenotypic character, allelochemical effects of clones of *A. tamarense* (at a concentration of 1500 cells mL^{-1}) towards the dinoflagellate predator *Oxyrrhis marina* (at a concentration of 500 cells mL^{-1}) were studied in 24 h short-term exposure experiments for 67 of the genotyped isolates, as described in detail in Tillmann *et al.* (2008). Allelochemical activity of *A. tamarense* isolates against *Oxyrrhis* was expressed as percent of target cells after treatment relative to the number of target cells in the negative control without addition of *A. tamarense* cells (% *Oxyrrhis*-T_{24}).

Inference of population structure by neutral genotypic markers

The software STRUCTURE 2.2 (Falush et al. 2007) was used to test for the existence of genetic population substructure. Two independent data sets were constructed for microsatellite and AFLP multilocus genotypic data, including the same 77 clonal lineages, such that results were fully comparable. Additionally, a more complete AFLP data set was built, consisting of all 86 clonal lineages that were genotyped by this marker. The computational model underlying our analyses was based on the assumption that population subgroups could be identified according to the partitioning of allele frequencies among them. For this purpose, multilocus genotypes were repeatedly assigned to one of a predefined number of hypothetical subpopulations in a Markov chain Monte Carlo (MCMC) simulation in an attempt to maximize the degree of genetic differentiation among all

hypothetical subpopulations (Pritchard et al. 2000a). Besides the assignment of individuals (or multilocus genotypes) to population subgroups, the program estimates the posterior probability of the resulting population structure, which allows comparison of independent runs with different numbers of assumed hypothetical subpopulations and for determination of the most probable number of subpopulations. At least three independent runs of the program were performed for each data set (consisting of either AFLP or microsatellite genotypes) and for each number of hypothetical subpopulations (*K*), ranging from one to ten. The initial 'burn-in' period of the MCMC scheme consisted of 50,000 iterations followed by an additional 300,000 iterations. The model assumptions were that complete multilocus genotypes could be assigned to differentiated hypothetical subgroups and that allele frequencies among subpopulations were allowed to be correlated (Falush et al. 2003).

In cases where STRUCTURE 2.2 results indicated population substructure, pairwise F_{ST} values and their significances were estimated among subpopulations in ARLEQUIN V. 2.0 (available at: http://anthro.unige.ch/software/arlequin/), to estimate the degree of differentiation among these hypothetical subpopulations. For this purpose, independent data sets were built for AFLP and microsatellite data, including 86 and 77 individuals, respectively, such that genotypes were arranged into four population subgroups according to the STRUCTURE 2.2 results obtained with AFLP data (86 isolates).

Phenotypic differentiation among population subgroups

Different phenotypic characters were utilized to assess if hypothetical subpopulations showed signs of phenotypic differentiation. Clonal isolates were grouped into population subgroups according to the STRUCTURE 2.2 results for the AFLP data set (a total of four hypothetical population subgroups). The group averages of cellular PSP toxin content and allelochemical effect towards *Oxyrrhis* and their standard deviations were then calculated. Among-group differences in cellular PSP toxin content (86 isolates included) and allelochemical effects (67 isolates included) were analysed with the software STATISTICA ™ V.6 (StatSoft®, Hamburg, Germany), including tests for homogeneity of variances and normal distribution of variables within groups by Levene's test and the Shapiro-Wilk *W*-test , respectively. Homogeneity of variances and normal distribution were assumed for the % *Oxyrrhis*-T_{24} values and existence of among-group differences was tested by ANOVA. Normal distribution of total cellular content of PSP toxins (fmol cell^{-1}) could not be assumed for all population subgroups because subgroup C (n = 34), the largest of the four population subgroups, showed a significant deviation ($p < 0.05$) from the expected normal distribution in the Shapiro-Wilk test. The non-parametric alternative to one-way ANOVA, the Kruskal-Wallis test, was therefore applied, and multiple two-tailed pairwise comparisons were performed after the non-parametric test to detect significant differences between groups.

The significance of phenotypic differentiation among subpopulations with respect to PSP toxin profiles (molar proportions of each toxin group) was assessed by ANOSIM with 9,999 bootstrapped replicates in the statistical software package BRODGAR V. 2.5.0 (Highland Statistics Ltd., Newsburgh, UK) using Euclidean dissimilarities as measure of relatedness among toxin profiles. An estimate of the significance of overall phenotypic differentiation among the four defined population subgroups was obtained by ANOSIM analysis, with 9,999 bootstrapped

replicates performed in BRODGAR V. 2.5.0 on a fused data set consisting of ranked similarities after separate estimation of pairwise Euclidean distances for the three different phenotypic characters − cellular PSP toxin content and molar percent composition and magnitude of allelochemical effect towards *Oxyrrhis* sp. The analysis was performed with BIONUMERICS V. 2.50 (Applied Maths, Sint-Martens-Latem, Belgium) statistical software. The significance level for all tests of phenotypic differentiation among population subgroups was defined as $p < 0.05$.

Analyses of multilocus linkage disequilibrium

The 'standardized index of association' (I^{s}_{A}) (Haubold et al. 1998) was calculated and its significance was estimated to assess if multilocus linkage disequilibrium was present within the population of *A. tamarense*. These computations were performed with the software LIAN V. 3.5 (Haubold and Hudson 2000) through its web interface (http://adenine.biz.fh-weihenstephan.de/cgi-bin/lian/lian.cgi.pl) by a simulation test (10,000 Monte Carlo resamplings). After STRUCTURE 2.2 computations, the I^{s}_{A} was calculated for each of the four subpopulations as they were defined for the AFLP data to check if the separation of individuals into these groups led to a reduction or even disappearance of multilocus LD. These calculations were performed separately for data sets for AFLP (total of 86 individuals) and microsatellites (total of 77 individuals).

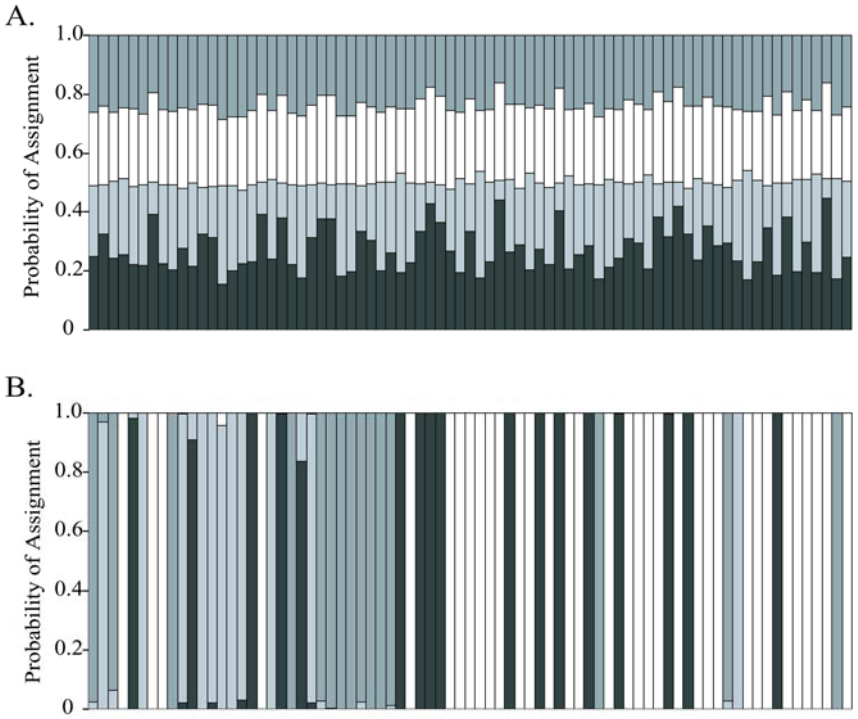

Fig. 2.6.1. Probability of assignment of genotypes (n = 77) of clonal isolates to one of the four population subgroups: (A) microsatellite genotypes; (B) AFLP genotypes. The probability for each subpopulation is expressed in a different shading code: dark-grey: subpopulation A; light-grey: subpopulation B; white: subpopulation C; medium-grey: subpopulation D.

2.6.4. Results

Genotypic and phenotypic data

The four selective primer combinations yielded a total of 482 well-scoreable polymorphic AFLP loci in the 86 analysed isolates. A total of 99 alleles were found at the 18 microsatellite loci and allele numbers per locus ranged from two to ten with an average of 5.5 (SD ± 2.3). No AFLP or microsatellite multilocus genotype was repeatedly sampled. Total cellular toxin quotas during early exponential growth ranged from 25.3 to 471.1 fmol cell^{-1}. Neosaxitoxin (NEO) was the most prominent (mol% ± S.D.: 52.3 ± 16.2) among the groups of saxitoxin (STX) derivatives, followed by C1/C2 (21.2 ± 9.8) and then STX (11.0 ± 7.0). The gonyautoxins GTX2/GTX3 (6.2 ± 9.7) and GTX1/GTX4 (5.6 ± 8.4) were minor components, followed by B2 (2.4 ± 4.3) and B1 (1.2 ± 2.0), the *N*-sulfocarbamoyl derivatives of NEO and STX, respectively. In the test assay for allelochemical activity of the *Alexandrium tamarense* isolates the effect against *Oxyrrhis* ranged from 1.2 to 100.9 % *Oxyrrhis*-T_{24}.

Inference of population structure

The computations for the estimation of genetic population substructure in STRUCTURE 2.2 gave substantially different results for two different genetic markers, AFLP and microsatellites. Whereas AFLP data analysis supported the formation of four genetically differentiated subpopulations ($K = 4$: $p < 0.0001$ for $n = 86$ and $n = 77$), the existence of population subdivision was not confirmed by microsatellite data. In the latter case, probabilities estimated for population subdivision from one to ten hypothetical subpopulations gave highest values for the existence of one homogenous population without substructure ($K = 1$: $p < 0.0001$). The individual genotypes consequently showed very high probabilities of falling into one of the four subpopulations in the case of AFLP genotypes, whereas microsatellite genotypes could not be successfully assigned to any of the subpopulations with high probability, when $K = 4$ was specified in the STRUCTURE 2.2 analysis (Fig. 2.6.1).

Genetic differentiation among the four subpopulations as defined for the AFLP data set was confirmed by highly significant F_{ST} values for all pairwise combinations of subpopulations by AFLP data (Table 2.6.1). The magnitude of F_{ST} values indicates that population subgroups were assigned to yield a moderate to great degree of genetic differentiation according to Wright (1978). However, the microsatellite data for the same population subgroups only yielded two significant F_{ST} values for the six pairwise comparisons among the four subpopulations (Table 2.6.1). Additionally, pairwise F_{ST} values estimated from microsatellites were considerably lower than those estimated from AFLP data and displayed at most moderate genetic differentiation.

Analysis of multilocus linkage disequilibrium

Significant multilocus linkage disequilibrium (LD) in the total population was detected for the AFLP data set with an I^{s}_{A} of 0.011 ($p < 0.0001$). The estimate of multilocus LD for the microsatellite data set resulted in a lower I^{s}_{A} value of 0.008 at a significance level of $p = 0.013$. Tests for

Table 2.6.1. Pairwise F_{ST} values and their significances for subpopulations as defined by STRUCTURE 2.2 computations with AFLP data from 86 clonal lineages. Upper right triangle: calculations performed with microsatellite data (77 isolates); lower left triangle: calculations performed with AFLP data (86 isolates).

Subpopulation	A	B	C	D
A	–	0.00	0.02	0.06 *
B	0.18 ***	–	0.01	0.01
C	0.15 ***	0.07 ***	–	0.06 **
D	0.36 ***	0.13 ***	0.13 ***	–

$p < 0.001$, **$p < 0.0005$, ***$p < 0.0001$

multilocus LD within the four population subgroups as defined by the STRUCTURE 2.2 analysis of the AFLP data set showed that I^{s}_{A} decreased considerably for both markers (Table 2.6.2). Significant I^{s}_{A} values were obtained within all four population subgroups for the AFLP data, but none of the subgroups yielded significant multilocus LD for the microsatellite loci.

Phenotypic differentiation among subpopulations

The mean cellular content of PSP toxins in exponentially growing *A. tamarense* isolates ranged from 117.3 to 184.7 fmol cell^{-1} among the four population subgroups (Table 2.6.3). The statistical test for phenotypic differentiation of population subgroups with respect to total PSP toxin content showed that significant differences existed among subgroups (Kruskal-Wallis, $p = 0.027$). Multiple pairwise tests for differences between groups indicated that only subgroups A and D were significantly different in the rank-based analysis ($p = 0.021$). The results for the test of phenotypic differentiation based on the relative composition of different PSP toxins were not significant (ANOSIM, $R = 0.041$, $p = 0.12$). Within the four population subgroups defined by STRUCTURE 2.2 analysis, the average allelopathic effect of *A. tamarense* isolates towards *Oxyrrhis* in 24 h co-incubation experiments ranged from 38.5 to 58.3 % *Oxyrrhis*-T_{24} (Table 2.6.3), when expressed as fraction of intact target cells of *Oxyrrhis* with respect to control treatment without addition of *A. tamarense* cells. No statistically significant differences in allelopathic properties among groups were found (ANOVA, $p = 0.15$). The analysis of the combined phenotypic data set, which consisted of the ranked pairwise similarities obtained for the data on cellular PSP toxin content and composition and magnitude of allelochemical effect towards *Oxyrrhis* did not give statistically significant results (ANOSIM, $R = 0.076$, $p = 0.07$).

2.6.5. Discussion

Our results revealed significant population substructure and multilocus linkage disequilibrium (LD), i.e. significant deviations from expectations of a single panmictic population, in a single planktonic population of *Alexandrium tamarense*. Yet the two molecular markers (AFLP and microsatellites)

provided different evidence regarding these phenomena. First, we provide here the background for this incongruence. Subsequently we interpret and link these unexpected patterns of genetic variation with life cycle characteristics of *A. tamarense* and phenotypic characteristics of population subgroups. Finally, integrating all our findings, we propose a conceptual model for the development of the observed population genetic patterns in planktonic populations of *A. tamarense*.

Evidence for genetic population substructure

The result of the analysis of the AFLP genotypic data strongly supports the idea that the population of *A. tamarense* under study is characterized by a significant substructure. The analysis of the microsatellite data does not give the same result, but one-third of the pairwise tests for population differentiation are still significant, when the substructure as obtained by AFLP is superimposed on the microsatellite data. This clearly indicates that the population substructure as revealed by AFLP data is also reflected to a considerable extent in the microsatellite data, although the genetic differentiation of subgroups is not high. General differences in the capabilities of AFLP and microsatellite markers to discriminate populations with weak genetic differentiation for example have been observed in previous studies, which led to the conclusion that AFLP in general is the more powerful marker for the identification of weak genetic population differentiation (see Campbell *et al.* 2003 and references therein). This might explain why the microsatellite data fail to detect the population subdivision as obtained for the AFLP data in the STRUCTURE 2.2 analysis, but still successfully distinguish between the two most differentiated pairs of population subgroups.

The demonstrated presence of significant genetic subdivision within a single, geographically and temporally defined population of *A. tamarense* could be explained by admixture of individuals from geographically distinct and/or genetically differentiated isolated populations. According to this interpretation, the four defined subgroups would correspond to a locally established resident population intermingled with individuals derived from three other populations that were recently admixed. Such admixture processes are expected to maintain their signature in populations for quite a long time, since sexual reproduction in *A. tamarense* is assumed to be a rather rare and discontinuous event − occurring annually during planktonic population development and involving only a limited subset of gamete-forming individuals (Anderson 1998). The pattern of sexual reproduction has not been followed in *A. tamarense* populations along the Scottish east coast, but this assumption is supported by observations of late timing of fusion of gametes and an obligatory dormancy period that lasts up to several months in *A. tamarense* hypnozygotes from other coastal regions (Anderson 1998).

In any case, the prerequisites for this explanation for the existence of population substructure is that significantly differentiated populations of *A. tamarense* exist along the Scottish North Sea coast and that admixture events can take place by advective transport. Studies on the hydrographical regime in the North Sea provide the physical mechanism to support this hypothesis, given the prevailing current and transport of water masses from north to south along the Scottish east coast (Backhaus and Maier-Reimer 1983; Davies 1983; Prandle 1984; Durance 1989). While this might enable the transport of individuals from northerly populations to the area sampled in our study, data on population differentiation of *A. tamarense* along the Scottish North Sea coast

Table 2.6.2. Standardised index of association (I^s_A) as measure of multilocus linkage disequilibrium in four hypothetical subpopulations as defined by STRUCTURE 2.2 computations with AFLP data from 77 clonal lineages.

Subpopulation	No. of isolates	I^s_A (AFLP)	I^s_A (microsatellites)
A	17	0.0025**	0.0057
B	11	0.0044**	0.0021
C	34	0.0007*	0.0077
D	15	0.0037**	–0.0052

$p < 0.005$; ** $p < 0.0001$; I^s_A, standardised index of association

argue against this explanation. Differentiation of cultured isolates of *A. tamarense* from geographically separated locations (up to several hundreds of kilometres apart) do not suggest the existence of genetically distinct populations along the eastern Scottish coast (Alpermann *et al.*, unpublished data). Moderately high levels of gene flow among geographically distant planktonic *A. tamarense* populations therefore can be assumed. Coupled with the prevailing current regime in the coastal waters of the Scottish North Sea, a continuous gradient of genetic differentiation rather than the existence of sharp population boundaries with highly differentiated local populations is most likely.

With a view towards the critical role of life-history transitions in *A. tamarense,* we therefore propose that creation of population substructure does not occur in space by simple admixture of genetically differentiated populations, but rather in time by the genetic differentiation of successive planktonic populations. A necessary assumption for this explanation is that local planktonic populations − hatched at the same location from the same locally established cyst bed − substantially differentiate within the course of the annual planktonic growth period due to over-representation of certain clonal lineages resulting from differential rates of growth, survival and/or sexual induction. Such genetic differentiation in the planktonic population would then be followed by consequent genetic differentiation in the hypnozygotes formed in this population after fusion of gametes. Genetic differentiation within annually formed subpopulations of cysts would be the source for a signature of admixture of subpopulations when the next planktonic population is seeded, since cyst populations remain viable for many years. Planktonic populations can consequently be assumed to be derived from several year classes (Wyatt and Jenkinson 1997; Nagai et al. 2007a). The validity of this latter assumption is essential for the development of our conceptual model on the formation of population genetic structure in *A. tamarense*.

Multilocus linkage disequilibrium

The finding of a low, but significant amount of multilocus LD within the *A. tamarense* population is unique and has important implications for the interpretation and modelling of population differentiation. The results of our analysis for multilocus LD in the total populations suggest that

the standardised index of association (I^{s}_{A}) is a valid measure for the relative effect of vegetative reproduction in partially clonal dinoflagellate species such as *A. tamarense*. However, although values of the I^{s}_{A} for the different genotypic markers were in the same range, their significance estimates differed notably, with that for the AFLP-based analysis being two orders of magnitude higher. An explanation for the considerably lower significance estimates of the I^{s}_{A} value for microsatellites relative to the AFLP analysis is that the number of microsatellite loci that were analysed was about ten times lower than the number of chromosomes (an estimated number of 143) known for this species (Hackett et al. 2004), making physical linkage among the studied loci relatively unlikely. Whereas multi-allelic markers, like most of the microsatellite loci in our analysis, generally enhance the chances of detecting LD, when compared with strictly bi-allelic markers such as AFLP, the number of markers − in our study more than 20 times higher for AFLP than for microsatellites − also greatly influences the statistical power of the test (Chapman and Wijsman 1998; Sham et al. 2000).

Although the values of the standardized index of association for AFLP (I^{s}_{A} = 0.011) and microsatellites (I^{s}_{A} = 0.008) for *A. tamarense* were very low when compared to those for the cyanobacterium *Microcoleus chthonoplastes* (maximum value of I^{s}_{A} = 0.036 for a population from the southern North Sea (Lodders et al. 2005)), they raise questions concerning the origin of multilocus LD in the planktonic population. In light of the finding on population substructure, this result is even expected, as pooling samples from differentiated populations is one of the most well known situations where multilocus LD arises due to simultaneous differences in allele frequencies at multiple loci, even in the absence of physical linkage (i.e. intra-chromosomal) among loci (Maynard Smith et al. 1993; Falush et al. 2003). However, in the case of AFLP the multilocus LD detected did not disappear even when the subpopulations were analysed separately, which indicates a deviation from a panmictic null model even within the subpopulations. The explanation for this finding and the absence of multilocus LD within population subgroups for microsatellites again most likely reflect the differences in the sets of markers. As reasoned above, the much smaller number of microsatellite loci makes it unlikely to detect physical LD, whereas the high number of AFLP loci implies that many of these are physically linked to a certain extent. The presence of intra-chromosomal multilocus LD within population subgroups as detected by AFLP indicates that another explanation for multilocus LD besides the existence of population substructure must be formulated. We suggest that the presence of multilocus LD within the genetically differentiated population subgroups are due to shifts in the genotypic composition of vegetatively growing *A. tamarense* populations. This consequently will influence the genetic structure of the population of resting cysts that are formed after fusion of planktonic gametes.

Phenotypic differentiation among genetically differentiated subgroups

Genetic differentiation over the course of the planktonic growth period − if not solely driven by stochastic processes − requires an ecological explanation, such as the existence of specific ecological traits that enable certain clonal lineages to grow better than others under specific environmental conditions. This explanation in turn presupposes the existence of phenotypic differences in natural populations of *A. tamarense*. Several studies on phenotypic diversity in

Table 2.6.3. Phenotypic characters in the four hypothetical subpopulations as defined by STRUCTURE 2.2 computations with AFLP data from 86 clonal lineages: Mean magnitude of allelochemical effects of clonal *A. tamarense* isolates towards *Oxyrrhis* (% *Oxyrrhis*-T_{24}) expressed as percent of intact target cells after 24 h of intact cells in the control (*Oxyrrhis* incubated without *A. tamarense*) and mean cellular PSP toxin content during early exponential growth.

Subpopulation	% *Oxyrrhis*-T_{24}	PSP toxin content (fmol cell^{-1})
A	42.5 (S.D. ± 27.7), *n* = 8	117.3 (S.D. ± 43.2), *n* = 17
B	38.5 (S.D. ± 24.6), *n* = 16	160.6 (S.D. ± 85.9), *n* = 19
C	58.3 (S.D. ± 17.7), *n* = 9	178.9 (S.D. ± 98.9), *n* = 16
D	52.2 (S.D. ± 23.3), *n* = 34	184.7 (S.D. ± 86.8), *n* = 34

S.D., standard deviation; *n*, number of individuals in the population subgroup for which data on the respective phenotypic character was obtained

marine dinoflagellates have proved that their planktonic populations can indeed be extremely diverse (e.g., Cembella et al. 1987; Ichimi et al. 2002; Loret et al. 2002). In *A. tamarense*, phenotypic traits such as growth under standardized laboratory conditions (Brand 1981) and PSP toxin composition (Cembella et al. 1987; Anderson et al. 1994; Ichimi et al. 2002) have been shown to vary considerably within natural populations.

It has already been hypothesized that the relative over-abundance of certain lineages is to be expected if selective processes act differentially on phenotypically diversified individuals within a population of a clonal species (Yoshida et al. 2003). Here, both types of processes, 'gain processes' such as the rate of vegetative growth and 'loss processes' such as predation, can lead to differences in the relative abundance of genotypes if they differ with respect to selectively advantageous phenotypic characters. In our analyses on the differences in phenotypic characters among the four hypothetical population subgroups formed according to the STRUCTURE 2.2 results for AFLP, only one of the tests yielded significant differences. Two population subgroups, A and D, differed significantly in their cellular PSP toxin content (Kruskal-Wallis, $p = 0.027$; multiple pairwise comparison, $p = 0.022$). However, we did not necessarily expect to be able to detect significant differences for this phenotypic character among the population subgroups, since we acknowledge that the toxin content in *Alexandrium* cells from batch cultures may show a high "growth stage variability" (Anderson et al. 1990b). Nevertheless, the randomized design by which the samples were cultured and collected, together with the fact that population subgroups were not determined at the time when culture experiments and toxin measurements were conducted, precludes the occurrence of a sampling artefact.

We therefore have to look for an ecological explanation for the found differences. Several studies have claimed that PSP toxins might play a role in grazer deterrence, and this may indeed be the case in certain circumstances (Turner et al. 1998; Selander et al. 2006), but this could not be confirmed by a number of experimental studies as a general hypothesis (see Cembella (2003) and references therein). True allelochemicals are assumed to play an important role in the success of

dinoflagellate populations, since they can reduce/modulate predation on more effective lineages or enhance competitive capabilities of the respective dinoflagellate species in relation to other microalgae that are negatively affected by their allelochemical properties (Smayda 1997; Cembella 2003; Legrand et al. 2003; Tillmann 2004). These negative effects on potential competitors or grazers have been demonstrated by several studies investigating species-species interactions (Arzul et al. 1999; Tillmann and John 2002; Tillmann et al. 2007; Tillmann et al. 2008a) as well as effects on natural planktonic communities (Fistarol et al. 2004b).

Ecological advantages and positive selection for those clonal lineages that express strong allelochemical properties is therefore expected, particularly at times when the grazing pressure by heterotrophic protists or macrozooplankton or the risk of bacterial pathogen or protistan parasite infection is high. However, even if significant differences for a specific phenotypic character are found, as in our case for cellular PSP toxin content, one cannot be sure that the respective trait has indeed been under selection in the different population subgroups. Non-random distribution of this phenotypic character in different subpopulations might also arise due to selection on other tightly coupled phenotypic traits. Diverse selective pressures might also have levelled out a stronger phenotypic differentiation based on the other two phenotypic characters investigated here − the relative PSP toxin composition and allelochemical properties. Many such phenotypic traits contribute to intra-specific fitness differences among clonal lineages. A much more extensive sampling than was available for our study might therefore be needed to identify significant phenotypic differentiation among population subgroups.

Conceptual model for the development and maintenance of population genetic structure and LD in Alexandrium tamarense *populations*

Integrating the information on population genetic characteristics of the population under study into the broad base of knowledge about life cycle peculiarities of *A. tamarense* leads us to propose the following conceptual model capable of explaining the observed deviations from panmictic expectations (population subdivision and the presence of multilocus LD even within subpopulations). Assuming an isolated planktonic population seeded by hypnozygotes from the previous year, we hypothesize that LD does not increase during the planktonic growth period unless clonal over-representation manifests itself as the consequence of either a stochastic process or − more likely in a large planktonic population − of natural selection acting differentially on clonal lineages that thrive by vegetative growth (Yoshida et al. 2003). Without shifts in the relative abundance of clonal lineages during planktonic growth, LD if it existed in the population at the start of planktonic growth would decrease due to meiotic recombination before the next planktonic population is started from the cysts they produced. In this model (which does not fit the data we obtained in this study) intra-chromosomal LD would become very low and eventually disappear with each cycle of sexual reproduction, whereas inter-chromosomal LD among loci would already have disappeared at the beginning of planktonic growth, since meiosis, which precedes the formation of vegetatively growing, planktonic cells, would have led to the complete reduction of LD by fortuitous segregation of chromosomes.

To account for the existence of multilocus LD that we showed in this study, we need to invoke the occurrence of clonal over-representation during planktonic growth. This explanation is independent of the mechanism by which clonal over-representation is achieved, i.e. whether or not phenotypic selection actually occurs. Whereas LD will increase in a planktonic population with the magnitude of shifts in the frequencies of clonal lineages during vegetative growth, independently of the chromosomal localization of loci, inter-chromosomal LD will disappear after sexual reproduction and before the seeding of the next planktonic generation. In this case, only intra-chromosomal LD would be observed in the next generation, but the separation of clonal lineages into population subgroups by means of an assortment of genotypes should result in a homogenous, panmictic population without formation of population subgroups.

To integrate our findings on the genetic population substructure and persistent multilocus LD within the population subgroups we therefore had to add another life cycle peculiarity to our conceptual model, enabling us to explain all observations concerning intra- and inter-chromosomal LD in this study. Only if we further assumed that planktonic populations are seeded by cysts from different year classes were we able to explain the existence of the observed population subgroups. This hypothesis not only explains the genotypically diversified population subgroups, as stemming from planktonic populations that responded differentially in different years to environmental determinants by shifts in frequencies of clonal lineages (and thereby alleles), but also the observation of multilocus LD within the population subgroups. According to this conceptual model, this portion of multilocus LD is attributable to intra-chromosomal LD that was maintained in the population subgroup after meiosis.

Our model relies on the idea that cyst beds are composed of a mixture of cohorts of year classes that to a variable degree are differentiated from the seeding population due to growth advantages of certain clonal lineages. Within each cohort the inter-chromosomal linkage is broken by meiosis before the next planktonic population is seeded, but the total planktonic population that is recruited from several such cohorts will display two kinds of LD: intra-chromosomal – maintained within each population subgroup/cohort – and also intra-chromosomal as well as inter-chromosomal LD from the coexistence of genetically differentiated population subgroups within the planktonic population. Both forms of LD should therefore also be present in the population of cysts in an established cyst bed.

2.6.6. Conclusions

Our results revealed significant population substructure and multilocus LD which do not support the model of a single panmictic population of *Alexandrium tamarense*. The unexpected patterns of genetic variation can be linked with life cycle characteristics of *A. tamarense* to suggest that the former is potentially a result of the latter. The observed genetic differentiation in populations in this planktonic organism is likely the consequence of selection on ecologically important phenotypic traits. Phenotypic characters such as cellular PSP toxin content or composition and allelochemical properties may contribute to the differentiation of annual planktonic populations. The lack of clear

phenotypic differentiation with respect to allelochemical activity might be the cause of the absence of strong selective pressure with respect to this trait or balancing selection acting on other traits.

Whereas the analyses of multilocus LD in the total population and the population subgroups suggest that frequency shifts in clonal lineages indeed influence the genetic structure of *A. tamarense* populations, two factors seem to be responsible for the maintenance of phenotypic and genotypic diversity: (1) planktonic populations of *A. tamarense* are seeded by benthic cyst beds presumably composed of cyst cohorts derived from several years; (2) genetic recombination that accompanies the seeding of new planktonic populations leads to the formation of new genotypes with different phenotypic characteristics. If this model for the maintenance of genetic and phenotypic diversity in populations of *A. tamarense* holds true, population-wide differentiation with respect to certain phenotypic traits can be expected over long-term evolutionary rather than ecological time-scales (the latter being the annual period of planktonic population growth and bloom development). Genetic diversity and phenotypic adaptability might be maintained in benthic cyst populations even after strong natural selection on the haploid clonal lineages that comprise the planktonic populations of *A. tamarense*. Thus life cycle transitions from vegetative growth of planktonic haploid lineages to diploid benthic resting stages can enhance the adaptability and genetic resilience of *A. tamarense* and other dinoflagellates with similar life history traits.

2.7. GENETIC AND PHENOTYPIC DIFFERENTIATION AMONG GLOBALLY DISPERSED POPULATIONS OF THE NORTH AMERICAN RIBOTYPE OF THE TOXIC DINOFLAGELLATE *ALEXANDRIUM TAMARENSE*

2.7.1. Abstract

Novel molecular approaches were employed to address questions on population differentiation at different geographical scales for the toxigenic dinoflagellate '*Alexandrium tamarense* species complex'. Samples of planktonic vegetative cells of *A. tamarense/A. fundyense* (North American ribotype) were obtained from regional locations where *Alexandrium* blooms are prominent and recurring along the north-eastern US coast, the North Sea coast of Scotland, and Hiroshima Bay in Japan. Both genotypic markers in this study, microsatellites and amplified fragment length polymorphism (AFLP), showed a high degree of gene diversity and genetic differentiation (highly significant F_{ST} values) among populations from different regions. Weak but significant genetic differentiation on smaller geographical scales, however, was only detected by AFLP for one of the three pairs of population samples from the North Sea. Phenotypic differentiation as displayed by the cellular content and composition of paralytic shellfish poisoning (PSP) toxins followed the pattern of genetic differentiation. Analysis of population structure and probabilistic assignment of individuals by genotype to potential source populations was achieved by Bayesian clustering. Whereas both genotypic markers gave adequate results to separate Western and Eastern North Atlantic isolates, the Western Pacific population sample was not readily distinguished from the North Sea samples nor were North Sea samples separated on the intra-regional scale. The assignment of individual AFLP and microsatellite genotypes to prospective source populations by region proved its validity for backtracking of dispersal and reconstruction of the dispersal history of planktonic microbes.

2.7.2. Introduction

The increase in documented events of harmful algal blooms (HABs) in recent decades has led to the formulation of possible explanations for this phenomenon (Anderson 1989; Hallegraeff 1993) as well as to an intensification of research efforts to unravel the causes (Hallegraeff 2003). Monitoring efforts at sites where algal blooms have frequently occurred have intensified over the last few decades, for reasons such as the development of the aquaculture industry and exploitation of new seafood resources in regions that were formerly not surveyed for the occurrence of HAB events and associated phycotoxins (see Hallegraeff (Hallegraeff 2003) and Anderson (Anderson 1989) and references therein). This *apparent* increase in HAB events is thus somewhat dependent upon increased awareness and monitoring efforts, thereby increasing the proportion of detected versus undetected bloom events and the total number of events recorded world-wide.

While this certainly is part of the explanation for the increase of recorded HABs, there is also abundant evidence of actual increases in the frequency, duration, magnitude and biogeographical distribution of these events over the last several decades. Detection of novel HAB species and their effects even at localities that have been intensively monitored (Lilly et al. 2002) or which have a long and complete anecdotal history, indicating that these areas were formerly free of such events, has led to the development of the "*Global Spreading Hypothesis*" (Wyatt 1995). More controversially, documentation of human-assisted means of introduction of HAB species to new areas, indicates that recent range extensions of some bloom-forming species may not only be due to naturally occurring dispersal, but in many cases are the consequence of human activities (Smayda 2007). Increasing intercontinental ship traffic, with the associated discharge of ship ballast water, and the translocation of infested seafood stock such as aquacultured shellfish are proposed as major vectors for human-mediated translocation of bloom-forming microalgal species, including those of HABs (Smayda 2007). In other cases, resident populations of harmful species may have been present at low cell concentration but remained undetected as a hidden component of the local phytoplankton community. Anthropogenically driven or natural regime changes in prevailing environmental conditions (e.g., enhanced nutrient input or elevated sea surface temperatures) may favour the development and expansion of cryptic populations of these harmful species, resulting in sudden mass occurrences of HABs (for a deeper discussion on the topic of colonization vs. hidden flora species see Smayda (2002)).

Strategies for avoidance of future introductions of HAB species and mitigation of their effects must include consideration of possible routes and means of translocation of potentially harmful species. Determining the causes and effects of *apparent* and/or *actual* spreading of HABs requires access to techniques to track the inherent diversity and biogeography of the implicated taxa at the cellular and population level. Recent natural- and human-assisted dispersal must be clearly distinguished from long existing but cryptic local populations of harmful species.

In earlier studies, mainly before the advent of genetic markers, phenotypic characters have been utilized to characterize locally established populations or to study the relatedness of populations of toxic species from different localities or regions (Cembella and Taylor 1986; Hayhome et al. 1989; Bolch et al. 1999). Among toxigenic dinoflagellates of the same species, toxin profiles have been proposed as biogeographical markers, e.g., the proportional toxin composition among strains of paralytic shellfish poisoning (PSP) causing dinoflagellate species such as *Gymnodinium catenatum* (Oshima et al. 1993; Holmes et al. 2002) or *Alexandrium* sp. (Hall 1982; Cembella et al. 1987; Anderson et al. 1994; Cembella and Destombe 1996). A prerequisite for the use of this biochemical character as a phenotypic marker has been its stability under standard culture conditions of clonal isolates; in most cases this criterion has been met (Hall 1982; Cembella et al. 1987; Ogata et al. 1987a). However, biogeographical surveys including isolates from different regions (usually based upon one or a few clones) did not typically yield a conclusive pattern of relatedness and causes for the existence of observed phenotypic differences among isolates from different source populations. It is therefore a dubious undertaking to base hypothetical provenance of newly detected populations of harmful species on this phenotypic character alone.

With the advent of neutral molecular markers such as microsatellites (or simple sequence repeats; SSR) (Tautz and Renz 1984) and amplified fragment length polymorphism (AFLP) (Vos et

al. 1995) and the application of these techniques to toxic dinoflagellate species (e.g., AFLP in *Alexandrium tamarense* (John et al. 2004) and SSR in *A. tamarense* (Nagai et al. 2004; Alpermann et al. 2006a)), *A. minutum* (Nagai et al. 2006a)) and *Karenia brevis* (Henrichs et al. 2008)) or toxic diatoms (e.g., SSR in *Pseudo-nitzschia multiseries* (Evans et al. 2004)), it is now possible to genotype individual clonal isolates and to characterize locally established populations or translocated individuals. Methods for the analysis of population genetic data provide elaborate means to answer unresolved questions concerning the geographical distribution patterns and the degree of relatedness among populations of harmful algal species. Together with the awareness of the existence of the aforementioned means of range extension and/or bloom promotion, we may be able to unravel the course of events that led to the appearance of recent blooms at new localities.

Alexandrium tamarense is probably the best studied harmful algal bloom species with respect to genetic variation among (Scholin et al. 1994; Scholin and Anderson 1994; John et al. 2003; Nagai et al. 2007a) and within populations (Alpermann et al. 2009; Alpermann et al. in press). Our objective for the current study was to test the utility of two different neutral markers (microsatellites and AFLP) for population genetic studies in *A. tamarense* across different spatial scales, ranging from less than a few hundred nautical miles to a larger scale across the extent of the North Atlantic and then to the Western Pacific (Japan). Although our study is subject to some of the same constraints as previous ones, i.e. a relatively small sample size due to limits in collection and culturing of clonal isolates, we were able to test the suitability of both markers to detect genetic population differentiation across the different geographical scales for the first time. We were also able to test the suitability of both markers to display genetic relatedness of individuals by a

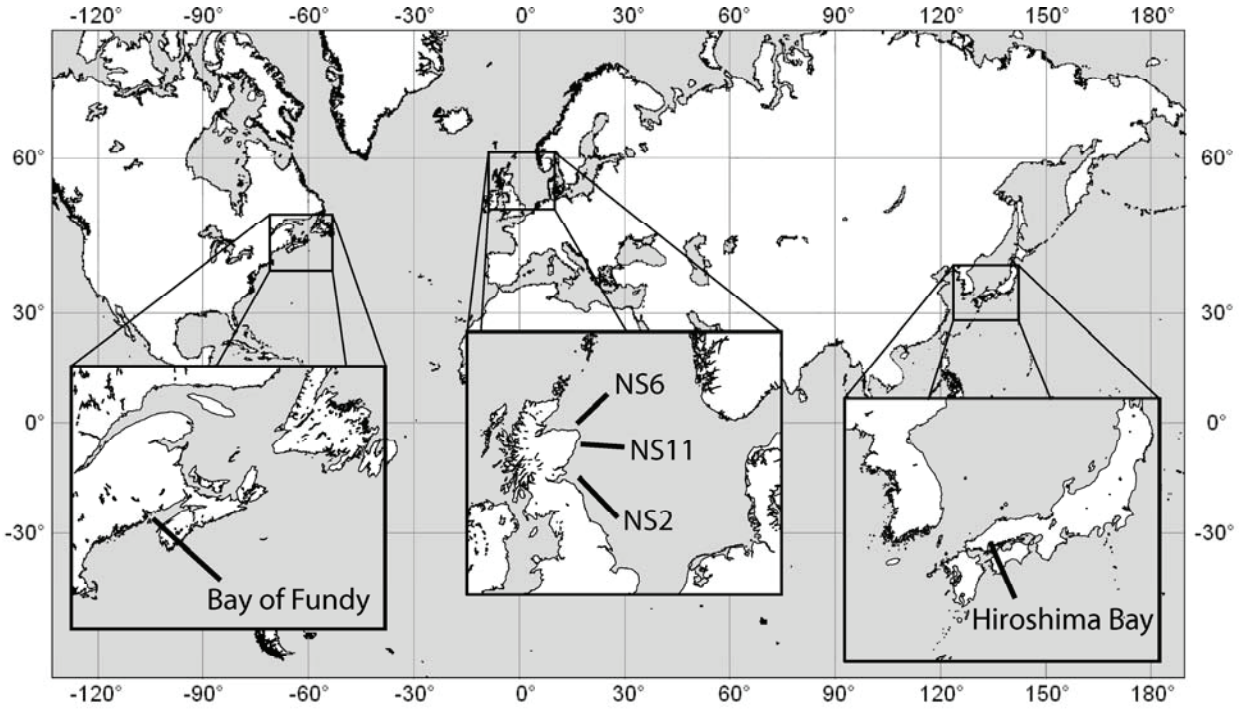

Fig. 2.7.1. Geographical origin of population samples of the ‚*Alexandrium tamarense* species complex' (NS = North Sea).

genotype-based approach using multivariate statistics. Further, we adopted a Bayesian clustering approach (Pritchard et al. 2000a) to probabilistically assign individuals of unknown status of genetic relatedness to one of several genetically differentiated population clusters. As a comparative approach, we utilized a classical phenotypic marker for the species, namely PSP toxin profiles, to test its power to reveal phenotypic differentiation of populations across the same geographical scales. Additionally, a small population sample that was available from the Western Pacific was used as an outgroup sample for the microsatellite based analysis and to provide preliminary information on the genetic relatedness of Western Pacific and Atlantic *A. tamarense* populations. This study provides clear insights into the extent of genotypic differentiation within and among geographically separated and presumably disjunct populations of *A. tamarense* and the means to identify source populations of recently established local populations after human-mediated or natural dispersal.

2.7.3. Materials and Methods

Origin and culture of Alexandrium tamarense *isolates*

Planktonic populations of the '*Alexandrium tamarense* species complex' were sampled from three different regions of the Northern Hemisphere: (A) one population of the *A. fundyense* morphotype was sampled in May 2005 from the Bay of Fundy (BoF), southeast of Grand Manan Island, (B) samples of three populations of the *A. tamarense* morphotype were obtained in May/June 2004 from the North Sea (NS) at three locations along the coast of Scotland, and (C) one population was sampled in Hiroshima Bay (HB), Japan, (Fig. 2.7.1, Table 2.7.1). From these surface water samples single *A. tamarense* cells were isolated under a stereo-microscope with a micropipette and transferred into individual wells of 96-well tissues culture plates containing 150 µL per well of diluted seawater-based f/2 medium (Guillard and Ryther 1962) or K medium (Keller et al. 1987) without silica, supplemented with selenite (Dahl et al. 1989). Isolated cells were then incubated at 10 °C under cool-white fluorescent light at a photon flux density of 100–150 µmol photons m^{-2} s^{-1} and a 14 to 16h day-length. After some weeks, the surviving isolates were transferred to larger containers containing undiluted growth medium. A total of 239 clonal isolates of *A. tamarense/A. fundyense* were obtained (Table 2.7.1).

DNA extraction and sequencing of the D1/D2 region LSU rDNA

Extraction of DNA from batch cultures of *A. tamarense/A. fundyense* was performed with a DNeasy Plant Kit (Qiagen, Hilden, Germany) Purity and concentration of the DNA sample was checked by UV-spectroscopy with a NanoDrop ND-1000 spectro-photometer (Peqlab, Erlangen, Germany). Integrity of DNA fragments of a molecular weight of about 20 kb was verified on a 0.8 % agarose gel.

Sequencing of the D1/D2 region of the large subunit (LSU) rDNA was performed for more than half of the individuals from each geographic population sample to confirm the membership of

Table 2.7.1. Strain designations and LSU rDNA sequences from different ribotypes of the *A. tamarense* 'species complex' used for the phylogenetic classification of *A. tamarense* isolates.

Clade / ribotype	Strain	Morphotype	GenBank accession no.
North American	OF844230	*A. fundyense*	AJ535364
	AFNFA3.1	*A. fundyense*	U44926
	AT-9	*A. tamarense*	AJ535365
	PW06	*A. tamarense*	U44927
Mediterranean	SZN19	*A. tamarense*	AJ535370
	SZN21	*A. tamarense*	AJ535374
Western European	Pgt183	*A. tamarense*	U44930
	UW42	*A. tamarense*	AJ303428
Temperate Asian	BAHME215	*A. catenella*	AJ535361
	ALexcat3	*A. catenella*	AF042818
Tasmanian	ATBB01	*A. tamarense*	U44933
Outgroup	TAMI2207	*A. tamiyavanichii*	AB088267
	Alexaffi	*A. affine*	U44935
	AOSH1	*A. ostenfeldii*	AJ535358

the North American ribotype (or Group I) of the species complex. After PCR amplification with the primers D1R (forward) and D2C (reverse) (Scholin et al. 1994) and subsequent cloning into the vector provided with the TOPO TA Cloning® kit (Invitrogen, Carlsbad, CA), one clone per *Alexandrium* isolate was sequenced from both ends with a standard sequencing chemistry on an ABI 3130 XL capillary sequencer (Applied Biosystems, Darmstadt , Germany) using universal M13 primers supplied with the cloning kit. Assembled sequences were then aligned in ClustalX 1.83 (Thompson et al. 1997) together with LSU rDNA sequences of strains belonging to the five different LSU ribotypes of the '*A. tamarense* species complex' (Scholin et al. 1994; John et al. 2003) and two other *Alexandrium* species as outgroup (Table 2.7.1). After exclusion of repeatedly occurring sequences phylogenetic relationships among the remaining unique sequences were analysed by the Kimura 2-parameter model of evolution in the software MEGA, version 3.1 (Kumar et al. 2004). In the same program a phylogenetic tree was constructed by the neighbour joining method and the reliability of tree topology was tested by 1,000 boot replicates.

Amplified fragment length polymorphism (AFLP) analysis

All cultured isolates of *A. tamarense/A. fundyense* (North American ribotype) available from the North Sea and the Bay of Fundy were genotyped by AFLP, with the exception of North Sea population sample NS2 (only 93 of 156 isolates). Samples from Hiroshima Bay lost their DNA integrity due to shipping to the laboratory in Germany and therefore could not be included in the AFLP analysis. The assessment of AFLP loci was carried out according to the protocol of John et al. (2004) (see also for sequences of adapters used in the ligation and primers used in the pre-amplification reactions) and modifications as in Alpermann et al. (in press). In brief, *Alexandrium* DNA (240 ng) was digested with EcoRI and MseI for 15h at 37 °C. After heat-inactivation the restriction mix was added to a ligation mix consisting of EcoRI and MseI adapters, T4 DNA ligase and ATP. The mix was incubated for 9 h at 16 °C and subsequently for 6 h at 21 °C. The ligated DNA was diluted and added to a preamplification PCR mix with EcoRI+A and MseI+C primers. After 22 PCR cycles the products were diluted and used as a template in final amplification reactions with four different combinations of selective primers, where each selective primer contained three selective bases (see Table 2.7.2). In these reactions the EcoRI primers were fluorescently labelled with 6'-Fam. One µL of the product of the amplification reaction in 15 µL HiDi™ formamide and 0.5 µL of the size-marker GeneScan™–500[ROX]™ (both reagents from Applied Biosystems, Darmstadt, Germany) were sized by a capillary sequencer ABI 3130XL (Applied Biosystems). Sizing and scoring of AFLP fragments in a range from 100 to 500 bp was carried out with the help of GENEMAPPER ® v4.0 (Applied Biosystems) with a signal intensity threshold of "250" for binning and a threshold of "50" for scoring of AFLP loci. Signal intensity was normalized over all samples for binning and scoring.

Table 2.7.2. Origin of *Alexandrium tamarense* (North American ribotype) isolates. N.D. = Not determined

Geographical population (code)	Origin of Isolate	Year	Latitude	Longitude	Sample size (*n*) (genotyped)	Gene diversity over all AFLP loci	Gene diversity over all SSR loci
North Sea S2 (NS2)	Scotland, U.K.	2004	56° 05' 47'' N	1° 42' 35'' W	156 (86)	0.17	0.56
North Sea S6 (NS6)	Scotland, U.K.	2004	57° 25' 18'' N	1° 41' 23'' W	17 (14)	0.17	0.56
North Sea S11 (NS11)	Scotland, U.K.	2004	57° 48' 79'' N	2° 07' 28'' W	13 (13)	0.16	0.52
Bay of Fundy (BoF)	USA	2005	44° 55' 98'' N	66° 45' 48'' W	16 (16)	0.17	0.46
Hiroshima Bay (HB)	Japan	2004	34° 27' 54" N	132° 26' 73" E	33 (5) [a]	N.D.	0.56

[a] microsatellites only.

Microsatellite genotyping

Eighteen previously characterized microsatellite loci for the *A. tamarense* North American ribotype (Table 2.7.3) (Nagai et al. 2004; Alpermann et al. 2006a) were amplified from the same DNA templates that were used for AFLP genotyping. Additionally, to the samples from cultured isolates, DNA from 33 isolates from Hiroshima Bay, Japan, were genotyped at all loci, though quantities of genomic DNA in these samples were much lower and routine amplification of microsatellite alleles under the conditions as described above could not be guarantied.

The PCR conditions were as described in Alpermann et al. (2006a). In brief, PCR reactions with a total volume of 11.4 µL were run for each pair of specific primers containing 10–20 ng of DNA, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.1 or 0.5 µM of each primer and 1 unit of HotMaster™ Taq DNA Polymerase (Eppendorf, Hamburg, Germany). The PCR was performed either with a constant annealing temperature for 40 PCR cycles (only for loci ATB1) or as a touchdown PCR with 20 cycles, in which the annealing temperature was reduced by 0.3°C every cycle followed by 25 cycles with constant annealing temperature (Table 2.7.3). One of the two primers used in each PCR was 5' terminally labelled with either 6-FAM, Ned or Hex (Applied Biosystems, Darmstadt, Germany). One µL of the product of the amplification reaction in 15 µL HiDi™ formamide and 0.5 µL of the size-marker GeneScan™–500[ROX]™ (both reagents form Applied Biosystems) were sized using a capillary sequencer ABI 3130XL (Applied Biosystems). If necessary, the PCR products were diluted appropriately to enable correct sizing. Sizing and scoring of microsatellite alleles was carried out with the help of the software GENEMAPPER® v3.7 (Applied Biosystems).

Analysis of AFLP and microsatellite genotypic data

Data sets for the analysis of AFLP genotypic data were composed of those isolates that proved to amplify PCR products in all four different primer combinations of the amplification reaction. Samples that failed to produce alleles for the majority of microsatellite loci were excluded from the SSR based analyses. The high number of non-amplifying DNA samples from isolates from Hiroshima Bay was attributed to low DNA concentration and loss of integrity of high molecular-weight DNA as a result of prolonged shipping and repeated freezing-thawing of samples. To prevent any influence of inadequately genotyped individuals on the outcome of subsequent analyses those individuals that did not result in unambiguous determination of alleles in at least half of the loci therefore were excluded from the microsatellite data sets. Further, all subsequent analyses were performed on data sets containing only population samples from the Eastern and Western Atlantic and additionally for a complete data set containing also those isolates from Japan, for which suitable samples for genotyping were available. Size range and allele number per population as well as the number of private alleles were retrieved for all microsatellite loci by use of the software GENALEX, version 6 (Peakall and Smouse 2006). Gene diversity according to Nei (Nei 1987) was estimated in the same software individually for each microsatellite locus and over all SSR and AFLP loci by population.

Genetic differentiation among populations was estimated by calculation of pairwise F_{ST} values in the software ARLEQUIN, version 2.000 (Schneider et al. 2000), using (A) the AFLP data for the North Atlantic populations, (B) the microsatellite data for the North Atlantic populations and

Table 2.7.3. Specifications and summary statistics of microsatellite loci for genotyping of *A. tamarense* isolates (Ann. Temp.-TD = annealing temperature at start of touch-down PCR, Ann. Temp. = constant annealing temperature).

Microsatellite Locus	Ann. Temp.-TD(°C)	Ann. Temp.(°C)	Total number of alleles
ATB1 [a]	–	53.0	3
ATB8 [a]	61.0	54.0	14
ATD8 [a]	61.0	54.0	5
ATF1 [a]	61	54.0	12
ATF11 [a]	68.5	61.5	8
ATG6 [a]	62.7	55.7	8
Atama04 [b]	61.0	54.0	3
Atama06 [b]	67.0	60.0	5
Atama13 [b]	60.0	53.0	8
Atama15 [b]	63.0	56.0	12
Atama16 [b]	63.0	56.0	8
Atama17 [b]	59.0	52.0	5
Atama23 [b]	59.0	52.0	4
Atama26 [b]	56.0	49.0	5
Atama27 [b]	58.0	51.0	16
Atama32 [b]	60.0	53.0	9
Atama39 [b]	59.0	52.0	5
Atama42 [b]	59.0	52.0	16

[a] Alpermann et al. (2006a). [b] Nagai et al. (2004).

(C) the microsatellite data for all Atlantic populations and the population sample from the Pacific population from Hiroshima Bay Japan.

Nei's (1978) unbiased minimum distance among population samples from the Western and Eastern Atlantic was calculated and graphically depicted by an UPGMA dendrogram in the software TFPGA (Tools for Population Genetic Analyses), version 1.3 (Miller 1997), for both genotypic markers (AFLP and microsatellites). For microsatellite data an additional analysis was performed including the Western Pacific population sample from Hiroshima Bay, Japan.

Principal coordinate analysis (PCoA) (Gower 1966) of AFLP and microsatellite data was performed in the statistical software BRODGAR, version 2.5.0 (Highland Statistics Ltd., Newsburgh, UK). Matrices of pairwise similarities were generated by the Simple Match index for AFLP and the

Dice index of similarity for microsatellite data as suggested by Kosman and Leonard (Kosman and Leonard 2005).

The AFLP data set included all genotyped isolates from the North Sea and the Bay of Fundy. The microsatellite based analysis was performed on the same population samples first. In a second analysis isolates from Hiroshima Bay were included in the microsatellite based PCoA.
Population assignment was performed with data sets of multilocus AFLP and microsatellite genotypes in the software STRUCTURE, version 2.2 (Falush et al. 2007), to estimate the potential of Bayesian clustering methods for the assignment of genotypes to their respective population of origin. Analyses were performed with the number of populations set to four (K = 4) by 100,000 iterations during the 'burn-in' period followed by 1,000,000 iterations during Markov Chain Monte Carlo simulations.

To test the ability of the Bayesian clustering approach implemented in the software STRUCTURE 2.2 to assign individuals from other population samples as included in the initial analysis to either one or several of the existing population clusters or to assign them to a separate cluster, we added five genotypes from the population sample that was available from Hiroshima Bay, Japan. STRUCTURE 2.2 analysis were performed with the same settings as previous stated, but this time ten independent runs were performed for four (K = 4) and five (K = 5) assumed populations. The relative composition of the four resulting 'STRUCTURE 2.2 populations' was then analysed.

Analysis of PSP toxin composition in A. tamarense *isolates*

Batch cultures of 16 clonal *A. tamarense* isolates originating from the US East coast as well as 94 isolates from the North Sea (NS2: 79 isolates, NS6: 7 isolates, NS11: 8 isolates) were collected by filtration over a 10 µm polyethylene Nitex® mesh or by centrifugation at 3,000 x g for 7 minutes. For cell enumeration a representative subsample of each culture containing at least 400 cells was counted in a settling chamber on an Axiovert (Zeiss, Jena, Germany) inverted microscope. The population samples from Hiroshima Bay, Japan were not available for PSP toxin analysis since isolates were not maintained in culture.

The sample preparation protocol of Hummert et al. (1997) was followed for extraction, detection and quantification of PSP toxins from the frozen cell pellets of clonal *A. tamarense* isolates. The toxin analyses were performed by automated high-performance liquid chromatography (HPLC) applying ion-pair chromatographic separation, post-column oxidation with periodic acid, and fluorescence detection (FD), based on the method of Thielert et al. (1991) with modifications as described in Hummert et al. (1997) and Yu et al. (1998). The 0.45 µm-filtered acetic acid lysates of cultures were injected into a HPLC equipped with a fluorescence detector and *N*-sulfocarbamoyl toxins were indirectly determined after their hydrochloric acid mediated hydrolysis by the difference of the peak areas to those obtained by acetic acid extract. Standard solutions of GTX2 and GTX3 included traces of dcGTX2 and dcGTX3, but the concentration of these toxins was not given. A standard for dcSTX was provided by the European Commission (BCR, The Community Bureau of Reference, Brussels, Belgium).

After determination of the concentration of individual PSP toxin derivatives, toxin concentrations were converted to molar quantities from the known molecular weight of each toxin.

The total per cell PSP toxin quota as well as the cellular content of the different saxitoxin derivatives was calculated after cell counts from Lugol's iodine fixed samples.

Since the determination of concentrations of *N*-sulfocarbamoyl toxins was based on an indirect method, tandem mass spectrometry was performed to directly confirm the presence of *N*-sulfocarbamoyl toxins in the unhydrolysed samples. Those *N*-sulfocarbamoyl toxins that could not be verified by tandem mass spectrometry were judged as measurement artefacts of the indirect method or as present below the detection limit and consequently were excluded from the following analysis. Since facile interconversions among epimeric toxins such as C1 and C2 are known to occur (Hall et al. 1990), the actual ratios for these pairs of toxin species at the moment of cell harvesting could not be inferred with certainty. Therefore, molar quantities of these epimeric pairs were fused in subsequent analyses into the following groups: 'C1/C2', 'GTX2/GTX3', 'GTX1/GTX4'. Neither dc-NEO nor any decarbamoyl gonyautoxins (dc-GTX1/dc-GTX4 and dc-GTX2/dc-GTX3) were detected, but traces of dc-STX, with a maximum value of 0.6 mol% of total toxin and an average of 0.12 mol% (S.D. 0.13), were found in some samples. Decarbamoyl toxins are primarily found in invertebrates such as bivalves that accumulate and modify PSP toxins. Hence, we presume that the small amounts of dc-STX found in some dinoflagellates samples were an artefact from spontaneous decarbamoylisation of either STX or B1 due to cleavage of the carbamoyl or sulfocarbamoyl moiety, respectively, and were excluded from further analyses as an unstable trait.

To equally weigh each of the toxin groups, 'normalized toxin composition ratios' were used for further analyses as suggested by (Cembella et al. 1987). The molar percentage of each toxin group in each individual was divided by the maximum molar percentage that was found in all individuals for the respective toxin. With this data, Euclidean distances for all pairs of isolates were calculated and a two-dimensional plot was generated by non-metric multi-dimensional scaling (nMDS). The significance of phenotypic differentiation among population samples with respect to PSP toxin profiles was then assessed in the software BRODGAR, version 2.5.0, by an 'analysis of similarities' (ANOSIM) (Clarke 1993) with 9,999 bootstrapped replicates using the Euclidean distance as measure of relatedness among toxin profiles. Significance levels for pairwise comparisons were corrected by Bonferroni's procedure (Sokal and Rohlf 1995). On the same data, a 'similarity percentage breakdown' (SIMPER) (Clarke 1993) was carried out to elude, which of the toxins contributed most to the pairwise differences of significantly distinct population samples. These analyses all were performed in the statistical software package PRIMER, version 6 (PRIMER-E Ltd., Ivybridge, UK).

2.7.4. Results

Phylogenetic analysis of population samples

Phylogenetic analysis of *A. tamarense* isolates from the Bay of Fundy, the North Sea and Hiroshima Bay based on the hypervariable D1/D2 region of LSU rDNA sequences allowed identification of all isolates as members of the North American ribotype *sensu* Scholin et al. (Scholin et al. 1994). This group is alternatively designated as "Group I" according to Lilly et al. (36), and exhibited clear separation from other ribotypes of this species with 100% bootstrap support (Fig. 2.7.2) (sequences and alignment are available at http://www.treebase.org).

AFLP genotypic data

The four combinations of primers used in the AFLP amplification reaction produced a total of 462 well-scoreable PCR products of distinct size (i.e. band alleles) in 121 clonal isolates from the population samples from the Western and Eastern Atlantic. The number of AFLP loci per primer pair ranged from 104 to 132 with an average of 115.5 (S.D. 11.7) (Table 2.7.5). The average gene diversity per population over all AFLP loci did not substantially vary (Table 2.7.2).

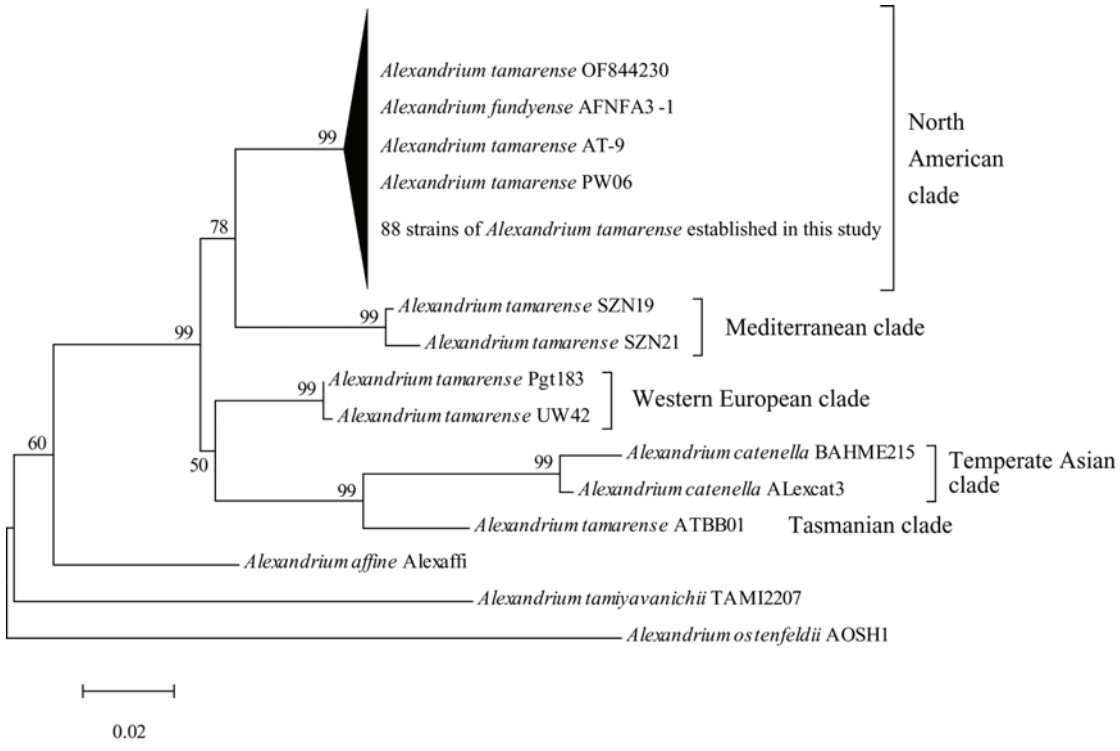

Fig. 2.7.2. Neighbour-Joining dendrogram depicting phylogenetic relationships of 88 *A. tamarense* isolates established in this study within the *Alexandrium tamarense* 'species complex' (Scholin et al. 1994, John et al. 2003). Scale bar indicates substitutions per site; bootstrap values (in percent) were obtained by 1,000 resamplings; the branch containing all *A. tamarense* isolates established in this study and those that were previously assigned to the North American clade was collapsed (alignment and original phylogenetic tree are available at http://www.treebase.org).

Table 2.7.4. Summary statistics for 18 microsatellite loci in *Alexandrium tamarense* populations (North American ribotype); N = Number of individuals genotyped; N_a = Number of alleles; N_p = Number of private alleles; R = size range of alleles; H_E = gene diversity (*sensu* Nei (1987)).

Marker		Bay of Fundy (BoF)	North Sea (NS2)	North Sea (NS6)	North Sea (NS11)	Hiroshima Bay (HB)
ATB1	N	14	71	9	1	10
	N_a	1	2	1	1	1
	N_p	1	1	0	0	0
	R	292	285–286	286	286	286
	H_E	0.000	0.028	0.000	0.000	0.000
ATB8	N	8	56	10	2	7
	N_a	4	9	4	2	3
	N_p	2	5	1	2	0
	R	386–395	377–492	337–412	386–412	407–437
	H_E	0.656	0.701	0.640	0.500	0.449
ATD8	N	15	79	10	4	10
	N_a	3	4	4	2	4
	N_p	0	0	0	1	0
	R	267–278	267–278	267–278	267–278	263–276
	H_E	0.551	0.694	0.700	0.500	0.640
ATF1	N	16	85	14	4	13
	N_a	5	8	6	4	5
	N_p	0	3	0	1	0
	R	154–169	163–189	154–187	163–187	161–184
	H_E	0.500	0.669	0.724	0.750	0.746
ATF11	N	15	83	14	4	13
	N_a	4	6	3	2	3
	N_p	1	2	0	1	0
	R	268–299	268–315	268–299	268–299	259–268
	H_E	0.436	0.281	0.439	0.375	0.462
ATG6	N	5	57	6	1	8
	N_a	3	5	2	1	3
	N_p	2	3	0	0	1
	R	180–220	179–212	179–180	179–214	184
	H_E	0.560	0.480	0.444	0.000	0.406
Atama04	N	16	86	14	5	13
	N_a	2	3	3	3	3
	N_p	0	0	0	0	0
	R	117–119	115–119	115–119	115–119	115–119
	H_E	0.117	0.578	0.622	0.640	0.639
Atama06	N	16	85	12	5	12
	N_a	1	3	3	4	3
	N_p	0	1	0	1	0
	R	188	188–191	188–190	188–190	185–190
	H_E	0.000	0.511	0.569	0.720	0.486
Atama13	N	13	83	14	3	12
	N_a	4	6	5	3	5
	N_p	1	1	0	1	0
	R	115–119	113–118	113–118	113–118	113–124
	H_E	0.627	0.710	0.745	0.667	0.681

Table 2.7.4. (continued)

Marker		Bay of Fundy (BoF)	North Sea (NS2)	North Sea (NS6)	North Sea (NS11)	Hiroshima Bay (HB)
Atama15	N	16	85	13	5	13
	N_a	4	10	4	5	5
	N_p	0	4	0	1	1
	R	238–250	234–250	234–248	234–273	240–250
	H_E	0.484	0.684	0.627	0.800	0.651
Atama16	N	13	80	14	4	13
	N_a	6	6	5	4	2
	N_p	0	1	0	1	0
	R	153–167	159–169	153–167	163–165	155–163
	H_E	0.793	0.465	0.551	0.750	0.260
Atama17	N	14	78	12	2	10
	N_a	4	5	4	2	3
	N_p	0	1	0	0	0
	R	134–140	130–140	134–140	134–140	134–136
	H_E	0.663	0.687	0.681	0.500	0.640
Atama23	N	16	86	13	2	13
	N_a	3	3	3	2	3
	N_p	0	0	0	1	0
	R	177–181	177–181	177–181	177–181	174–179
	H_E	0.656	0.492	0.379	0.500	0.544
Atama26	N	12	72	11	3	10
	N_a	2	3	2	2	2
	N_p	1	1	0	0	1
	R	277–301	246–283	277–283	273–283	277–283
	H_E	0.153	0.335	0.298	0.444	0.180
Atama27	N	12	80	12	4	12
	N_a	4	11	6	3	6
	N_p	3	3	0	1	1
	R	151–166	156–174	156–172	158–178	157–170
	H_E	0.417	0.802	0.750	0.625	0.694
Atama32	N	14	73	11	5	10
	N_a	3	7	4	4	6
	N_p	0	1	0	2	0
	R	156–158	144–160	144–158	144–158	148–159
	H_E	0.500	0.785	0.645	0.720	0.820
Atama39	N	16	84	14	5	13
	N_a	2	3	3	4	3
	N_p	3	2	3	2	0
	R	138–140	138–143	138–143	138–143	136–150
	H_E	0.305	0.471	0.439	0.720	0.462
Atama42	N	8	84	14	5	13
	N_a	6	7	6	5	4
	N_p	0	0	0	3	0
	R	222–241	220–256	230–270	230–256	218–266
	H_E	0.813	0.673	0.786	0.800	0.663

Table 2.7.5. Combinations of primers in the AFLP amplification reaction and results on polymorphic loci and average number of band alleles.

Primer combination	EcoRI-Primer	MseI-Primer	Number of polymorphic loci	Mean number of bands (± SD)
1	EcoRI+AAG	MseI+CTA	104	20.4 (±21.2)
2	EcoRI+AGG	MseI+CCG	105	16.6 (±19.6)
3	EcoRI+AAG	MseI+CTT	132	24.9 (±23.2)
4	EcoRI+AGG	MseI+CCT	121	21.1 (±23.0)

Microsatellite genotypic data

The number of alleles for a specific microsatellite marker varied considerably and ranged from three to sixteen (Table 2.7.3) with an average of 8.1 (S.D. 4.1). Gene diversity among microsatellite ranged from 0 to 0.82 (Table 2.7.4). The average gene diversity per population over all microsatellite loci reached similar values for the three population samples from the North Sea and the isolates from Hiroshima Bay, but was slightly lower for the population sampled from the Bay of Fundy (Table 2.7.2).

Analysis of genotypic data

Genetic differentiation. Independent estimation of pairwise F_{ST} with both genotypic markers, AFLP and microsatellites, showed that the Western Atlantic population from the Bay of Fundy is highly genetically differentiated from the North Sea populations (Table 2.7.6). Whereas F_{ST} values were generally higher for microsatellite data for the same population pairs by AFLP, the significance levels ($p < 0.001$) was always very high for pairwise comparisons of the Western Atlantic population with the Eastern Atlantic populations. Only one significant pairwise F_{ST} was detected among the Eastern Atlantic populations from the North Sea; the AFLP-based analysis showed that populations NS2 and NS6 were weakly genetically differentiated compared to the pairwise differentiation found between the Bay of Fundy and either of the North Sea populations. The inclusion of the Pacific population sample from Hiroshima Bay, Japan, to the microsatellite-based analysis indicated a high level of genetic differentiation from the Bay of Fundy population and moderate levels of genetic differentiation from the three North Sea populations.

Nei's unbiased minimum distance (Nei 1987) allowed depicting the relatedness among population samples from the Bay of Fundy and the North Sea by UPGMA dendrograms for AFLP (Fig. 2.7.3A) and microsatellite (Fig. 2.7.3B) markers. High bootstrap support was obtained by both markers for the branch separating populations of transatlantic origin. All AFLP and microsatellite loci were informative for this separation. The arrangement of the four Atlantic population samples

Table 2.7.6. Genetic differentiation estimated as pairwise F_{ST} of *A. tamarense* populations as defined by sampling locations for (1) microsatellites data (lower diagonal) and (2) AFLP data (upper diagonal). BoF, Bay of Fundy; NS, North Sea; HB, Hiroshima Bay

	BoF	**NS11**	**NS2**	**NS6**
BoF		0.072 [a]	0.094 [a]	0.081 [a]
NS11	0.344 [a]		0.015	−0.008
NS2	0.297 [a]	0.003		0.023 [a]
NS6	0.269 [a]	−0.012	0.006	
HB	0.263 [a]	0.118 [a]	0.119 [a]	0.075

[a] significant at $p < 0.01$ after Bonferroni correction.

in the dendrogram was not markedly influenced when the small Pacific population sample from Hiroshima Bay, Japan, was added to the analysis (Fig. 2.7.3C). The separation of the Japanese population from North Atlantic populations was also supported by high bootstrap values, though only 50 percent of loci supported the node as depicted in the dendrogram.

Principal coordinate analysis (PCoA) of AFLP data revealed that isolates from the four population samples were not unambiguously separated on the first axis that explained most of the variation in the data (Fig. 2.7.43A). However, along the second principal coordinate the Bay of Fundy isolates separated from the residual population samples. Although the isolates from the North Sea stations NS6 and NS11 lay within the coordinate space occupied by isolates from North Sea station NS2, they are found in only half of that area, leaving a considerable proportion of the total coordinate space to isolates from NS2 alone. The microsatellite-based PCoA showed a clear separation of Bay of Fundy clones from the residual population samples. No isolates from other populations were within the coordinate space occupied by the Bay of Fundy isolates (Fig. 2.7.4B). Isolates from the North Sea were found in another area of the coordinate space and did not show a tendency to segregate. The Japanese isolates that were included in the second PCoA with microsatellite data, did not clearly separate from the North Sea isolates, but showed a tendency to segregate on the second principal coordinate (Fig. 2.7.4C).

Population assignment. In nine of ten STRUCTURE 2.2 runs with AFLP data for the populations from the Bay of Fundy and the three North Sea populations the following picture emerged: individuals were only assigned to three of the four hypothetical populations. One population with individual numbers from 13 to 21 was formed with an average probability of assignment of 0.98, to which the majority of individuals from the Bay of Fundy population were assigned (ranging from 75 to 93.8 %, Fig. 2.7.5A). This population always included a small fraction of individuals from one of the other populations (ranging from 1.2 to 3.6 % for NS2, from 0 to 15.4 % for NS6 and from 0 to 11.1 % for NS11). The residual two populations to which individuals were assigned contained individuals from all of the three NS populations with a characteristic

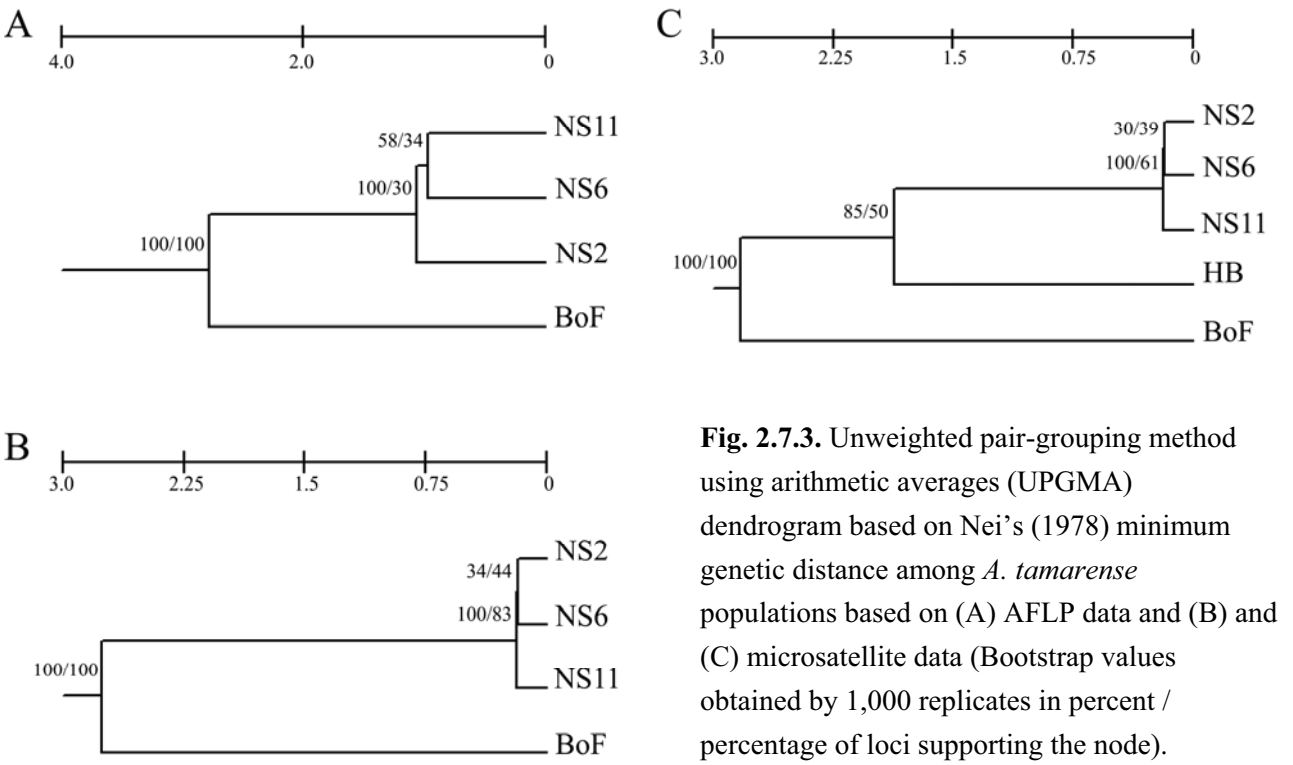

Fig. 2.7.3. Unweighted pair-grouping method using arithmetic averages (UPGMA) dendrogram based on Nei's (1978) minimum genetic distance among *A. tamarense* populations based on (A) AFLP data and (B) and (C) microsatellite data (Bootstrap values obtained by 1,000 replicates in percent / percentage of loci supporting the node).

proportional composition. The larger of these two populations (from 65 to 70 individuals) always contained the majority of individuals from NS2 (from 66.3 to 68.7 percent) and NS6 (from 53.8 to 69.2 percent), but only a smaller proportion of the individuals from population NS11 (from 33.3 to 44.4 percent). The smaller of these two populations (from 35 to 38 individuals) always included the same individuals from NS11 (55.6 percent), NS2 (always 30.1 percent) and NS6 (30.8 percent). Individuals from the Bay of Fundy population that were not included in the same hypothetical population as the majority of individuals from this population were always found in the smaller of the residual two hypothetical populations that included individuals (ranging from 6.3 to 25 % of Bay of Fundy individuals). Average assignment probabilities for the larger and the smaller population was 0.98 and 1, respectively. The only STRUCTURE 2.2 run that did not fit this general picture obtained the lowest Ln posterior probability (−19915.2) of all runs (average −19745.5 ± S.D. 73.7) and therefore resembles a population assignment that is less likely than the others. Here individuals of all populations were assigned almost proportionally to only two hypothetical subpopulations.

Ln posterior probabilities of the ten STRUCTURE 2.2 runs with microsatellite data for the populations from the Bay of Fundy and the three North Sea populations were comparable among runs and ranged from −2525.4 to −2468.8 (average −2491.3 ± S.D. 16.8). In nine of the ten runs individuals were assigned to all four hypothetical populations. One of these always included all individuals from the Bay of Fundy and the same individual from the North Sea population NS2. Average assignment probabilities of individuals to this population across runs ranged from 0.99 to 1. The formation of the residual three clusters was not supported by high probabilities of

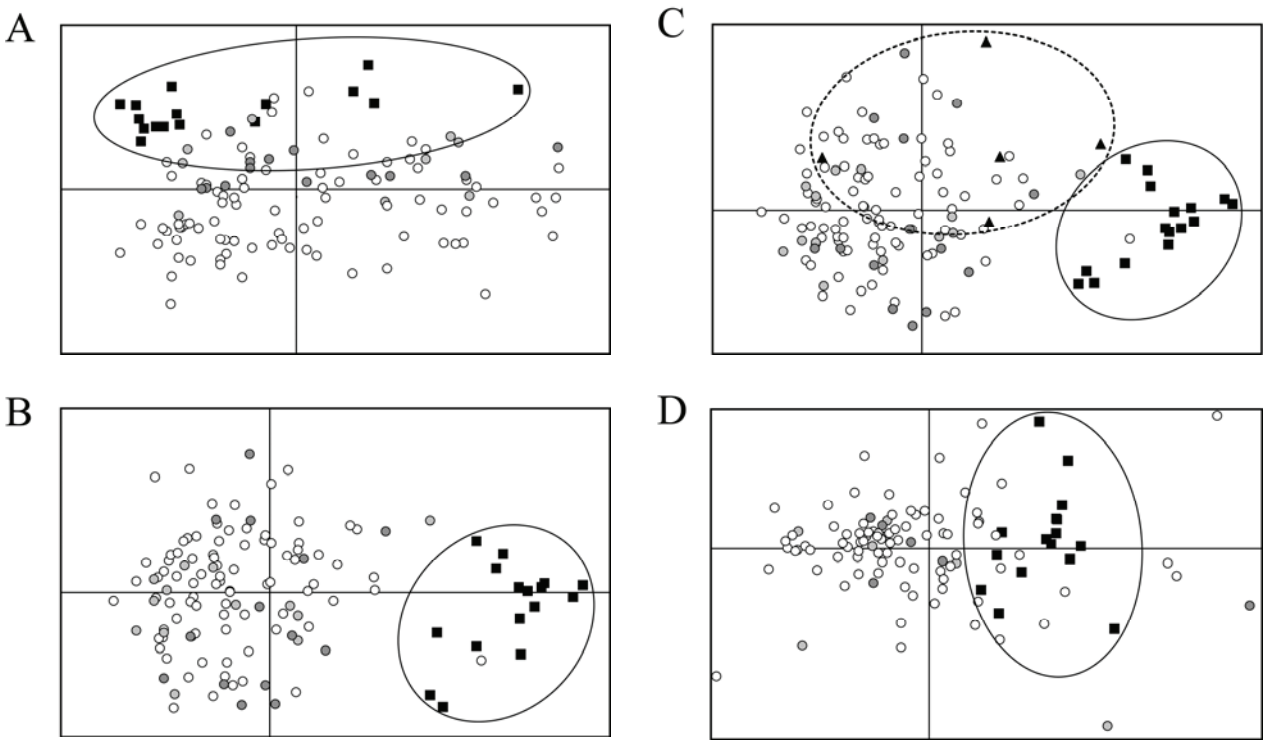

Fig. 2.7.4. Principal coordinate analysis PCoA for (A) AFLP data and (B) microsatellite data for Bay of Fundy (BoF) and North Sea (NS) population samples and (C) the same microsatellite data including the population sample from Hiroshima Bay (HB); (D) non-metric multi-dimensional scaling (nMDS) of PSP toxin profiles expressed as 'normalized toxin composition ratios' (stress 0.02). BoF = squares (within solid ellipse), NS2 = open circles, NS6 = dark-grey circles, NS11 = light-grey circles, HB = triangles (within dashed ellipse).

assignment, ranging across runs from 0.34 to 0.54. Consequently, the formed clusters did not reoccur in the same arrangement of individuals in different runs. In one of the ten STRUCTURE 2.2 runs only two additional populations were formed. The population that included all individuals from Bay of Fundy, however, was still composed the same, including the individuals from North Sea population NS2.

Ln posterior probabilities for the STRUCTURE 2.2 analyses of microsatellite data that included the five individuals from Hiroshima Bay, Japan, were very consistent across runs and ranged from −2633.7 to −2628 (average −2631.7 ± S.D. 1.6). Accordingly, the formation of clusters was the same in eight of ten runs and differed for the residual two runs in distinct assignment of only one individual. As in the analyses without the Japanese individuals, one cluster was always composed of all isolates from the Bay of Fundy plus the same individual from the NS population NS2. This cluster had with a value of 0.97 the highest average assignment probability of all clusters. The smallest of the three remaining clusters included 20 to 21 individuals from the three NS populations (16.3 percent of individuals from NS2, 28.6 to 35.7 percent of individuals from NS6 and 15.4 percent of individuals of NS11) plus all of the five Japanese individuals. The larger of the last two

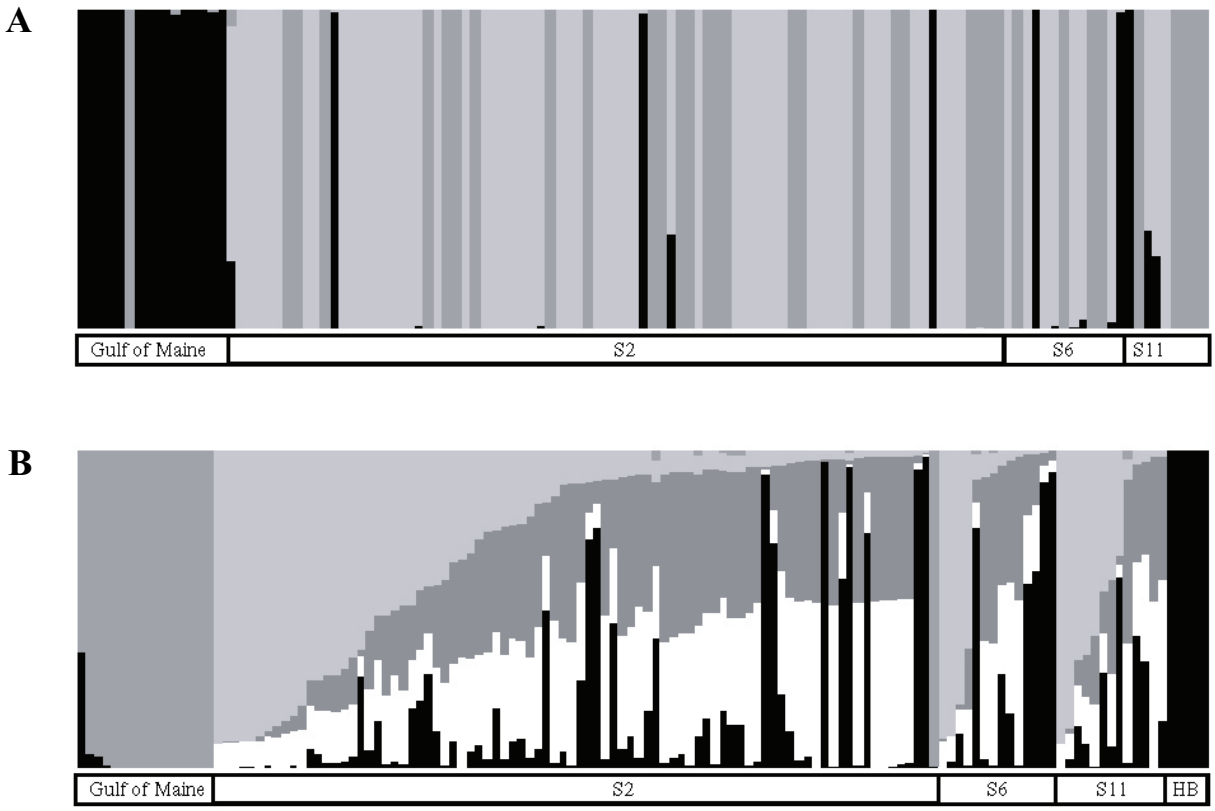

Fig. 2.7.5. Assignment probabilities of (A) AFLP and (B) microsatellite genotypes as determined by the most probable STRUCTURE 2.2 run. Probabilities to fall into one of the four (A) respectively five (B) population clusters are depicted in different shades from white over grey to black for every individual of the four (A) respectively five (B) source populations.

clusters contained always the same 47 individuals (41.9 percent coming from NS2, 28.6 percent from NS6 and 53.8 percent from NS11). The smaller contained 44 to 45 individuals (40.7 percent coming from NS2, 35.7 to 42.9 percent from NS6 and 30.8 percent from NS11). These two populations had considerably lower average assignment probability of 0.73 and 0.72, respectively, for the larger and the smaller one.

Ln posterior probabilities for the STRUCTURE 2.2 analyses that assumed five hypothetical populations ranged from −2619.4 to −2610.2 (average −2614.6 ± S.D. 2.4). The formation of the five population clusters was similar across runs, but in one out of the ten runs only four population clusters were formed instead of five. As in the analyses with four assumed populations one of the clusters always included all Bay of Fundy individuals plus the one additional individual from NS population NS2, which was always the same (Fig. 2.7.5B). This cluster also had the highest average assignment probability with 0.97. Another cluster that was formed in all runs contained the five individuals from Hiroshima Bay and others from the three NS populations (15.1 % of individuals from NS2, 35.7 to 42.9 to percent of individuals from NS6 and 15.4 to 23.1 percent of individuals of NS11). Here average assignment probabilities were relatively high with an average of 0.77. The average assignment probabilities for the individuals from Japan to this population cluster even

reached a value of 1. The residual two to three population clusters varied in the composition of individuals. The considerably high variability was reflected in a low average assignment probability of 0.5. Population clusters always contained individuals of the largest North Sea population sample NS2 and varying numbers of isolates from NS6 and NS11, but no individuals from the Bay of Fundy or Japan.

Analysis of PSP toxin composition in A. tamarense isolates

The clonal isolates from the North Sea and the Bay of Fundy populations showed a remarkable variation with respect to the relative amounts of the different toxin groups. Neosaxitoxin (NEO) was the most abundant PSP toxin in the three population samples from the North Sea (Table 2.7.7). In the population sample from the Bay of Fundy the relative contribution of NEO to toxin profiles was considerably lower, not exceeding that of C1/C2. While the average contribution of most toxin groups to the regional predominating PSP toxin profiles were quite similar, the epimeric pairs of gonyautoxins GTX1/GTX4, GTX2/GTX3 were considerably more relatively abundant in the profiles from the Bay of Fundy then in those from the North Sea.

The global statistics of the ANOSIM analysis (R = 0.231) was highly significant with $p <$ 0.001, indicating that toxin profiles grouped by their origin were significantly differentiated (Table 2.7.8). The graphical representation of pairwise Euclidean distances among isolates from all population samples by multi-dimensional scaling allowed to identify the uneven distribution of isolates in the coordinate space (Fig. 2.7.4D). Here, the population sample from the Bay of Fundy occupied only a relatively small sub-area of the coordinate space. Accordingly, pairwise comparisons of toxin profiles by origin showed that only the profiles from the Bay of Fundy were

Table 2.7.7. Average molar percentage of the cellular content of PSP toxins among all isolates from populations (BoF, Bay of Fundy; NS, North Sea) included in this study

Toxin	**BoF (n=16)** **Mean% (± SD)**	**NS2 (n=79)** **Mean% (± SD)**	**NS6 (n=7)** **Mean% (± SD)**	**NS11 (n=8)** **Mean% (± SD)**
STX	11.6 (± 8.8)	11.6 (± 7.5)	9.8 (± 6.0)	12.2 (± 4.4)
NEO	22.6 (± 7.6)	52.3 (± 16.1)	49.6 (±15.4)	50.2 (±18.6)
GTX1/GTX4	16.0 (± 6.1)	5.6 (± 8.2)	9.7 (±18.2)	3.5 (± 2.9)
GTX2/GTX3	22.4 (± 9.5)	6.4 (± 9.8)	5.0 (± 6.6)	4.9 (± 5.1)
B1	2.9 (± 4.1)	1.1 (± 2.0)	1.6 (±1.7)	3.9 (± 8.6)
B2	1.3 (± 0.8)	2.4 (± 4.2)	1.6 (± 2.0)	3.6 (± 6.1)
C1/C2	23.2 (±.9.0)	20.6 (± 9.9)	22.7 (± 7.1)	21.8 (± 8.6)

Table 2.7.8. Phenotypic differentiation of *A. tamarense* isolates by sampling location with respect to relative proportions of PSP toxin groups as expressed by *R* values obtained by 9,999 permutations ANOSIM (lower diagonal) and percentage of contribution to sample dissimilarity of neosaxitoxin for the statistically differentiated population samples as obtained by the SIMPER analysis (upper diagonal)

	BoF	**NS2**	**NS6**	**NS11**
BoF		33.3	29.5	27.9
NS2	0.42 [a]			
NS6	0.52 [a]	−0.06		
NS11	0.50 [a]	0.05	−0.07	

[a] significant at $p < 0.001$ after Bonferroni correction

statistically differentiated when compared to NS2, NS6 and NS11, but no significant differentiation was detected among the three population samples from the North Sea (see Table 2.7.8). The SIMPER analysis showed that for all three significant pairwise population comparisons of PSP toxin profiles always NEO contributed the most to the observed differences (Table 2.7.8). Interestingly, the second most contributing PSP toxin was different for all pairwise comparisons (BoF–NS2: STX with 15.4 %, BoF–NS6: GTX1/GTX4 with 26.2 %, BoF–NS11: B1 with 20.5 %).

2.7.5. Discussion

Suitability of genotypic markers in population studies

In spite of the fact that methodological tools are now available to answer questions concerning the dispersal history, distributional patterns and biodiversity for some protistan plankters, conclusive studies on harmful species are rare. Although a few studies (e.g., Nagai et al. 2007a; Masseret et al. 2009) have dealt with toxic dinoflagellates, most applications deployed to study genetics of natural populations have focussed on diatoms (Rynearson and Armbrust 2000; Evans et al. 2004; Rynearson and Armbrust 2004; Evans et al. 2005). Conclusive studies on harmful flagellate species are rare, partially because many such taxa are both highly cosmopolitan and genetically diverse, leading to the problem of describing patterns of genetic diversity and differentiation based upon a few isolates from a few populations. In any case, it is not valid to simply extrapolate population genetic models from diploid spring bloom diatom populations to haploid dinoflagellates, with complex and often poorly known life histories, and which may tend to remain as background taxa, other than when forming exceptional high biomass blooms. Furthermore, with noteworthy exceptions (Frommlet and Iglesias-Rodríguez 2008; Henrichs et al. 2008) , population genetic studies on microalgal species have depended on the availability of cultured isolates as a prerequisite to gain genetic material for genotyping studies. This has limited such studies to those species that

could be adequately cultured. In a few cases, fine-scale patterns of population differentiation have been described, e.g. by analysis of microsatellite markers for populations of *A. tamarense* in waters around Japan and the Eastern Korean coast (Nagai et al. 2007a), but the general suitability of the respective marker to detect population differentiation across different geographical scales over a larger geographical range has not yet been shown for any HAB species.

Despite these limitations, among the dinoflagellates, the genus *Alexandrium*, and particularly members of the *A tamarense/fundyense/catenella* complex have been the most intensively studied at the combined population genetic and genomic level (Scholin et al. 1994; Scholin and Anderson 1994; John et al. 2003; John et al. 2004; Nagai et al. 2007a). This species complex therefore provides the best available model system for further explorations. In our current study, the genetic differentiation of populations across the transatlantic distributional area was consistent for both genotypic markers, but there was some slight difference concerning the genetic differentiation among the three North Sea populations. With microsatellites no significant genetic differentiation was detected, but the AFLP based analysis of pairwise F_{ST} yielded significant differentiation for one population pair among the three populations. Similar, for AFLP the genetic distance was considerably higher among the North Sea population samples, but not between the North Sea samples and the population sample from the Bay of Fundy, when compared to microsatellites.

These findings could be due to the higher power of AFLP, as applied in this study, to detect weak genetic differentiation. Similar observations already have been made when the comparative potential of AFLP and microsatellites to detect genetic differentiation across different geographical scales have been explicitly tested (Gaudeul et al. 2004). Most likely the higher sensitivity to detect weak genetic population differentiation can be attributed to the considerably higher number of biallelic AFLP markers investigated in most studies (more than 400 loci in this study) in comparison to the relatively low number of multiallelic microsatellite markers (18 loci in this study).

The principal analytical disadvantage of the application of dominant genetic markers (such as AFLP) to diploid or polyploid organisms does not impair the usefulness of this marker in studies on haploid individuals such as the vegetatively dividing life cycle stages of planktonic dinoflagellates. Here no additional assumptions must be made to carry out allele frequency or genotype based analyses. On the contrary, the power of differentiating capacity of multiallelic co-dominant markers such as microsatellites is reduced in haploids because only one allele per locus is scored.

Under consideration of the distinct nature of the two genotypic markers in this study, for population studies on *A. tamarense* over different geographical scales we generally conclude the following: (A) both markers are well suited to detect genetic differentiation on the larger geographical scale, and (B) AFLP is the better marker to detect lower levels of genetic differentiation among closely located populations, if a considerably higher number of markers is analysed.

Influence of autecological peculiarities on population differentiation

To interpret the weak genetic differentiation found among the population samples of *A. tamarense* from the North Sea, we have to consider the complex life cycle that includes a vegetatively

dividing, planktonic life form and a sexually formed, resting cyst that remains immotile in the sediment. Low levels of genetic differentiation in vegetatively proliferating populations of planktonic protists may be the result of temporally distinct environmental conditions favouring the growth of only a small subset of clonal lineages that comprise the local population. The existence of broad population-wide variation in vegetative growth rates, as investigated by Tillmann et al. (2009) under nutrient replete conditions on multiple clonal strains from population sample NS2, indicates that relative abundances of clonal lineages within planktonic populations are prone to rapid changes. Weak genetic differentiation such as that detected by AFLP among the North Sea population samples NS2 and NS6 might therefore be the result of short term differentiation of these populations as the consequence of phenotypic adaptation on the population level (Alpermann et al. 2009). Such genetic and phenotypic differentiation in planktonic populations, however, might have little influence on the long term differentiation of local populations of dinoflagellates such as those of the genus *Alexandrium*. Here, local benthic seed beds of resting cysts (hypnozygotes) that were sexually derived by fusion of gametes from (potentially differentiated) planktonic populations over several consecutive years can be expected to enhance the genetic resilience of populations. A great proportion of vegetatively dividing lineages that are released into the plankton in the next period of recruitment from a particular cyst bed most likely have a very similar level of variation concerning genotypic and phenotypic characters to that from the previous year, since all but last year's cyst cohorts have already contributed to the recruitment of the planktonic population in the previous year.

Patterns of phenotypic differentiation

The pattern of genetic differentiation among population samples analysed here is accompanied by a similar pattern of phenotypic differentiation with respect to PSP toxin profiles. Our results emphasize the potential of PSP toxin profiles to detect significant phenotypic differentiation (if any exists) among populations. Whereas the Western Atlantic population from the Bay of Fundy was clearly phenotypically distinguished from the North Sea populations by their toxin profiles, there was no phenotypic differentiation among the North Sea populations. Differences in PSP toxin profiles among the three North Sea populations and the Western Atlantic population were always primarily based on the relative proportions of neosaxitoxin (NEO), although the sub-dominant toxin group (as mol% of total toxins) was different in all cases. Whereas the range of proportional content of NEO in toxin profiles from the Bay of Fundy and adjacent areas has been reported to range from 0 to 40 mol% (Anderson et al. 1994), in this study the average value for the Western Atlantic isolates was considerably lower than the average values for the three North Sea population samples.

The potency of saxitoxin derivatives differs considerably – by over two orders of magnitude in mammalian bioassays (Oshima 1995a). When comparing relative toxicity of saxitoxin derivatives, the "average North Sea toxin profile" contains a slightly more toxic cocktail due to the higher molar toxicity of NEO (as determined in a mouse bioassays) than for the gonyautoxins (Oshima et al. 1992). However, with respect to toxicity of natural *A. tamarense* populations and the associated potential for shellfish toxicity in the different regions, these relatively small differences in molar toxicity might not crucially influence the magnitude of PSP outbreaks. The cellular content

of PSP toxins as a result of enhanced toxin production moreover might be a much more critical parameter. Differences in PSP toxin content are not only known to be dependent on ambient conditions, but also are found among different strains at equal ambient conditions and therefore reflect characteristic phenotypic traits of clonal lineages of *A. tamarense* (Anderson 1990).

Although PSP toxin profiles have been used as a phenotypic population marker, the ecological significance of differences in the PSP toxin composition is not understood, since knowledge of the function is lacking (Cembella 2003). Analysis of population subgroups from a large sub-structured population sample of *A. tamarense* gave some indication that PSP toxin content (but not toxin profiles) were under recent selection (Alpermann et al. 2009). The observed phenotypic differences among populations from different geographical origin still might be the consequence of a long history of natural selection upon lineages with certain PSP toxin profiles. Nevertheless, the eco-evolutionary significance of the expression of a certain toxin profile remains unresolved until a selective advantage can be shown. Neutral evolution of PSP phenotypes therefore cannot be excluded as a hypothesis for the development of phenotypic differences among geographically separated populations with a high degree of genetic differentiation.

Genotypic differentiation

The multivariate analysis of genotypic data conformed to the results obtained by the PSP toxin profiles as phenotypic marker, in that *Alexandrium tamarense* isolates from the Western Atlantic (Bay of Fundy) were grouped together, apart from individuals from the Eastern Atlantic (North Sea). Yet the inter-population genotypic variability was represented by the scattering of individuals over a relatively broad area of the coordinate space. In the case of isolates from the three North Sea populations the absence of strong genetic differentiation as detected by gene-frequency based measures (F_{ST} and genetic distance) was represented by the absence of distinct population clusters in the two dimensional display of PCoA results. However, the observation of isolates from the North Sea population samples NS6 and NS11 in only a subarea of the coordinate space that was occupied by individuals from station NS2 in the AFLP based analysis indicates that the former two population samples only shared parts of the genotypic variation of population NS2. In other words, a considerable proportion of individuals from population sample NS2 possessed AFLP genotypes that were quite different from those observed in all other population samples. In contrast to this, the similar distributional pattern of individuals of the three North Sea populations in the microsatellite based PCoA shows that similar microsatellite genotypes were found in all three populations. In general, the display of genotypes in the two dimensional coordinate space reflected the outcome of the analysis of genetic differentiation among populations, where only AFLP could detect significant genetic differentiation (although very weak) among two of the population samples from the North Sea.

Genetic structure and population assignment

In comparison with genotype-multivariate methods based on similarity indices, the Bayesian clustering approach in this study has the advantage that it provides a probability of assignment of each individual to either of the population clusters that were constructed in the analysis. The

clusters that were formed by the STRUCTURE 2.2 software uncontestedly conformed to the pattern of genetic differentiation of populations with transatlantic origin. In all STRUCTURE 2.2 analyses one cluster was almost exclusively composed of individuals from the Bay of Fundy population. In comparison with 462 biallelic AFLP loci, the analysis of 18 microsatellite loci lead to a sharper distinction between individuals (clones) of Western and Eastern Atlantic provenance. In this analysis, the advantage of AFLP over microsatellites by providing a much larger number of loci for subsequent analysis seems to be negatively compensated by its slow evolution. While AFLP seems to be generally more sensitive to population differentiation based on allele frequency shifts in recently differentiated populations, the acquisition of new alleles occurs far less frequently than in microsatellites due to considerably lower mutation rates (Mariette et al. 2002). The high number of private alleles for some of the microsatellite loci (Table 2.7.4) could be the consequence of mutation events after separation of populations from a common ancestral population. An extreme is locus ATB1 with one allele exclusive to the Bay of Fundy isolates and another one exclusive to all North Sea and all Hiroshima Bay isolates (with the exception of one North Sea strain). The exclusion of such discriminative markers from the data set would certainly result in drastically reduced differentiation capacity of the remaining set of markers. Relatively slower mutation rates in AFLP thus presumably contribute to a slight reduction of the capability of the marker to separately cluster Western and Eastern Atlantic populations.

A yet finer level of genetic differentiation among the North Sea populations, however, as could be indicated by the formation of clearly separated clusters exclusively consisting of individuals from any of the three North Sea populations, was not achieved by Bayesian clustering with either AFLP or microsatellites. Nevertheless, the general applicability of the Bayesian clustering approach to assign individuals probabilistically to different source populations becomes clear from our analyses. In the analysis of the AFLP data set the probabilities of Bay of Fundy genotypes to be assigned to the cluster that contained most of the genotypes from this population were very high, and a high proportion of the individuals from this population were assigned to the respective cluster. For the microsatellite data, a slightly better success of assignment of individuals to the comparative cluster was reached. Here, not only the average probability of assignment of individuals to the respective population cluster was higher, but also the assignment rate, i.e. more (in this case all) individuals from the Bay of Fundy populations were assigned unambiguously to the same population cluster. However, whether or not microsatellites in general are superior genotypic markers for Bayesian clustering approaches in haploid organisms such as dinoflagellates needs to be further tested.

When multilocus microsatellite genotypes from the population sample from Hiroshima Bay in the Eastern Pacific were added to the initial STRUCTURE 2.2 analysis with the same number of assumed population clusters, the overall pattern, i.e. the initial subclusters, were not changed to great extent. The separation of Western and Eastern Atlantic populations and the formation of almost mutually exclusive clusters were maintained. Moreover, all Japanese isolates fell into one population cluster that was more clearly separated from other clusters than those that exclusively were composed of individuals from different North Sea populations (as indicated by average assignment probabilities). The genetic differentiation of the population sample from Japan to those from Europe and North America were confirmed by significant F_{ST} values. The analysis of genetic

distance can therefore be interpreted as an indication of comparatively close genetic relatedness of Japanese and European populations. Results of the STRUCTURE 2.2 analysis of the same data, in which the number of populations was set to five, equaling the number of actual sampling locations, isolates from Hiroshima Bay still did not form a cluster by themselves. Owing to the signs of genetic differentiation (given by significant F_{ST} values for all pairwise comparisons of the Japanese population with others) we interpret these results such that a clear distinction of populations by the Bayesian clustering approach requires not only at least a moderate level of genetic differentiation, but also a substantial number of individuals from each population included in the analysis. We therefore assume that a sharper distinction between Japanese and North Sea isolates would have been possible if more individuals from Japanese waters were included. For the general applicability of the clustering approach to resolve questions of provenance of single or a few isolates, as might be available from ballast water or from locations were *A. tamarense* newly occurred, this would mean that unambiguous assignment of individuals (clones) will only be possible if the respective population of origin is represented by a large number of individuals. In any case, our results show that Bayesian clustering is adequate to successfully assign individuals to their presumptive population of origin with probabilities depending on the genetic differentiation of the populations included in the study, and is also capable of describing population structure on the different geographical scales investigated here.

Implications for studies on biogeography and dispersal history

Previous approaches (Scholin et al. 1994; Scholin and Anderson 1994) have helped to advance the understanding of phylogenetic relationships among strains from various regions displaying one or more morphotypes, but belonging to different ribotypes. Since morphotype and ribotype do not form the same groups, the hypothesis that ribotypes rather than morphotypes represent true "biological species" was formulated (Scholin et al. 1994). This generalized hypothesis of a speciation event as the consequence of geographical separation and genetic divergence of populations of the common ancestor of the *A. tamarense* species complex was later more explicitly expressed by John et al. (John et al. 2003), who suggested that the geographical origin of the North American clade of the *A. tamarense* species complex was located in the Eastern Pacific region, from which the species then could have spread to other coastal regions to finally reach its present day distribution. According to Lilly et al. (Lilly et al. 2007), the regions where the North American clade of the *A. tamarense* species complex (termed "*A. tamarense* Group I") presently occurs includes the western and eastern coasts of the temperate to sub-polar North Pacific, as well as the Western coast of the Northern Atlantic and Northern European waters . This ribotype was however also reported from the Pacific and the Atlantic coasts of temperate South America and from South Africa.

The nuclear sequence marker in these studies, the hypervariable region of the large subunit rDNA, did not allow the secure reconstruction of the dispersal history of the ribotype or to determine whether or not the current distribution is due to natural expansion or a consequence of human-mediated translocation. Certainly, more variable markers such as AFLP and microsatellites are needed to unravel the nature of these processes. Though our data are obtained from only a

limited number of populations and samples and therefore do not allow more refined population genetic analyses, the utility of both markers in studies concerning the biogeographical history of the NA clade of the *A. tamarense* species complex is evident. The results from our analyses suggest indeed a closer proximity for Japanese and European populations than for Western Atlantic populations to either of the two other regions.

If the NA clade had its origin in the Eastern Pacific close to the Eastern Pacific North American west coast, as previously speculated by Scholin et al. (1995) and John et al. (2003), natural dispersal and range extension of the species could have occurred via eastwards currents north of North America into the Atlantic ocean and via westward currents to the Western Pacific Asian coasts. In this case we would expect a closer genetic relationship among the North Sea and Western North Atlantic populations, which is in contrast to our findings. An alternative scenario of natural dispersal, which would better explain the degree of inter-population genetic differentiation found in this study, hypothesizes the origin of the ancestral *A. tamarense* population in the Western Pacific region. Long range dispersal via the Eastern Pacific coasts to the northern and eastern coasts of North America and via Northern Asian coastal waters to Europe could then have resulted in the observed intermediate position of the Japanese population sample in the analysis of inter-population genetic distance. However, these speculations do not help in answering a principal question: do the observed patterns in genetic relationships result from natural dispersal or are they due to human-mediated long range dispersal of regional genetic lineages (ribotypes). To conclusively answer such questions regarding natural dispersal routes of this species/clade over space and time, geographically intermediate populations need to be analysed to reveal whether or not the population genetic structure of globally dispersed *A. tamarense* is disjunct, as expected under a scenario of human-mediated dispersal.

Our analyses of individual genotypes identified two isolates from the North Sea, which always are clustered with the Bay of Fundy group. These two isolates may well be recent immigrants (or their descendants), introduced via human activity (e.g., from ballast water discharge) to the Scottish coast. While this is a legitimate speculation, there is no formal way to prove the real status of these two isolates. Another interpretation of these findings could be that the Bayesian methods applied here do not guarantee one hundred percent assignment success. In case of such an inconsistency these two individuals would represent outliers that were miss-assigned.

Conclusion

The results of the different analyses show that principally genotypic data from either of the two markers is suited to form consistent clusters for differentiated populations of *A. tamarense*. Results on inter-laboratory reproducibility of genotyping analyses (Jones et al. 1997) suggest that both genotypic markers, AFLP and microsatellites, are generally well suited for the creation of genotype data collections. Genotypic data that once was obtained by either of the two markers from any natural population, in principle can be used in future analyses, when more samples from other populations become available. A prerequisite would be that strictly the same protocol is followed (especially in the case of AFLP) and that suitable genotypic samples are at hand to be used as allele standards. By increasing the number of populations for which comparable genotypic data are

available, one could proceed substantially towards the aim of reconstructing the migration and dispersal history of the species. However, due to the broad dispersal of *A. tamarense* and other planktonic microbes such an approach has to cover the complete distributional range of the respective species and therefore must be based on a collaborative research scheme, including the maintenance of a growing genotypic population data base. Future studies on the evolutionary ecology and recent dispersal history of harmful algal species certainly would benefit from such collaborative efforts.

3. SYNTHESIS

3.1. INTER-SPECIES DIVERSITY IN *ALEXANDRIUM*

Species discrimination of potentially harmful algal species is an important issue in HAB detection, monitoring and forecast. Analysis of cryptic species diversity not recognisable by the hitherto utilised morphological descriptors, such as thecal plate patterns and gross morphological appearance or tendency for chain formation, proved not to resolve the evolutionary relationships among toxigenic and non-toxic ribotypes of the *A. tamarense* species complex (Scholin et al. 1994; Lilly et al. 2007) and other species complexes in the genus *Alexandrium* (Lilly et al. 2005). Nevertheless, adaptive processes can be assumed to underlie this cryptic diversity leading to differences in ecological characteristics of the different ribotypes. Inter-species genetic differences in the genus *Alexandrium*, as in other dinoflagellates, have mainly been investigated by analyses of ribosomal genes (e.g., SSU by Scholin and Anderson (1994), LSU by Scholin *et al.* (1994) and ITS by Adachi *et al.* (1994)). The hypervariable region of the LSU gene has proven best suited to reveal membership of individual isolates in one of the five ribotypes within the *A. tamarense* species complex (Scholin et al. 1994; John et al. 2003). The usefulness of this sequence marker as a first cut for distinction of evolutionary units within the species complex has also been shown in this study **(Publication 2, 3, 4, 6 and 7)**. With respect to the much debated question as to whether or not the different ribotypes of the species complex represent biological species (e.g., Scholin et al. 1994; John et al. 2003; Leaw et al. 2005; Lilly et al. 2007) additional molecular support was obtained in this thesis from other markers **(Publication 3)**. Although sequence markers other than ribosomal genes (e.g., other nuclear or mitochondrial genes) have not yet been applied in molecular phylogenetic analyses of the species complex, AFLP was used in a previous study for discrimination of clonal isolates from different ribotypes of the species complex (John et al. 2004). Whereas in this earlier study no good resolution of genetic relatedness for isolates from different ribotypes (i.e., putative species) was obtained, the present study showed that inter-species differences can be resolved by AFLP analyses **(Publication 3)**. The genetic divergence of the studied species or ribotypes (*A. tamarense* NA ribotype, *A. tamutum* and *A. ostenfeldii*) was assessed in this thesis by estimation of 'evolutionary genome divergence', a simple but apparently robust evolutionary model (Mougel et al. 2002). Taking up the conclusion of Leaw *et al.* (2005) that analyses of ribosomal genes have "probably reached a bottleneck" in molecular genetic analyses of species complexes, such as the *A. tamarense* species complex, this is obviously also true for the '*A. minutum* species complex' (Lilly et al. 2005). Given the limitations of ribosomal genetic analysis, AFLP might be the best suited additional phylogenetic marker, particularly in dinoflagellates because the haploid genome of vegetative stages enables the application of simple models to estimate evolutionary relationships. Nevertheless, the finding of seemingly higher rates of nucleotide substitutions and more unique loci in *A. tamutum*, when compared to the *A. tamarense*

NA ribotype, might be an artefact of the sampled population **(Publication 3)** and generalisations on the nature of evolutionary divergence only can be made by integrating samples from other populations under such circumstances. Comparative application of AFLP, a marker system assumed to cover the whole genome, in conjunction with sequence markers, however, will enable even more meaningful interpretation in inter-species studies than possible by the sole analyses of slow evolving rDNA genes. The application of the model adopted in this thesis is especially recommended in cases where molecular evolution of the ribosomal genes did not allow for discrimination of phenotypically differentiated species, such as *Scrippsiella hangoei* and *Peridinium aciculiferum* from the Baltic Sea and Northern temperate lakes, respectively (Logares et al. 2007). In such cases the approach followed in this thesis should allow for the definition of the evolutionary units (or "evolutionary species") even in absence of divergence of ribosomal genes.

In contrast to AFLP, the microsatellites established for the *A. tamarense* NA ribotype cannot be used for inter-species analyses in *Alexandrium*. All of the newly established loci failed to yield amplification products for other ribotypes within the species complex such as the *A. tamarense* Temperate Asian or Western European ribotypes and other *Alexandrium* species (**Publication 2)**. A considerable number of mutations in the flanking regions of microsatellite loci, containing the primer binding sites, can be assumed to be responsible for the failure of cross-amplification in other species. The fact that already other ribotypes within the species complex show these mutations therefore is in good congruence with the hypothesis of phylogenetic separation as supported by rDNA sequence analyses.

This thesis study yielded the following novel insights into variation and divergence within *Alexandrium*:

- the first application of AFLP in a phylogenetic study of dinoflagellates showed that this genome-wide distributed marker system was able to resolve inter-species differences;
- estimates of evolutionary genome divergence obtained for individuals (clones) and species clusters (morphospecies and ribotypes) opened the way to more detailed analyses of species evolution by providing a measure for the rate of nucleotide substitutions across the genome, and proved capable of defining the relevant biological units from an evolutionary perspective;
- the failure of cross-amplification of microsatellite loci confirmed the advanced genetic divergence of ribotypes within the *A. tamarense* species complex.

With respect to population growth and aptitude for bloom development, this thesis addressed the topic of phenotypic differentiation of species from the genus *Alexandrium* to achieve a general understanding of phenotypic differences among species and ribotypes and their evolutionary causes. At the inter-species level, phenotypic characters were investigated by detailed analyses of allelopathic potency towards different protistan target species, assumed to reflect specific production of lytic substances. Results on allelopathic potency of different *Alexandrium* species indicate that lytic properties are a common but not universal feature in the genus **(Publication 1)**. Potential competitors and grazers from different phylogenetic groups were all heavily affected by the more lytic *Alexandrium* strains. These findings, together with previous reports on the wide

distribution of allelopathic properties in *Alexandrium* (e.g., Tillmann and John (2002)) underscore the suggested importance of allelopathy in the ecology of bloom formation in dinoflagellates (Smayda 1997).

From an ecological point of view, it is interesting to compare the relative importance of the two allelochemical strategies proposed by Smayda (1997), i.e., allelochemically enhanced inter-species competition and allelopathic anti-predation defence. The present findings indicate that potential grazers such as ciliates and heterotrophic dinoflagellates may be heavily impacted by allelochemical properties **(Publication 1;** Tillmann and John 2002). Diatoms, as potential competitors of dinoflagellates for limiting nutrients or trace elements, have shown to be less sensitive to allelochemicals than flagellate grazers in laboratory analyses **(Publication 1),** as well as in mesocosm experiments with samples from natural plankton communities (Fistarol et al. 2004b). The explanation for the weakness of allelopathic effects on diatoms might be that *Alexandrium* and diatoms do not compete for the same ecological niches, and thus allelopathy did not evolve as a result of beneficial effects to *Alexandrium* by inhibiting diatoms. In fact, in habitats in which blooms of dinoflagellates such as *Alexandrium* occur, rapid population growth or bloom development of diatoms already has typically taken place earlier in the year (see e.g., Smayda 1980). The decline of diatom populations is rather related to limitation in essential macro- and micronutrients and grazing by herbivorous mesozooplankton such as copepods and their nauplii (see e.g., Margalef 1978; Smayda 1997). Allelopathic strategies alone seem obviously not suited to suppress growth of diatoms and results on target specificity suggest that they are not primarily directed towards this group.

Since neither the compounds nor the mode of action is known, the factors that determine the difference in allelopathic effect in different target organisms remain speculative. A direct effect on membrane function with consequences for cytoskeleton integrity is one of the possible explanations. Such membrane targeted mode of action has been proposed for allelochemical activity of karlotoxins from the mixotrophic dinoflagellate genus *Karlodinium* (Adolf et al. 2006; Adolf et al. 2007). The stabilisation of diatom cell structure by silica frustules could thus explain a weaker effect on this group. However, to make generalisations on the sensitivity of certain groups of marine protists, more species from different groups need to be tested.

What became evident from the comparison of allelopathic effects of different *Alexandrium* species on different target organisms was that exist both quantitative and qualitative differences in allelochemical potential **(Publication 1)**. A plausible explanation is that lytic effects in different *Alexandrium* species are mediated by different substances with differing effects. This might be achieved by synthesis of an allelochemical compound with a slightly modified chemical structure. As other dinoflagellate secondary metabolites (e.g., saxitoxins, spirolides and yessotoxins), allelochemicals of *Alexandrium* might be complex suites of substances, derived from a common chemical structure with subsequent modifications. The existence of a species- (or strain-) specific suite of derivatives of varying potencies would be a sound explanation for the observed qualitative differences, if different species express a certain suite of allelochemicals.

A potential role in mixotrophic food acquisition might be added to the list of proposed ecological functions for allelochemicals. Mixotrophy has been reported to occur in *Alexandrium* as in many other dinoflagellates (Nygaard and Tobiesen 1993; Jacobson and Anderson 1996; Jeong

et al. 2005). The finding that *Fragilidium*, well known for its mixotrophic mode of nutrition (Skovgaard 1996; Hansen and Nielsen 1997; Jeong et al. 1997), also displays previously unknown allelopathic properties **(Publication 1)** goes in hand with that supposition.

Over the evolutionary time scales in which this phenotypic trait has evolved, species-specific allelochemical properties certainly have undergone co-evolutionary processes. A "watery arms race" proposed to have shaped niches of phytoplankton taxa (Smetacek 2001) might have taken place by chemical warfare. Diversification of allelochemical "arms", driven by co-evolution then can be assumed to have played an important role in species divergence by imposing disruptive selection pressures. With respect to the paradigm that environmental variables such as nutrient availability, light and turbulence are the major forces that rule species diversity in phytoplankton communities (e.g., Margalef 1978), findings of broadly distributed allelopathic properties urge for a reflection on the general truth or completeness of traditional concepts. Specific adaptations that determine the allelochemical activity against certain competitors or grazers certainly add to a more complete description of ecological niches of phytoplankton organisms such as *Alexandrium tamarense*. These species-specific attributes will to a considerable extent add to the determination of which species of closely related dinoflagellate species will eventually dominate the phytoplankton, even if these species possess similar or almost identical niche requirements concerning environmental variables. The apparent *stochasticity* of selection of which species will dominate the plankton community numerically at a certain time (e.g., Smayda and Reynolds 2001) should be drastically reduced by incorporating descriptors of allelopathic characteristics in multi-species models of phytoplankton development.

The insights provided by this thesis work allow for the following general conclusions:

- allelopathy is a common if not ubiquitous principle in the genus *Alexandrium* because all species tested expressed allelopathic activity in combination with certain protistan target species;
- differences in effect strength on different target groups suggest that allelopathic properties are not primarily directed towards diatoms, but rather towards other microalgae and heterotrophic protists such as ciliates;
- qualitative differences in allelopathic potency support the idea that diversification of these phenotypic characters are the result of co-evolutionary processes.

3.2. INTRA-SPECIES DIVERSITY IN THE *A. TAMARENSE* NA RIBOTYPE

At this uppermost hierarchical level of intra-species diversity, the hypervariable region of the LSU rDNA already has been shown to be of only limited value for detection of genetic differentiation (Scholin et al. 1994; Medlin et al. 1998; John et al. 2003; Lilly et al. 2007). In this thesis, more variable molecular marker systems were shown to be appropriate to investigate genetic differentiation among populations of the globally distributed *A. tamarense* North American ribotype. The comparative analysis of populations from the different regions with AFLP and microsatellite markers – specifically established for this purpose **(Publication 3)** – showed that they were markedly genetically differentiated. Though a close evolutionary relationship of Eastern and Western Atlantic populations of this ribotypes has been hypothesised (Medlin et al. 1998), both markers support a closer genetic relationship of East Atlantic (specifically North Sea) and the Western Pacific population sample **(Publication 7)**. The previously hypothesised biogeographical route of dispersal of the genotype from the Pacific to the West and then to the East Atlantic (John et al. 2003), appears less likely given this new data. A more plausible biogeographic scenario now seems that natural dispersal of the ribotype to the Atlantic regions occurred on different routes from a common ancestral population in the Pacific via polar waters **(Publication 7)**. From the lower genetic distance and lower degree of differentiation, the dispersal from Pacific to the European coasts should have occurred in more recent times than that to the American East coast, perhaps only possible after the end of the last glaciation period. Molecular clock analyses of the genus *Alexandrium* and the ribotypes of the *A. tamarense* species complex estimate the species complex to have evolved between 23 to 45 million years ago (John et al. 2003). Taking this estimate, the dispersal from Western to Eastern North American coasts might already have taken place long before the closure of the Isthmus of Panama via the open Meso-american connection. While such biogeographic scenarios would explain better the intermediate position of the Japanese population from the Pacific, a final picture of the dispersal history of the NA ribotype in the Northern hemisphere cannot yet be drawn. One reason for this uncertainty is that the route of natural dispersal can only be followed if more intermediate populations are included in such analyses. Another reason is that the influence of human activities in the creation of the recent distribution patterns of the ribotype has to be carefully examined. This issue of human mediated dispersal of HAB species has already received much attention and awareness because the need for mitigation of bloom events and investigation of mechanisms and processes that govern bloom formation is still growing.

Population assignment as performed in this study **(Publication 7)** proved to be a promising tool for the analysis of human-mediated spreading, by providing the means for assigning isolates from novel bloom populations to long established populations of bloom sites with long time records. By such approaches, 'novel' populations of *A. tamarense* NA might either be identified as new to a region or as a well established and differentiated (but earlier unrecognised), hidden component of the local community. Such information in turn allows for refined understanding of factors governing bloom development, e.g., under scenarios of environmental change. Furthermore,

and of equal importance, the Bayesian procedure allows identifying source populations of recent immigrants within population samples from established populations. By this latter possibility, the novel probabilistic approach of the Bayesian procedure proved superior to conventional multivariate ordination techniques or standard measures of genetic population differentiation. With respect to the two population genetic marker systems in this study microsatellites and AFLP performed almost equally well in Bayesian population assignment **(Publication 7)**. The high number of unique population- or region-specific alleles at microsatellite loci, however, seems to be responsible for the slight analytical advantages observed for this marker system. The relatively higher average mutation rate of microsatellite loci compared to AFLP is likely the cause for this observation. Whereas within the North Sea region, AFLP, as applied in this study, even was able to detect weak genetic differentiation between the two geographically most distant populations, microsatellites were not. Gene flow among sampled populations within the North Sea seems to be high enough to distribute region-specific microsatellite alleles, acquired by new mutations, among the populations within the region. However, with respect to the global perspective of the issue of spreading of HAB species, here specifically studied with the *A. tamarense* NA ribotype, another important feature suggests that microsatellites are the genotypically superior marker in collaborative investigations involving experts in several laboratories. While the reproducibility of AFLP and microsatellites seems to be equally high when performed in different laboratories under strict consideration of optimised experimental protocols (Jones et al. 1997), recent developments in experimental procedures suggest that microsatellites will become the marker of choice in future studies of many single-celled plankton organisms. Although a relatively large amount of genomic DNA is necessary for AFLP analyses, restricting its application to cultured specimen, single-cell PCR techniques opens up the possibility of microsatellite genotyping from fresh and even preserved single cells (Henrichs et al. 2008). Simultaneous "multiplexed" amplification of multiple microsatellite loci by single-cell PCR would not only tremendously reduce culture work for population genetic studies of dinoflagellates, but also would open the avenue for studies on those species that are difficult to grow or up to now uncultivable.

The finding of genetic differentiation among *A. tamarense* populations within relatively short distance in the North Sea region is not unexpected, since the existence of prominent inter-regional population structure was recently reported from an extensive survey of *A. tamarense* NA ribotype populations in Japanese waters (Nagai et al. 2007a).

With respect to the hypothesis that "everything is everywhere – the environment selects" (Finlay and Fenchel 2004; Fenchel 2005), this thesis and other recent studies explicitly show that distinct biogeographies exist for planktonic dinoflagellates **(Publication 7;** Nagai et al. 2007a; Nagai et al. 2009). There is clear evidence for restrictions in the dispersal of microalgal species. In *Alexandrium* such constraints that determine long range dispersal patterns include the requirement for suitable cyst bed habitats to establish populations over inter-annual cycles. Such cyst beds coupled with advective or human-mediated dispersion can serve as "stepping stones" for natural biogeographic range extension. The connectivity of established cyst bed populations is therefore determined by dispersal of the planktonic fraction of the population primarily by oceanographic factors.

In this thesis biallelic AFLP markers and the set of highly polymorphic microsatellite markers developed from tandemly repeated sequences enriched in a genomic sequence library successfully established the following:

- these new markers are valuable tools for population genetic analyses, as they proved suitable for detection of inter-population genetic differentiation and genotypic diversity;
- genetic differentiation among populations of the globally dispersed North American ribotype enabled a re-defined scenario of natural dispersal of the North American ribotype of the *A. tamarense* species complex;
- Bayesian assignment of clonal lineages to respective source populations showed that the majority of genotypes could be distinguished by their geographic origin, thereby demonstrating a promising tool for identification of human-mediated dispersal of *A. tamarense* and other HAB species;
- both markers, microsatellites and AFLP, perform well at the level of inter-population analyses in *A. tamarense*. Microsatellites, however, seem to have the higher potential for application in larger scale studies, as technical advances might enable genotyping from single cells.

Genetic differentiation at the inter-population level within a species raises the question of whether or not the observed differences in neutral genetic markers are also reflected in functional or physiological differentiation of populations, as an adaptive response to natural selection. In this study, PSP toxins were one of the chosen phenotypic population markers since the strain-specific expression of a certain toxin profile has been reported to be rather stable under a range of experimental conditions (see e.g., Anderson (1990) and references therein). The phenotypic analysis of multiple isolates from the same populations confirmed that intra-species variation may be easily over-looked when analysing only single or a few clonal isolates – a fact that in many past studies has been neglected. Although considerable inter-population variation in toxin profiles was observed, the regional (i.e. inter-populational) differences were highly statistically significant **(Publication 7)**. This finding gives additional support to the hypothesis of long term separation of populations, based on genetic analyses. Furthermore, these results confirm that a higher potential for phenotypic differentiation at the population level can be expected for those populations with lower levels of gene flow among them. The use of PSP toxin profiles as a biochemical phenotypic population marker was herewith validated at least for genetically well differentiated populations such as those from both sides of the northern Atlantic Ocean. However, an ecological meaningful explanation for the source of this inter-population differentiation is not readily at hand. Whereas grazer deterrence or inhibition of competitors has been commonly accepted as an ecological explanation for allelopathic properties (e.g., Smayda 1997; Cembella 2003; Legrand et al. 2003; Tillmann 2004), a general ecological explanation for expression of a PSP toxin phenotype is lacking and experimentally observed effects appear highly dependent on certain combinations of species or strains (Cembella 2003). Whether or not inter-population differences in PSP toxin phenotypes evolved due to selective advantage cannot be answered without resolving the question of the significance of this phenotypic character. The stability of PSP toxin profiles (i.e. the proportional

composition, characteristic for a certain strain), however, make it a useful chemotaxonomic marker at the inter-population level (Cembella et al. 1987; Anderson et al. 1994; Cembella and Destombe 1996).

With respect to toxin profiles and phenotypic differentiation, in this thesis the following general conclusions can be drawn:

- genetically differentiated population from the Western and Eastern North Atlantic were highly significantly differentiated with respect to PSP toxin profiles, giving support for long time separation of populations;
- although the use of PSP toxin profiles as a population marker was generally validated, the specific mode of population differentiation (adaptive vs. neutral) and ecological significance remains unclear.

3.3. INTRA-POPULATION DIVERSITY IN THE *A. TAMARENSE* NA RIBOTYPE

Several studies in this thesis confirmed that genotypic diversity within the natural populations sampled was extremely high because no genotype was repeatedly sampled **(Publication 2, 3, 4, 6 and 7)**. The broad genotypic diversity found in all populations under study at the intra-population level is remarkable because most studies of genotypic diversity in microalgae report repeated sampling of genotypes (e.g., Shankle et al. 2004; Rynearson and Armbrust 2005; Nagai et al. 2007a). Resolution of clonal identity by genotypic markers, however, largely depends on the differentiating capacity of the utilised marker system, and not all genotypic markers adequately differentiate among distinct but similar clonal lineages. A sufficiently large set of polymorphic loci as applied in this thesis, therefore, is an indispensible prerequisite for studies aiming at detection of clonal diversity at the population level. This current work indicates that underestimation of clonal diversity in marine phytoplankton has often likely occurred in previous studies because of the selection of inappropriate genotypic markers, thereby creating a distorted picture of intra-species and intra-population diversity. As a consequence, this has led to fundamental underestimations and perhaps misinterpretations of selective processes during vegetative growth of clonal lineages. Such selective processes, although very likely an inherent feature of microalgal populations, can never be consequently followed in any natural population of marine phytoplankton by assessment of repeatedly sampled genotypes. This interpretation is evident from the high clonal diversity found in this study **(Publication 4, 6 and 7**), as well as from previous work (e.g., (Iglesias-Rodríguez et al. 2006; Nagai et al. 2007a) involving large enough sets of polymorphic markers.

In this thesis, the utility of different genotypic marker systems was explicitly demonstrated with a high number of genotyped clonal isolates and numerical simulations with the obtained genotypic data. Both the set of microsatellite and of AFLP loci used in this study proved to be sufficiently suited to discriminate among different clonal lineages, even in the largest population sample from the North Sea **(Publication 4 and 6)**. With respect to ordination of clonal isolates by genotypic similarity, the set of AFLP loci performed better than the smaller number of microsatellite loci. While the resulting pattern of genotypic similarity as obtained by AFLP can be expected to have already stabilised, the pattern resulting from microsatellite based pairwise similarity certainly would change after inclusion of other microsatellite loci. Detailed analyses with sub-sampled sets of the two marker systems, however, indicate that with equal numbers of loci, multiallelic microsatellites perform much better than biallelic AFLP **(Publication 4)**. The experimental ease of generation of dozens or hundreds of AFLP loci explains why this marker system, in spite of its limiting dominant nature, is used for the assessment of genetic diversity, even in diploid and polyploid species or agriculturally relevant breeds and cultivars. In haploid organisms such as dinoflagellates the limitations in interpretation of dominant markers are not apparent as the haplotypic state can be directly inferred. Microsatellite loci, however, lose half their information content for haploid organisms when compared to diploids because only one of the two alleles is assessed per locus.

With respect to the utility of genotypic markers at the intra-population level in *A. tamarense* the following can be concluded:

- genotypic diversity within the large population sample under study was extremely high, as evidenced by no repeated detection of individual genotypes;
- underestimation of clonal diversity by inadequate genotyping approaches most likely has occurred in many past studies on planktonic microalgae, generating a distorted picture of real clonal diversity in large microalgae populations;
- the larger set of biallelic AFLP loci resulted in more stable ordination of clonal isolates by genotypic differences, though multiallelic microsatellites showed a higher power to generate stable patterns at equal numbers of loci.

The broad genotypic diversity found in *A. tamarense* populations was accompanied by an equally broad phenotypic diversity at the intra-population level **(Publication 4 and 6)**. Whereas diversity of PSP toxin profiles already was known at the intra-population level, the population-wide assessment of the allelopathic phenotype provides novel and unique insights into the nature of this important phenotypic trait. Allelopathic traits have never before been surveyed for intra-population distribution for any dinoflagellate or indeed within any other microalgal species. All the quantitative phenotypic characters that were representatively assessed at the population level were normally distributed, as is characteristic for quantitative genetic traits. The expression of these phenotypic characters is therefore likely the result of the interaction of allelic variants at multiple gene loci. Attempts to approach the genetic nature of the quantitative differences were followed by analysis of global correlations of genotypic similarities and patterns **(Publication 4)**. A global phenotype-genotype correlation was not found and could only be expected under certain circumstances, e.g., after admixture of populations that were differentiated for both, phenotypic and genotypic, characteristics. The joint analysis of genotypic characters in North American and North Sea populations, as presented in **Publication 4**, probably would have revealed such a global correlation. However, a global correlation of this kind would not have helped in the detection of causative genetic elements for phenotype expression, since the majority of loci responsible for genetic population differentiation must be assumed to be neutral with respect to phenotype expression (Pritchard et al. 2000b; Sham 2001; Rosenberg and Nordborg 2006).

The analysis of associations of individual loci with phenotype expression is a widely used approach in detection of genomic regions with functionally important genetic elements (see e.g., Slate 2005). Though association studies are widely utilised in the study of gene defects in humans and natural populations of other organisms (see e.g., Pritchard et al. 2000b; Flint-Garcia et al. 2003; Erickson et al. 2004), the approach followed here is the first association study in a marine microalga. Association testing in this study, however, did not result in detection of phenotype-associated AFLP loci **(Publication 4)**. Improvement of association testing might be obtained by inclusion of more individuals, by enhancing the precision of phenotype determination or by generation of experimental populations by crossing of phenotypically divergent clonal lineages. The general assumptions underlying association testing that certain genes are causative for the observed phenotypic differences are supported by the long time stability of the phenotypic characters chosen

for analysis. In the case of allelopathic properties, this was shown by highly significant correlations obtained by quantitative analyses of effects towards *Oxyrrhis marina*, with more than one year between the two series of experiments **(Publication 4 and 5)**. In the subsample of the large population from the North Sea, in which detailed analyses of allelopathic properties were preformed, no qualitative differences in the effect towards target organisms were observed **(Publication 5)**. In contrast to the results at the inter-species level, where such qualitative differences among species can be explained by differences in the expression of specific allelochemical substances **(Publication 1)**, the failure to detect such qualitative differences **(Publication 4 and 5)** may indicate that no disruptive or diversifying selection is presently shaping the population under study. The finding that just two of 67 tested isolates did not display allelopathic properties is also remarkable. The complete absence of allelopathic properties in *A. tamarense* NA populations must be seen as a rare exception rather than as the rule. Studies on multiple isolates of *A. tamutum* (Tillmann et al., unpublished) indicate that this conclusion might be generally true for other *Alexandrium* species. The absence of obvious metabolic costs such as reduction of growth under nutrient-replete conditions **(Publication 5)** may partly explain why the expression of the allelopathic phenotype is not more strongly selected against in the population. In theory, negative selection against the allelopathic phenotype should be stronger when allelopathic properties do not provide an advantage for growth or survival, e.g. against competitors or predators. On the other hand, the group selective benefit of expression of lytic effects by conspecifics within the same population can also be considerable for weakly allelopathic active clonal lineages, resulting in a broad distribution of that character as observed in the studied population.

The idea of a group-selective advantage of allelopathic properties, perhaps mediated in conjunction with the formation of a phycosphere associated with concentrated bioactive metabolites, is a possible explanation for survival of members of clonal lineages with low allelopathic potencies. Expression of high allelopathic potency by the majority of the population and formation of an enhanced phycosphere as a result of active cell migration and aggregation behaviour might be a combined mechanism for creation of such a group-selective effect. Dense thin-layers of cells of *A. fundyense* (Townsend et al. 2005b) and *A. catenella* (Sullivan et al. in press) – both members of the *A. tamarense* species complex – have been observed in coastal waters. Modification of the chemical micro-environment by exudation of algal secondary metabolites can be expected to occur in such thin layers and expression of allelopathic properties by the majority of the population might benefit the population as a whole.

Several novel aspects of evolutionarily relevant phenotypic traits of *A. tamarense* were elaborated at the intra-population level:

- an enormous phenotypic diversity exists within a single population for all phenotypic characters under study – cellular PSP toxin content, toxin profiles and allelopathic potency;
- global phenotype-genotype correlations, as demonstrated by joint analysis of AFLP loci and phenotypic markers, cannot be expected unless admixture of highly differentiated populations has occurred;
- the absence of association of AFLP loci with phenotypic characters might be explained by

the large genome size characteristic of dinoflagellates (including *A. tamarense*) and consequently low genome coverage;

- the stability of the allelopathic potency in multiple *A. tamarense* isolates suggests a strong genetic component in the expression of this phenotypic character;
- the absence of allelopathic properties in only very few isolates indicates that this character is important for the survival and selection of clonal lineages, and that this phenotypic trait may play a crucial role in population growth and bloom development;
- formation of phycospheres around individual cells and within thin-layers of aggregated cells at certain water depths by active migratory behaviour may enhance the effect of allelochemical exudates and even provide a group-selective advantage.

Allelopathy may play an important selective role only at certain stages of population development. In this case, highly allelopathic phenotypes might be out-performed by conspecifics with lower allelopathic activity but superior adaptation via other phenotypic traits (e.g. nutrient uptake, feeding strategies, intrinsic growth rate, etc.) thereby favouring higher rates of vegetative reproduction or survival. The existence of such differences in the net growth of clonal lineages has been concluded in this study **(Publication 6)** from multilocus linkage disequilibrium, a genetic peculiarity, indicative of clonal reproduction (Halkett et al. 2005). The conceptual model developed herein **(Publication 6)** is capable of integrating all population genetic peculiarities such as population sub-structure and population-wide LD, and allows for formulating further hypotheses on the nature of the development of populations of species. While some proportion of population wide LD could be attributed to the existence of population substructure, which also became apparent from the AFLP based analysis of population structure, the residual LD in the four population subgroups indicated vegetative growth and clonal over-representation **(Publication 6)**. In contrast to studies that approached the assessment of effects clonality by sampling repeated genotypes, the application of standard models of population genetics in this thesis has proven suitable in assessing the extent of clonal over-representation and yielded a better understanding of its population genetic consequences. Despite the remaining uncertainties respective to the evolutionary forces causing genetic differentiation of population subgroups, a plausible explanation for the existence of the observed population structure was provided by linking genetic factors to life history peculiarities of meroplanktonic dinoflagellates **(Publication 6)**.

As the population genetic model presented here might be applicable to meroplanktonic dinoflagellates in general, similar observations on the development of populations of other HAB dinoflagellates with similar life cycles (especially those from the genus *Alexandrium*) can be expected. The maintenance of broad genetic diversity in spite of annually occurring planktonic population differentiation is especially important in dinoflagellates, since selective effects on haploid stages can not be balanced by the existence of an alternate allelic variant as is the case in diploids. Selection therefore can be expected to lead to sharper differentiation of annual planktonic populations in haploid organisms such as dinoflagellates, when compared to diploid microalgae such as diatoms. The maintenance of genetically diverse benthic cyst beds seems a remarkable

strategy able to explain the long term evolutionary success of meroplanktonic dinoflagellates, which becomes evident by the bloom formation capabilities of many dinoflagellate genera.

Even as standard population genetic models have to be adapted to specifics of the organisms studied, they also apply to planktonic dinoflagellates, as can be seen by findings of "isolation by distance (Nagai et al. 2007a), genetic "bottle necks" (Masseret et al. 2009) and population-wide linkage disequilibrium as a result of clonal over-representation **(Publication 7)**. Though meroplanktonic dinoflagellates show intriguing population genetic peculiarities, the study of their evolutionary ecology provides general insights into evolutionary processes in planktonic microalgae.

In this thesis, population genetic data are related to biological characteristics of *A. tamarense* to enhance the current understanding of the evolutionary ecology of the species at the intra-population level:

- population genetic peculiarities found for the *A. tamarense* NA ribotype can be explained by a conceptual population genetic model. Besides genetic factors this model integrates life cycle characteristics such as vegetative population growth and annual formation of sexual resting cysts;
- the population genetic model can only explain the observations and data sets if the conclusion of high level of genetic and phenotypic differentiation of planktonic populations is valid. The cause of recurring population differentiation is most likely due to clonal selection on phenotypically better-adapted clonal lineages;
- the model predicts that a vast genetic reservoir is retained in the benthic cyst beds, enabling the population to maintain genetic and phenotypic diversity in the long run.

3.4. CONCLUSIONS

Variability in phenotypic traits and genotypic variation was investigated among closely related species of the dinoflagellate genus *Alexandrium* and within and among natural populations of the species, *A. tamarense*, with a view towards understanding the significance of variation in accounting for the ecological success and evolution of microalgal species. The potential for strain differences was thereby explicitly acknowledged in formulating generalisations and conclusions about species-specific phenotypic characteristics. Questions regarding phenotypic variation at the population level in dinoflagellates have only been addressed in a few other studies. In this thesis the interpretation of phenotypic data, such as toxin profiles in *Alexandrium*, was shown to greatly depend on the capacity to adequately represent the extent of phenotypic variation within a population. By studying a large number of isolates from different *Alexandrium* species, and from naturally co-occurring or geographically separated populations of the globally dispersed *A.*

tamarense North American ribotype, both genotypic and phenotypic variation was addressed at different levels of biological organisation.

The results indicate that phenotypic traits, such as toxin production and expression of allelochemical properties against competitors or grazers, are under directional selection if the prevailing environmental conditions favour the growth and reproduction of those individuals that display the better adapted phenotypes. This leads to selective shifts in the frequency of individuals from different clonal lineages. The current study of variation in phenotypic traits contributes to a better understanding of the interaction among selective processes, such as directional or balancing selection, which can promote the fixation of a certain phenotype for a particular trait. The findings of broad phenotypic variation within the natural *Alexandrium* populations indicate the absence of strong selective pressure on any of the phenotypic traits studied.

Neutral genetic markers, specifically AFLP, did not yield insights into the relation of functional genetic elements and particular phenotypic traits. The analysis of neutral markers, however, allowed assessment of genetic variation among related species or among natural populations of the same species by displaying either genotypic uniformity or diversity. At the inter-species level, AFLP markers proved capable of revealing the degree of genome divergence among closely related species and can be expected to yield more detailed information with respect to the evolution of species complexes within the genus *Alexandrium*. Under the assumption that high genetic diversity reflects the adaptive capacity of a species, the dinoflagellate populations under study seem well prepared to withstand frequent environmental change and accompanying selective pressures. Results on neutral genetic diversity showed a high potential for maintenance of phenotypic diversity by genetic reorganisation through sexual reproduction and existence of a genetic reservoir represented by the fraction of the dinoflagellate population present in benthic cyst beds. Considered in the context of a new conceptual model for population genetics of *Alexandrium*, this thesis contains the first published work considering the consequences of clonality in microalgae by population genetic methods and model development. Finally, neutral genetic markers enabled the determination of inter-population differentiation of geographically separated populations and provided support for hypotheses concerning the dispersal of populations on a global scale integrated with novel findings on the relationships of regionally defined populations.

3.5. PERSPECTIVES FOR FUTURE RESEARCH

To further enhance our understanding of HAB development and to determine the causes and effects of *apparent* and/or *actual* spreading of HABs requires assessing the inherent diversity and biogeography of the implicated taxa at different levels of biological organisation. Most importantly, we have to clarify which biological entities are in fact the evolutionarily significant evolving units. In the case of the *Alexandrium tamarense* species complex, which apparently includes several genetically differentiated biological species, markers other than the conventional morphological characters have to be used for species discrimination in an ecologically and evolutionarily meaningful way. Application of AFLP in a phylogenetic context can provide additional support for molecular phylogenies that are usually based on sequences of single genes (e.g., LSU and SSU rDNA, ITS, etc.). Additionally, crossing experiments for the assessment of mating compatibility with a focus on ribotypes, not morphospecies, can also provide a more detailed understanding of species boundaries.

Strategies for avoidance of future introductions of HAB species and mitigation of their effects must include considerations of possible routes and means of translocation of potentially harmful species. Therefore, recent natural- and human-assisted dispersal must be clearly distinguished from long existing but cryptic local populations of harmful species. By increasing the number of populations for which comparable genotypic data is available, substantial progress could be made towards reconstructing the migration and dispersal history of the North American ribotype of the *A. tamarense* species complex. However, due to the broad dispersal of *A. tamarense* and other planktonic microbes such an approach has to cover the complete distributional area of the respective species, and therefore must be based on a collaborative research scheme, including the maintenance of a growing base of genotypic population data. Future studies on the evolutionary biology and recent dispersal history of harmful algal species certainly would benefit from such collaborative efforts.

Although the studies published in this thesis contribute to a better understanding of the population-wide distribution and existence of qualitative differences in allelopathic potency, several aspects concerning allelopathy in *Alexandrium* remain to be elucidated. Very importantly, the chemical nature of the active compounds needs to be characterised. Structural elucidation would greatly help in unravelling the mode of action on different groups of target organisms. Such analyses that lead to a more complete picture of the sensitivity of different target species will result in a better understanding of the co-evolutionary forces and the ecological advantages that might drive evolution of allelochemical properties in *Alexandrium*. Generally such analyses will benefit from investigating allelochemical effects in a larger number of co-occurring marine protists from selected clades, thereby allowing for generalisations of effects on the different taxa due to better representation of each. Besides the variation in allelopathic potency of the donor species (as in this study analysed by inclusion of multiple isolates of *Alexandrium*), the variation in sensitivity of isolates from certain target taxa should also receive some attention. The hypothesis that the ecological significance of allelochemical properties is related to mixotrophic nutrition of

Alexandrium should be tested in further studies. A step from controlled laboratory experiments, such as the test assays applied in this study, to more natural experimental conditions is also needed. For this purpose, mesocosm experiments with the well characterised clonal isolates are currently performed and analysed with the aim to better understand the ecological significance of the allelopathic phenotype in *Alexandrium*. Here, specific microsatellite markers that were developed and applied in this thesis can be applied in determining frequency shifts of different – allelochemically active and non-active – *A. tamarense* isolates in experimental plankton communities of different initial species composition. Enumeration of single, pre-characterised clones is performed in multiplex single cell PCR microsatellite assays and by estimation of amplification product ratios of single microsatellite alleles specific to the respective isolates from bulk plankton DNA extractions. Studies focussing on the role of allelochemicals at different stages of bloom formation (e.g., in mesocosms or natural populations over longer time periods) will further increase our knowledge and understanding of the mechanisms of bloom formation in toxic dinoflagellates. A further step to determine allelopathic activity in natural or experimental populations is the development of specific genetic markers. While pre-characterised isolates can be identified by e.g., single cell PCR for detection of their specific multilocus microsatellite genotypes, the identification of functionally important genes with relevance to the allelopathic genotype could enable estimation of allelochemical properties in previously uncharacterised isolates from natural populations. Such markers could be identified by association analysis or similar approaches making use of experimental populations obtained by crossing strains with reciprocal phenotypic traits. Molecular approaches for unravelling the genetic basis of phenotypic expression in *Alexandrium* are also possible by simultaneous screening of large numbers of expressed genes, such as expressed sequence tag (EST)-based DNA microarrays. Such future studies will shed more light on the evolution of these traits in *Alexandrium*, as well as in other toxigenic eukaryotic microalgae responsible for harmful algal blooms.

The development of a conceptual population genetic model that is capable of explaining the observed population genetic peculiarities (multilocus LD and population substructure) in this thesis provides the basis for further investigations in this direction. The present model should be further validated by analysing the genetic structure of cyst beds of *Alexandrium* populations from different regions. Analysis on population substructure could confirm the postulated hypothesis that cyst beds contain differentiated cohorts of sexually formed hypnozygotes from different years. Another approach to confirm the formation of annually differentiated cyst cohorts would be to directly follow the deposition of differentiated hypnozygotes in consecutive years. Coupling of such genetic analysis with the study of phenotypic markers as in the present study has the potential to further elucidate the ecological significance of phenotypic characters presumed to mediate adaptive clonal shifts in natural plankton populations.

4. REFERENCES

Adachi, M., Sako, Y., Ishida, Y. (1994) Restriction fragment length polymorphism of ribosomal DNA internal transcribed spacer and 5.8S regions in Japanese *Alexandrium* species (Dinophyceae). *Journal of Phycology*, 30, 857–863.

Adachi, M., Sako, Y., 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, 424–432.

Adachi, M., Sako, Y., Ishida, Y. (1997) Analysis of *Gymnodinium catenatum* (Dinophyceae) using sequences of the 5.8S rDNA-ITS regions and random amplified polymorphic DNA. *Fisheries Science*, 63, 701–707.

Adachi, M., Sako, Y., Ishida, Y., Anderson, D.M., Reguera, B. (1993) Cross-reactivity of five monoclonal antibodies to various isolates of *Alexandrium* as determined by an indirect immunofluorescence method. *Nippon Suisan Gakkaishi*, 59, 1807.

Adolf, J.E., Bachvaroff, T.R., Krupatkina, D.N., Nonogaki, H., Brown, P.J.P., Lewitus, A.J., Harvey, H.R., Place, A.R. (2006) Species specificity and potential roles of *Karlodinium micrum* toxin. *African Journal of Marine Science*, 28, 415–419.

Adolf, J.E., Krupatkina, D., Bachvaroff, T., Place, A.R. (2007) Karlotoxin mediates grazing by *Oxyrrhis marina* on strains of *Karlodinium veneficum*. *Harmful Algae*, 6, 400–412.

Alam, M.I., Hsu, C.P., Shimizu, Y. (1979) Comparison of toxins in three isolates of *Gonyaulax tamarensis* (Dinophyceae). *Journal of Phycology*, 15, 106–110.

Alpermann, T.J., Beszteri, B., John, U., Tillmann, U., Cembella, A.D. (2009) Implications of life history transitions on the population genetic structure of the toxigenic marine dinoflagellate *Alexandrium tamarense*. *Molecular Ecology*, 18, 2122–2133.

Alpermann, T.J., Beszteri, B., Tillmann, U., Cembella, A.D., John, U. (2008) Species discrimination in the genus *Alexandrium* by amplified fragment length polymorphism In: Moestrup, Ø., et al. [Eds.] *Proceedings of the 12th International Conference on Harmful Algae*. International Society for the Study of Harmful Algae and Intergovernmental Oceanographic Commission of UNESCO, Copenhagen, pp. 51–54.

Alpermann, T.J., John, U., Medlin, L.K., Edwards, K.J., Hayes, P.K., Evans, K.M. (2006a) Six new microsatellite markers for the toxic marine dinoflagellate *Alexandrium tamarense*. *Molecular Ecology Notes*, 6, 1057–1059.

Alpermann, T.J., John, U., Tillmann, U., Evans, K.M., Nagai, S., Anderson, D.M., Cembella, A.D. (2006b) Genetic differentiation and phenotypic characteristics of geographically separated populations of the *Alexandrium tamarense* North American ribotype. In: *Book of Abstracts, 12th International Conference on Harmful Algae, Copenhagen, Denmark, 4–8 September 2006*. International Society for the Study of Harmful Algal Blooms, pp. 53–54.

Alpermann, T.J., Tillmann, U., Beszteri, B., Cembella, A.D., John, U. (in press) Phenotypic variation and genotypic diversity in a planktonic population of the toxigenic marine dinoflagellate *Alexandrium tamarense*. *Journal of Phycology*.

Althoff, D.M., Gitzendanner, M.A., Segraves, K.A. (2007) The utility of amplified fragment length polymorphisms in phylogenetics: A comparison of homology within and between genomes. *Systematic Biology*, 56, 477–484.

Amato, A., Montresor, M. (2008) Morphology, phylogeny, and sexual cycle of Pseudo-nitzschia mannii sp nov (Bacillariophyceae): a pseudo-cryptic species within the P-pseudodelicatissima complex. *Phycologia*, 47, 487–497.

Anderson, D.M. (1989) Toxic algal blooms: A global perspective. In: Okaichi, T., Anderson, D.M., Nemoto, T. [Eds.] *Red tides: Biology, environmental science, and toxicology. Proceedings of the 1st International Symposium on Red Tides*. Elsevier, New York, NY, pp. 11–16.

Anderson, D.M. (1990) Toxin variability in *Alexandrium* species. In: Granéli, E., Sundström, B., Edler, L., Anderson, D.M. [Eds.] *Toxic Marine Phytoplankton*. Elsevier, New York, pp. 41–51.

Anderson, D.M. (1998) Physiology and bloom dynamics of toxic *Alexandrium* species, with emphasis on life cycle transitions. In: Anderson, D.M., Cembella, A.D., Hallegraeff, G.M. [Eds.] *Physiological Ecology of Harmful Algal Blooms*. Springer, Berlin, Heidelberg, New York, pp. 29–48.

Anderson, D.M., Cheng, T.P.-O. (1988) Intracellular localization of saxitoxins in the dinoflagellate *Gonyaulax tamarensis*. *Journal of Phycology*, 24, 17–22.

Anderson, D.M., Kulis, D.M., Binder, B.J. (1984) Sexuality and cyst formation in the dinoflagellate *Gonyaulax tamarensis*: Cyst yield in batch cultures. *Journal of Phycology*, 20, 418–425.

Anderson, D.M., Kulis, D.M., Doucette, G.J., Gallagher, J.C., Balech, E. (1994) Biogeography of toxic dinoflagellates in the genus *Alexandrium* from the northeastern United States and Canada. *Marine Biology*, 120, 467–478.

Anderson, D.M., Kulis, D.M., Sullivan, J.J., Hall, S. (1990a) Toxin composition variations in one isolate of the dinoflagellate *Alexandrium fundyense*. *Toxicon*, 28, 885–893.

Anderson, D.M., Kulis, D.M., Sullivan, J.J., Hall, S., Lee, C. (1990b) Dynamics and physiology of saxitoxin production by the dinoflagellates *Alexandrium* spp. *Marine Biology*, 104, 511–524.

Anderson, D.M., Lindquist, N.L. (1985) Time-course measurements of phosphorus depletion and cyst formation in the dinoflagellate *Gonyaulax tamarensis* Lebour. *Journal of Experimental Marine Biology and Ecology*, 86, 1–13.

Anderson, D.M., Morel, F.M.M. (1979) Toxic dinoflagellate blooms in the Cape Cod region of Massachusetts. In: Taylor, D.L., Seliger, H.H. [Eds.] *Toxic Dinoflagellate Blooms, Proceedings of the Second International Conference on Toxic Dinoflagellate Blooms*. Elsevier, New York, pp. 145–150.

Anderson, D.M., Rengefors, K. (2006) Community assembly and seasonal succession of marine dinoflagellates in a temperate estuary: The importance of life cycle events. *Limnology and Oceanography*, 51, 860–873.

Anderson, D.M., Stolzenbach, K.D. (1985) Selective retention of two dinoflagellates in a well-mixed coastal embayment: the importance of diel migration and surface avoidance. *Marine Ecology – Progress Series*, 25, 39–50.

Anderson, D.M., Wall, D. (1978) Potential importance of benthic cysts of *Gonyaulax tamarensis* and *G. excavata* in initiating toxic dinoflagellate blooms. *Journal of Phycology*, 13, 224–234.

Arzul, G., Seguel, M., Guzman, L., Erard-LeDenn, E. (1999) Comparison of allelopathic properties in three toxic *Alexandrium* species. *Journal of Experimental Marine Biology and Ecology*, 232, 285–295.

Backhaus, J.O., Maier-Reimer, E. (1983) On seasonal circulation patterns in the North Sea. In: Sundermann, J., Lenz, W. [Eds.] *North Sea Dynamics*. Springer-Verlag, Heidelberg, pp. 63–84.

Baker, T.R., Doucette, G.J., Powell, C.L., Boyer, G.L., Plumley, F.G. (2003) GTX4 imposters: characterization of fluorescent compounds synthesized by *Pseudomonas stutzeri* SF/PS and *Pseudomonas*/Alteromonas PTB-1, symbionts of saxitoxin-producing *Alexandrium* spp. *Toxicon*, 41, 339–347.

Balech, E. (1985) The genus *Alexandrium* or *Gonyaulax* of the tamarensis group. In: Anderson, D.M., White, A.W., Baden, D.G. [Eds.] *Toxic Dinoflagellates, Proceedings of the Third International Conference on Toxic Dinoflagellates*. Elsevier, New York, pp. 33–38.

Balech, E. (1995) *The genus* Alexandrium *Halim (Dinoflagellata)*. Sherkin Island Marine Station Publication, Sherkin Island, Co. Cork, Ireland, 151 pp.

Banse, K. (1982) Cell volumes, maximal growth rates of unicellular algae and ciliates, and the role of ciliates in the marine pelagial. *Limnology and Oceanography*, 27, 1059–1071.

Barker, G.L., Handley, B.A., Vacharapiyasophon, P., Stevens, J.R., Hayes, P.K. (2000) Allele-specific PCR shows that genetic exchange occurs among genetically diverse *Nodularia* (cyanobacteria) filaments in the Baltic Sea. *Microbiology*, 146, 2865–2875.

Barker, G.L.A., Green, J.C., Hayes, P.K., Medlin, L.K. (1994) Preliminary results using the RAPD analysis to screen bloom populations of *Emiliania huxleyi* (Haptophyta). *Sarsia*, 79, 301–306.

Barrett, R.D., Schluter, D. (2008) Adaptation from standing genetic variation. *Trends in Ecology and Evolution*, 23, 38–44.

Benavides, H., Prado, L., Diaz, S., Carreto, J.J. (1995) *An exceptional bloom of Alexandrium catenella in the Beagle Channel, Argentina*. Lavoisier, Paris (France), 113–119 pp.

Bergkvist, J., Selander, E., Pavia, H. (2008) Induction of toxin production in dinoflagellates: the grazer makes a difference. *Oecologia*, 156, 147–154.

Beszteri, B., John, U., Medlin, L.K. (2007) An assessment of cryptic genetic diversity within the *Cyclotella meneghiniana* species complex (Bacillariophyta) based on nuclear and plastid genes, and amplified fragment length polymorphisms. *European Journal of Phycology*, 42, 47–60.

Blanco, J., 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.

Boczar, B.A., Beitler, M.K., Liston, J., Sullivan, J.J., Cattolico, R.A. (1988) Paralytic shellfish toxins in *Protogonyaulax tamarensis* and *Protogonyaulax catenella* in axenic culture. *Plant Physiology*, 88, 1285–1290.

Bolch, C.J.S., Blackburn, S.I., Hallegraeff, G.M., Vaillancourt , R.E. (1999) Genetic variation among strains of the toxic dinoflagellate *Gymnodinium catenatum* (Dinophyceae). *Journal of Phycology*, 35, 356–367.

Bomber, J.W., Tindall, D.R., Miller, D.M. (1989) Genetic variability in toxin potencies among seventeen clones of *Gambierdiscus toxicus* (Dinophyceae). *Journal of Phycology*, 25, 617–625.

Boyer, G.L., Schantz, E.J., Schnoes, H.K. (1978) Characterization of 11-hydroxysaxitoxin sulphate, a major toxin in scallops exposed to blooms of the poisonous dinoflagellate *Gonyaulax tamarensis*. *Journal of the Chemical Society (London) Chemical Communications*, 474, 889–890.

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*. *Marine Biology*, 96, 123–128.

Boyer, G.L., Sullivan, J.J., Andersen, R.J., Taylor, F.J.R., Harrison, P.J., Cembella, A.D. (1986) Use of high-performance liquid chromatography to investigate the production of paralytic shellfish toxins by *Protogonyaulax* spp. in culture. *Marine Biology*, 93, 361–369.

Braarud, T. (1945) A phytoplankton survey of the polluted waters of inner Oslo Fjord. *Hvalrades skrifter – Scientific Results of Marine Investigations, Det Norske Videnskapsaakademi i Oslo*, 28, 1–142.

Braarud, T. (1951) Taxonomical studies of marine dinoflagellates. *Nytt. Mag. Naturv. Vidensk.*, 88, 43–48.

Brand, L.E. (1981) Genetic variability in reproduction rates in marine phytoplankton populations. *Evolution*, 35, 1117–1127.

Brand, L.E. (1982) Genetic variability and spatial patterns of genetic differentiation in the reproduction rates of the marine coccolithophores *Emiliana huxleyi* and *Gephyrocapsa oceanica*. *Limnology and Oceanography*, 27, 236–245.

Brand, L.E. (1985) Low genetic variability in reproduction rates in poluations of *Prorocentrum micans* Ehrenb. (Dinophyceae) over Georges Bank. *Journal of Experimental Marine Biology and Ecology*, 88, 55–65.

Brand, L.E. (1988/1989) Review of genetic variation in marine phytoplankton species and the ecological implications. *Biological Oceanography*, 6, 397–409.

Brand, L.E., Murphy, L.S., Guillard, R.R.L., Lee, H. (1981) Genetic variability and differentiation in the temperature niche component of the diatom *Thalassiosira pseudonana*. *Marine Biology*, 62, 103–110.

Bravo, I., Vila, M., Masó, M., Figueroa, R.I., Ramilo, I. (2008) *Alexandrium catenella* and *Alexandrium minutum* blooms in the, Mediterranean Sea: Toward the identification of ecological niches. *Harmful Algae*, 7, 515–522.

Brosnahan, M.L., Kulis, D.M., Solow, A.R., Erdner, D.L., Percy, L., Lewis, J., Anderson, D.M. (in press) Outbreeding lethality between toxic Group I and nontoxic Group III *Alexandrium tamarense* spp. isolates: Predominance of heterotypic encystment and implications for mating interactions and biogeography. *Deep-Sea Research Part II: Topical Studies in Oceanography*.

Burkholder, J.M., Glibert, P.M. (2006) Intraspecific variability: An important consideration in forming generalisations about toxigenic algal species. *African Journal of Marine Science*, 28, 177–180.

Campbell, D., Duchesne, P., Bernatchez, L. (2003) AFLP utility for population assignment studies: Analytical investigation and empirical comparison with microsatellites. *Molecular Ecology*, 12, 1979–1991.

Carreto, J.I., Benavides, H.R., Negri, R.M., 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. *Journal of Plankton Research*, 8, 15–28.

Cavalier-Smith, T. (1999) Principles of protein and lipid targeting in secondary symbiogenesis: euglenoid, dinoflagellate and sporozoan plastid origins and the eukaryote family tree. *Journal of Eukaryotic Microbiology*, 46, 347–366.

Cavalier-Smith, T. (2003) Protist phylogeny and the high-level classification of Protozoa. *European Journal of Protistology*, 39.

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, Berlin, Heidelberg, New York, pp. 381–403.

Cembella, A.D. (2003) Chemical ecology of eukaryotic microalgae in marine ecosystems. *Phycologia*, 42, 420–447.

Cembella, A.D., Destombe, C. (1996) Genetic differentiation among *Alexandrium* populations from eastern Canada. In: Yasumoto, T., Oshima, Y., Fukuyo, Y. [Eds.] *Harmful and Toxic Algal Blooms*. Intergovernmental Oceanographic Commission of UNESCO, Paris, pp. 447–450.

Cembella, A.D., Quilliam, M.A., Lewis, N.I., Bauder, A.G., Dell'Aversano, C., Thomas, K., Jellett, J., Cusack, R.R. (2002) The toxigenic marine dinoflagellate *Alexandrium tamarense* as the probable cause of mortality of caged salmon in Nova Scotia. *Harmful Algae*, 1, 313–325.

Cembella, A.D., Sullivan, J.J., Boyer, G.L., Taylor, F.J.R., Andersen, R.J. (1987) Variation in paralytic shellfish toxin composition within the *Protogonyaulax tamarensis*/*catenella* species complex; red tide dinoflagellates. *Biochemical Systematics and Ecology*, 15, 171–186.

Cembella, A.D., Taylor, F.J.R. (1985) Biochemical variability within the *Protogonyaulax tamarensis*/*catenella* species complex. In: Anderson, D.M., White, A.W., Baden, D.G. [Eds.] *Toxic Dinoflagellates, Proceedings of the Third International Conference on Toxic Dinoflagellates*. Elsevier, New York, pp. 55–60.

Cembella, A.D., Taylor, F.J.R. (1986) Electrophoretic variability within the *Protogonyaulax tamarensis*/*catenella* species complex: Pyridine-linked dehydrogenases. *Biochemical Systematics and Ecology*, 14, 311–323.

Cembella, A.D., Taylor, F.J.R., Therriault, J.-C. (1988a) Cladistic analysis of electrophoretic variants within the toxic dinoflagellate genus *Protogonyaulax*. *Botanica Marina*, 31, 39–51.

Cembella, A.D., Therriault, J.C., Béland, P. (1988b) Toxicity of cultured isolates and natural populations of *Protogonyaulax tamarensis* from the St. Lawrence Estuary. *Journal of Shellfish Research*, 7, 611–621.

Chapman, N.H., Wijsman, E.M. (1998) Genome screens using linkage disequilibrium tests: Optimal marker characteristics and feasibility. *American Journal of Human Genetics*, 63, 1872–1885.

Chen, C., Durand, E., Forbes, F., François, O. (2007) Bayesian clustering algorithms ascertaining spatial population structure: A new computer program and a comparison study. *Molecular Ecology Notes*, 7, 747–756.

Chinain, M., Germain, M., Sako, Y., Pauillac, S., Legrand, A.M. (1997) Intraspecific variation in the dinoflagellate gambierdiscus toxicus (Dinophyceae) .1. Isozyme analysis. *Journal of Phycology*, 33, 36–43.

Cho, H.J., Matsuoka, K. (2000) Cell lysis of a phagotrophic dinoflagellate, *Polykrikos kofoidii* feeding on *Alexandrium tamarense*. *Plankton Biology and Ecology*, 47, 134–136.

Cho, S.Y., Nagai, S., Nishitani, G., Han, M.S. (2009) Development of compound microsatellite markers in red-tide-causing dinoflagellate *Akashiwo sanguinea* (Dinophyceae). *Molecular Ecology Resources*, 9, 915–917.

Clark, A.G., Lanigan, C.M.S. (1993) Prospects for estimating nucleotide divergence with RAPDs. *Molecular Biology and Evolution*, 10, 1096–1111.

Clarke, K.R. (1993) Non-parametric multivariate analysis of changes in community structure. *Australian Journal of Ecology* 18, 117–143.

Cohen-Fernandez, E.J., Meave Del Castillo, E., Ugarte, I.H.S., Pedroche, F.F. (2006) Contribution of external morphology in solving a species complex: The case of *Prorocentrum micans*, *Prorocentrum gracile* and *Prorocentrum sigmoides* (Dinoflagellata) from the Mexican Pacific Coast. *Phycological Research*, 54, 330–340.

Corander, J., Waldmann, P., Sillanpaa, M.J. (2003) Bayesian analysis of genetic differentiation between populations. *Genetics*, 163, 367–374.

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, Berlin, pp. 383–405.

Davies, A.M. (1983) Application of a three-dimensional shelf model to the calculation of North Sea currents. In: Sundermann, J., Lenz, W. [Eds.] *North Sea Dynamics*. Springer, Heidelberg, pp. 44–62.

Dawson, K.J., Belkhir, K. (2001) A Bayesian approach to the identification of panmictic populations and the assignment of individuals. *Genetical Research*, 78, 59–77.

Deeds, J.R., Place, A.R. (2006) Sterol-specific membrane interactions with the toxins from *Karlodinium micrum* (Dinophyceae) – a strategy for self-protection? *African Journal of Marine Science*, 28, 421–425.

Després, L., Gielly, L., Redoutet, W., Taberlet, P. (2003) Using AFLP to resolve phylogenetic relationships in a morphologically diversified plant species complex when nuclear and chloroplast sequences fail to reveal variability. *Molecular Phylogenetics and Evolution*, 27, 185–196.

Doucette, G.J., Cembella, A.D., Martin, J.L., Michaud, J., Cole, T.V.N., Rolland, R.M. (2006) Paralytic shellfish poisoning (PSP) toxins in North Atlantic right whales *Eubalaena glacialis* and their zooplankton prey in the Bay of Fundy, Canada. *Marine Ecology Progress Series*, 306, 303–313.

Doucette, G.J., Kodama, M., Franca, S., Gallacher, S. (1998) Bacterial interaction with harmful algal bloom species: Bloom ecology, toxigenesis and cytology. In: Anderson, D.M., Cembella, A.D., Hallegraeff, G.M. [Eds.] *Physiological Ecology of Harmful Algal Blooms*. Springer, Berlin, Heidelberg, New York, pp. 619–647.

Doyle, J.J., Doyle, J.L. (1990) Isolation of plant DNA from fresh tissue. *Focus* 12, 13–15.

Doyle, R.W. (1975) Upwelling, clone selection and the characteristic shape of nutrient uptake curves. *Limnology and Oceanography*, 20, 487–489.

Du Yoo, Y., Jeong, H.J., Kim, M.S., Kang, N.S., Song, J.Y., Shin, W., Kim, K.Y., Lee, K. (2009) Feeding by phototrophic red-tide dinoflagellates on the ubiquitous Marine diatom *Skeletonema costatum*. *Journal of Eukaryotic Microbiology*, 56, 413–420.

Durance, J.A. (1989) A model of pollutant dispersal in the North Sea. *Deutsche Hydrographische Zeitschrift*, 42, 271–278.

Durbin, E., Teegarden, G., Campbell, R., Cembella, A.D., Baumgartner, M.F., Mate, B.R. (2002) North Atlantic right whales, *Eubalaena glacialis*, exposed to paralytic shellfish poisoning (PSP) toxins via a zooplankton vector, *Calanus finmarchicus*. *Harmful Algae*, 1, 243–251.

Edwards, K.J., Barker, J.H.A., Daly, A., Jones, C., Karp, A. (1996) Microsatellite libraries enriched for several microsatellite sequences in plants. *Biotechniques*, 20, 758–760.

Elbrächter, M. (2003) Dinophyte reproduction: Progress and conflicts. *Journal of Phycology*, 39, 629–632.

Erickson, D.L., Fenster, C.B., Stenoien, H.K., Price, D. (2004) Quantitative trait locus analyses and the study of evolutionary process. *Molecular Ecology*, 13, 2505–2522.

Estoup, A., Jarne, P., Cornuet, J.M. (2002) Homoplasy and mutation model at microsatellite loci and their consequences for population genetics analysis. *Molecular Ecology*, 11, 1591–1604.

Evans, K.M., Bates, S.S., Medlin, L.K., Hayes, P.K. (2004) Microsatellite marker development and genetic variation in the toxic marine diatom *Pseudo-nitzschia multiseries* (Bacillariophyceae). *Journal of Phycology*, 40, 911–920.

Evans, K.M., Kühn, S.F., Hayes, P.K. (2005) High levels of genetic diversity and low levels of genetic differentiation in North Sea *Pseudo-nitzschia pungens* (Bacillariophyceae) populations. *Journal of Phycology*, 41, 506–514.

Falkowski, P.G., Katz, M.E., Knoll, A.H., Quigg, A., Raven, J.A., Schofield, O., Taylor, F.J.R. (2004) The evolution of modern eukariotic phytoplankton. *Science*, 305, 354–360.

Falush, D., Stephens, M., Pritchard, J.K. (2003) Inference of population structure using multilocus genotype data: Linked loci and correlated allele frequencies. *Genetics*, 164, 1567–1587.

Falush, D., Stephens, M., Pritchard, J.K. (2007) Inference of population structure using multilocus genotype data: Dominant markers and null alleles. *Molecular Ecology Notes*, 7, 574–578.

Fauchot, J., Levasseur, M., Roy, S., Gagnon, R., Weise, A.M. (2005) Environmental factors controlling *Alexandrium tamarense* (Dinophyceae) growth rate during a red tide event in the St. Lawrence Estuary (Canada). *Journal of Phycology*, 41, 263–272.

Felsenstein, J. (1985) Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, 39, 783–791.

Felsenstein, J. (1993) PHYLIP: Phylogenetic Inference Package, version 3.5c. University of Washington, Seattle.

Fenchel, T. (2005) Cosmopolitan microbes and their 'cryptic' species. *Aquatic Microbial Ecology*, 41, 49–54.

Field, C.B., Behrenfeld, M.J., Randerson, J.T., Falkowski, P. (1998) Primary production of the biosphere: Integrating terrestrial and oceanic components. *Science*, 281, 237–240.

Figueroa, R.I., Bravo, I., Garcés, E. (2006) Multiple routes of sexuality in *Alexandrium taylori* (Dinophyceae) in culture. *Journal of Phycology*, 42, 1028–1039.

Figueroa, R.I., Garcés, E., Bravo, I. (2007) Comparative study of the life cycles of *Alexandrium tamutum* and *Alexandrium minutum* (Gonyaulacales, Dinophyceae) in culture. *Journal of Phycology*, 43, 1039–1053.

Finlay, B.J., Fenchel, T. (2004) Cosmopolitan metapopulations of free-living microbial eukaryotes. *Protist*, 155, 237–244.

Fisher, R.A. (1930) *The genetical theory of natural selection*, 1st ed. Oxford University Press, Oxford, 318 pp.

Fistarol, G.O., Legrand, C., Granéli, E. (2005) Allelopathic effect on a nutrient-limited phytoplankton species. *Aquatic Microbial Ecology*, 41, 153–161.

Fistarol, G.O., Legrand, C., Rengefors, K., Graneli, E. (2004a) Temporary cyst formation in phytoplankton: A response to allelopathic competitors? *Environmental Microbiology*, 6, 791–798.

Fistarol, G.O., Legrand, C., Selander, E., Hummert, C., Stolte, W., Granéli, E. (2004b) Allelopathy in *Alexandrium* spp.: Effect on a natural plankton community and on algal monocultures. *Aquatic Microbial Ecology*, 35, 45–56.

Flint-Garcia, S.A., Thornsberry, J.M., Buckler, E.S.I. (2003) Sructure of linkage disequilibrium in plants. *Annual Review of Plant Biology*, 54, 357–374.

Fraga, S., Figueroa, R.I., Bravo, I., Sampedro, N., Franco, J.M., Penna, A., Ramilo, I., Fernández-Villamarín, A. (2006) How many different species are in the *Alexandrium tamarense* / *catenella* / *fundyense* complex? In: *Book of Abstracts,12th International Conference on Harmful Algae, Copenhagen, Denmark, 4-8 September 2006*, p. 64.

François, O., Ancelet, S., Guillot, G. (2006) Bayesian clustering using hidden Markov random fields in spatial population genetics. *Genetics*, 174, 805–816.

Frommlet, J.C., Iglesias-Rodríguez, M.D. (2008) Microsatellite genotyping of single cells of the dinoflagellate species *Lingulodinium polyedrum* (Dinophyceae): A novel approach for marine microbial population genetic studies. *Journal of Phycology*, 44, 1116–1125.

Gallacher, S., Flynn, K.J., Franco, J.M., Brueggemann, E.E., Hines, H.B. (1997) Evidence for production of paralytic shellfish toxins by bacteria associated with *Alexandrium* spp. (Dinophyta) in culture. *Applied and Environmental Microbiology*, 63, 239–245.

Gallacher, S., Smith, E.A. (1999) Bacteria and paralytic shellfish toxins. *Protist*, 150, 245–255.

Gallagher, J.C. (1980) Population genetics of *Skeletonema costatum* (Bacillariophyceae) in Narragansett Bay. *Journal of Phycology*, 16, 464–474.

Gallagher, J.C. (1982) Physiological variation and electrophoretic banding patterns of genetically different seasonal populations of *Skeletonema costatum* (Bacillariophyceae). *Journal of Phycology*, 18, 148–162.

Garcia, A.A.F., Benchimol, L.L., Barbosa, A.M.M., Geraldi, I.O., Souza Jr., C.L., de Souza, A.P. (2004) Comparison of RAPD, RFLP, AFLP and SSR markers for diversity studies in tropical maize inbred lines. *Genetics and Molecular Biology*, 27, 579–588.

Gaudeul, M., Till-Bottraud, I., Barjon, F., Manel, S. (2004) Genetic diversity and differentiation in *Eryngium alpinum* L. (Apiaceae): comparison of AFLP and microsatellite markers. *Heredity*, 92, 508–18.

Genenah, A.A., Shimizu, Y. (1981) Specific toxicity of paralytic shellfish poisons. *Journal of Agricultural and Food Chemistry*, 29, 1289–1291.

Ghazarossian, V., Schantz, E., Schnoes, H., Strong, F. (1974) Identification of a poison in toxic scallops from a *Gonyaulax tamarensis* red tide. *Biochemical and Biophysical Research Communications*, 59, 1219–1225.

Ghazarossian, V.E., Schantz, E.J., Schnoes, H.K., Strong, F.M. (1976) A biologically active acid hydrolysis product of saxitoxin. *Biochemical and Biophysical Research Communications*, 68, 776–780.

Glibert, P.M., Kana, T.M., Anderson, D.M. (1988) Photosynthetic response of *Gonyaulax tamarensis* during growth in a natural bloom and in batch culture. *Marine Ecology Progress Series*, 42, 303–309.

Godhe, A., McQuoid, M.R., Karunasagar, I., Karunasagar, I., Rehnstam-Holm, A.-S. (2006) Comparison of three common molecular tools for distinguishing among geographically separated clones of the diatom *Skeletonema marinoi* Sarno et Zingone (Bacillariophyceae). *Journal of Phycology*, 42, 280–291.

Gottschling, M., Knop, R., Pl, ouml, tner, J., ouml, rg, Kirsch, M., Willems, H., Keupp, H. (2005) A molecular phylogeny of *Scrippsiella sensu* lato (Calciodinellaceae, Dinophyta) with interpretations on morphology and distribution. *European Journal of Phycology*, 40, 207–220.

Gower, J.C. (1966) Some distance properties of latent root and vector methods used in multivariate analysis. *Biometrika*, 53, 325–338.

Graham, L.E., Graham, J., Wilcox, L.W. (2008) *Algae*. Benjamin-Cummings Publishing Company, San Francisco, 720 pp.

Granéli, E., Hansen, P.J. (2006) Allelopathy in harmful microalgae: A mechanism to compete for resources? In: Granéli, E., Turner, J.T. [Eds.] *Ecology of harmful algae*. Springer, Berlin, Heidelberg, pp. 189–201.

Gross, E.M. (2003) Allelopathy of aquatic autotrophs. *Critical Reviews in Plant Sciences*, 22, 313–339.

Guillard, R.R.L., Ryther, J.H. (1962) Studies on marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervaceae* (Cleve) Gran. *Canadian Journal of Microbiology*, 8, 229–239.

Guillot, G., Estoup, A., Mortier, F., Cosson, J.F. (2005) A spatial statistical model for landscape genetics. *Genetics*, 170, 1261–1280.

Hackett, J., Scheetz, T., Yoon, H., Soares, M., Bonaldo, M., Casavant, T., Bhattacharya, D. (2005) Insights into a dinoflagellate genome through expressed sequence tag analysis. *BMC Genomics*, 6, 80.

Hackett, J.D., Anderson, D.M., Erdner, D.L., Bhattacharya, D. (2004) Dinoflagellates: A remarkable evolutionary experiment. *American Journal of Botany*, 91, 1523–1534.

Halim, Y. (1960) *Alexandrium minutum* nov. g. nov. sp. dinoflagellé provocant des 'eaux rouges'. *Vie et Milieu*, 11, 102–105.

Halkett, F., Simon, J.C., Balloux, F. (2005) Tackling the population genetics of clonal and partially clonal organisms. *Trends in Ecology and Evolution*, 20, 194–201.

Hall, S. (1982) *Toxins and toxicity of* Protogonyaulax *from the Northeast Pacific*. Ph.D. dissertation, University of Alaska, Fairbanks, 196 pp.

Hall, S., Neve, R.A., Reichardt, P.B., Swisher, J., G. A. (1979) Chemical analysis of paralytic shellfish poisoning in Alaska. In: Taylor, D.L., Seliger, H.H. [Eds.] *Toxic Dinoflagellate Blooms, Proceedings of the Second International Conference on Toxic Dinoflagellate Blooms*. Elsevier, New York, pp. 345–350.

Hall, S., Reichardt, P.B., Neve, R.A. (1980) Toxins extracted from an Alaskan isolate of *Protogonyaulax* sp. *Biochemical and Biophysical Research Communications*, 97, 649–653.

Hall, S., Strichartz, G., Moczydlowski, E., Ravindran, A., Reichardt, P.B. (1990) The saxitoxins. Sources, chemistry and pharmacology. In: Hall, S., Strichartz, G. [Eds.] *Marine toxins: Orgin, structure and molecular pharmacology*. American Chemical Society Symposium Series 418, Washington, DC, pp. 29–65.

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

Hallegraeff, G.M. (2003) Harmful algal blooms: A global overview. In: Hallegraeff, G.M., Anderson, D.M., Cembella, A.D. [Eds.] *Manual on Harmful Marine Microalgae*. UNESCO Publishing, Paris, pp. 25–49.

Hallegraeff, G.M., Bolch, C.J. (1991) Transport of toxic dinoflagellate cysts via ships' ballast water. *Marine Pollution Bulletin*, 22, 27–30.

Hallegraeff, G.M., Bolch, C.J., Bryan, J., Koerbin, B. (1990) Microalgal spores in ship's ballast water: A danger to aquaculture. In: Graneli, E., Sundström, B., Edler, L., Anderson, D.M. [Eds.] *Toxic Marine Phytoplankton*. Elsevier, New York, pp. 475–480.

Hallegraeff, G.M., Steffensen, D.A., Wetherbee, R. (1988) Three estuarine Australian dinoflagellates that can produce paralytic shellfish toxins. *Journal of Plankton Research*, 10, 533–541.

Han, M.S., Jeon, J.K., Kim, Y.O. (1992) Occurrence of dinoflagellate *Alexandrium tamarense*, a causative organisms of paralytic shellfish poisoning in Chinhae Bay, Korea. *Journal of Plankton Research*, 14, 1581–1592.

Hancock, J.M. (1999) Microsatellites and other simple sequences: genomic context and mutational mechanisms. In: Goldstein, D.B., Schlötterer, C. [Eds.] *Microsatellites*. Oxford University Press, Oxford, pp. 1–9.

Hansen, G., Daugbjerg, N., Franco, J.M. (2003) Morphology, toxin composition and LSU rDNA phylogeny of *Alexandrium minutum* (Dinophyceae) from Denmark, with some morphological observations on other European strains. *Harmful Algae*, 2, 317–335.

Hansen, P.J. (1989) The red tide dinoflagellate *Alexandrium tamarense*: Effects on behaviour and growth of a tintinnid ciliate. *Marine Ecology Progress Series*, 53, 105–116.

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. *Journal of Phycology*, 28, 597–603.

Hansen, P.J., Nielsen, T.G. (1997) Mixotrophic feeding of *Fragilidium subglobosum* (Dinophyceae) on three species of *Ceratium*: Effects of prey concentrations, prey species and light intensity. *Marine Ecology Progress Series*, 147, 187–196.

Haubold, B., Hudson, R.R. (2000) LIAN 3.0: Detecting linkage disequilibrium in multilocus data. *Bioinformatics*, 16, 847–848.

Haubold, B., Travisano, M., Rainey, P.B., Hudson, R.R. (1998) Detecting linkage disequilibrium in bacterial populations. *Genetics*, 150, 1341–1348.

Hayhome, B.A., Anderson, D.M., Kulis, D.M., Whitten, D.J. (1989) Variation among congeneric dinoflagellates from the northeastern United States and Canada. I. Enzyme electrophoresis. *Marine Biology*, 101, 427–435.

Henrichs, D.W., Renshaw, M.A., Santamaria, C.A., Richardson, B., Gold, J.R., Campbell, L. (2008) PCR Amplification of microsatellites from single cells of *Karenia brevis* preserved in Lugol's iodine solution. *Marine Biotechnology*, 10, 122–127.

Hille, B. (1968) Pharmacological modifications of sodium channels of frog nerve. *Journal of General Physiology*, 51, 199–219.

Hillis, D.M., Dixon, M.T. (1991) Ribosomal DNA – Molecular evolution and phylogenetic inference. *Quarterly Review of Biology*, 66, 411–453.

Ho, A.Y.T., Hsieh, D.P.H., Qian, P.Y. (2006) Variations in paralytic shellfish toxin and homolog production in two strains of *Alexandrium tamarense* after antibiotic treatments. *Aquatic Microbial Ecology*, 42, 41–53.

Hobbie, J.E., Daley, R.J., Jasper, S. (1977) Use of nuclepore filters for counting bacteria by fluorescence microscopy. *Applied and Environmental Microbiology*, 33, 1225–1228.

Hold, G.L., Smith, E.A., Birkbeck, T.H., Gallacher, S. (2001) Comparison of paralytic shellfish toxin (PST) production by the dinoflagellates *Alexandrium lusitanicum* NEPCC 253 and *Alexandrium tamarense* NEPCC 407 in the presence and absence of bacteria. *FEMS Microbiology, Ecology*, 36, 223–234.

Holmes, M.J., Bolch, C.J.S., Green, D.H., Cembella, A.D., Teo, S.L.M. (2002) Singapore isolates of the Dinoflagellate *Gymnodinium catenatum* (Dinophyceae) produce a unique profile of paralytic shellfish poisoning toxins. *Journal of Phycology*, 38, 96–106.

Huisman, J., Weissing, F.J. (2001) Fundamental unpredictability in multispecies competition. *American Naturalist*, 157, 488–494.

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 chromatographic separation, post-column oxidation, and fluorescence detection. *Chromatographia*, 45, 312–316.

Hutchinson, G.E. (1961) The paradox of plankton. *American Naturalist*, 95, 137–145.

Ichimi, K., Suzuki, T., Ito, A. (2002) Variety of PSP toxin profiles in various culture strains of *Alexandrium tamarense* and change of toxin profile in natural *A. tamarense* population. *Journal of Experimental Marine Biology and Ecology*, 273, 51–60.

Igarashi, T., Aritake, S., Yasumoto, T. (1998) Biological activities of Prymnesin-2 isolated from a red tide alga *Prymnesium parvum*. *Natural Toxins*, 6, 35–41.

Iglesias-Rodríguez, M.D., Saéz, A.G., Groben, R., Edwards, K.J., Batley, J., Medlin, L.K., Hayes, P.K. (2002) Polymorphic microsatellite loci in global populations of the marine coccolithophorid *Emiliania huxleyi*. *Molecular Ecology Notes*, 2, 495–497.

Iglesias-Rodríguez, M.D., Schofield, O.M., Batley, J., Medlin, L.K., Hayes, P.K. (2006) Intraspecific genetic diversity in the marine coccolithophore *Emiliania huxleyi* (Prymnesiophyceae): The use of microsatellite analysis in marine phytoplankton population studies. *Journal of Phycology*, 42, 526–536.

Innan, H., Terauchi, R., Kahl, G., Tajima, F. (1999) A method for estimating nucleotide diversity from AFLP data. *Genetics*, 151, 1157–1164.

Ishida, I., Kim, C.-H., Sako, Y., Hirooka, N., Uchida, A. (1993) PSP toxin production is chromosome dependent in *Alexandrium* spp. In: Smayda, T.J., Shimizu, Y. [Eds.] *Toxic Phytoplankton Blooms in the Sea*. Elsevier, Amsterdam, pp. 881–887.

Ishida, Y., Uchida, A., Sako, Y. (1998) Genetic and biochemical approaches to PSP toxin production of toxic dinoflagellates. In: Anderson, D.M., Cembella, A.D., Hallegraeff, G.M. [Eds.] *Physiological Ecology of Harmful Algal Blooms.* Springer, Berlin, Heidelberg, New York, pp. 49–58.

Jackim, E., Gentile, J. (1968) Toxins of a blue-green alga: Similarity to saxitoxin. *Science*, 162, 915–916.

Jacobson, D.M., Anderson, D.M. (1996) Widespread phagocytosis of ciliates and other protists by marine mixotrophic and heterotrophic thecate dinoflagellates. *Journal of Phycology*, 32, 279–285.

Jeong, H.J., Du Yoo, Y., Park, J.Y., Song, J.Y., Kim, S.T., Lee, S.H., Kim, K.Y., Yih, W.H. (2005) Feeding by phototrophic red-tide dinoflagellates: five species newly revealed and six species previously known to be mixotrophic. *Aquatic Microbial Ecology*, 40, 133–150.

Jeong, H.J., Lee, C.W., Yih, W.H., Kim, J.S. (1997) *Fragilidium* cf. *mexicanum*, a thecate mixotrophic dinoflagellate which is prey for and a predator on co-occurring thecate heterotrophic dinoflagellate *Protoperidinium* cf. *divergens*. *Marine Ecology Progress Series*, 151, 299–305.

John, U., Fensome, R.A., Medlin, L.K. (2003) 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). *Molecular Biology and Evolution*, 20, 1015–1027.

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

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., Vázquez, A., Karp, A. (1997) Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. *Molecular Breeding*, 3, 381–390.

Keafer, B.A., Buesseler, K.O., Anderson, D.M. (1992) Burial of living dinoflagellate cysts in estuarine and nearshore sediments. *Marine Micropaleontology*, 20, 147–161.

Keller, M.D., Selvin, R.C., Claus, W., Guillard, R.R.L. (1987) Media for the culture of oceanic ultraphytoplankton. *Journal of Phycology*, 23, 633–638.

Kim, C.-J., Kim, C.-H., Sako, Y. (2005) Paralytic shellfish poisoning toxin analysis of the genus *Alexandrium* (Dinophyceae) occurring in Korean coastal waters. *Fisheries Science*, 71, 1–11.

Kim, C.H., Sako, Y., Ishida, Y. (1993) Comparison of toxin composition between populations of *Alexandrium* spp. from geographical distant areas. *Nippon Suisan Gakkaishi*, 59, 641–646.

Kim, K.Y., Yoshida, M., Fukuyo, Y., Kim, C.H. (2002) Morphological observation of *Alexandrium tamarense* (Lebour) Balech, *A. catenella* (Whedon et Kofoid) Balech and one related morphotype (Dinophyceae) in Korea. *Algae*, 17, 11–19.

King, G., Nienhuis, J., Hussey, C. (1993) Genetic similarity among ecotypes of Arabidopsis thaliana estimated by analysis of restriction fragment length polymorphisms. *Theoretical and Applied Genetics*, 86, 1028–1032.

Kodama, M., Doucette, G.J., Green, D.H. (2006) Relationship between bacteria and harmful algae. In: Granéli, E., Turner, J.T. [Eds.] *Ecology of Harmful Algae.* Springer, Berlin, Heidelberg, New York, pp. 243–258.

Kooistra, W., Gersonde, R., Medlin, L.K., Mann, D. (2007) The origin and evolution of the Diatoms: Their adaptation to a planktonic existence. In: Knoll, A.H., Falkowski, P.G. [Eds.] *Evolution of Primary Producers in the Sea*. Elsevier Academic Press, pp. 207–250.

Kooistra, W.H., Sarno, D., Balzano, S., Gu, H., Andersen, R.A., Zingone, A. (2008) Global diversity and biogeography of *Skeletonema* species (bacillariophyta). *Protist*, 159, 177–193.

Kosman, E., Leonard, K.J. (2005) Similarity coefficients for molecular markers in studies of genetic relationships between individuals for haploid, diploid and polyploid species. *Molecular Ecology*, 14, 415–424.

Kremp, A., Lindholm, T., Dressler, N., Erler, K., Gerdts, G., Eirtovaara, S., Leskinen, E. (2009) Bloom forming Alexandrium ostenfeldii (Dinophyceae) in shallow waters of the Aland Archipelago, Northern Baltic Sea. *Harmful Algae*, 8, 318–328.

Krock, B., Seguel, C.G., Cembella, A.D. (2007) Toxin profile of *Alexandrium catenella* from the Chilean coast as determined by liquid chromatography with fluorescence detection and liquid chromatography coupled with tandem mass spectrometry. *Harmful Algae*, 6, 734–744.

Kumar, S., Tamura, K., Nei, M. (2004) MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics*, 5, 150–163.

Lagos, N. (1998) Microalgal blooms: A global issue with negative impact in Chile. *Biological Research*, 31, 375–386.

Lakeman, M.B., von Dassow, P., Cattolico, R.A. (2009) The strain concept in phytoplankton ecology. *Harmful Algae*, 8, 746–758.

Leaw, C.P., Lim, P.T., Ng, B.K., Cheah, M.Y., Ahmad, A., Usup, G. (2005) Phylogenetic analysis of *Alexandrium* species and *Pyrodinium bahamense* (Dinophyceae) based on theca morphology and nuclear ribosomal gene sequence. *Phycologia*, 44, 550–565.

Lebour, M.V. (1925) The dinoflagellates of the Northern seas. *Journal of the Marine Biological Association of the United Kingdom*, 13, 250 pp.

Lee, S.G., Park, J.S., Kim, H.G. (1993) Taxonomy of marine toxic flagellates occurring in the southern coastal waters of Korea. *Bulletin of National Fisheries Research and Development Agency (Korea). Yangsan*, 1–23.

Legrand, C., Rengefors, K., Fistarol, G.O., Granéli, E. (2003) Allelopathy in phytoplankton – Biochemical, ecological and evolutionary aspects. *Phycologia*, 42, 406–419.

Levinson, G., Gutman, G.A. (1987) Slipped-strand mispairing: A major mechanism for DNA sequence evolution. *Molecular Biology and Evolution*, 4, 203–221.

Lilly, E.L., Halanych, K.M., Anderson, D.M. (2005) Phylogeny, biogeography and species boundaries within the *Alexandrium minutum* group. *Harmful Algae*, 4, 1004–1020.

Lilly, E.L., Halanych, K.M., Anderson, D.M. (2007) Species boundaries and global biogeography of the *Alexandrium tamarense* complex (Dinophyceae). *Journal of Phycology*, 43, 1329–1338.

Lilly, E.L., Kulis, D.M., Gentien, P., Anderson, D.M. (2002) Paralytic shellfish poisoning toxins in France linked to a human-introduced strain of *Alexandrium catenella* from the western Pacific: Evidence from DNA and toxin analysis. *Journal of Plankton Research*, 24, 443–452.

Lodders, N., Stackebrandt, E., Nubel, U. (2005) Frequent genetic recombination in natural populations of the marine cyanobacterium *Microcoleus chthonoplastes*. *Environmental Microbiology*, 7, 434–442.

Logares, R., Rengefors, K., Kremp, A., Shalchian-Tabrizi, K., Boltovskoy, A., Tengs, T., Shurtleff, A., Klaveness, D. (2007) Phenotypically different microalgal morphospecies with identical ribosomal DNA: A case of rapid adaptive evolution? *Microbial Ecology*, 53, 549–561.

Logares, R.E. (2006) Does the global microbiota consist of a few cosmopolitan species? *Ecología Austral*, 16, 85–90.

Loret, P., Tengs, T., Villareal, T.A., Singler, H., Richardson, B., McGuire, P., Morton, S., Busman, M., Campbell, L. (2002) No difference found in ribosomal DNA sequences from physiologically diverse clones of *Karenia brevis* (Dinophyceae) from the Gulf of Mexico. *Journal of Plankton Research*, 24, 735–739.

Lundholm, N., Moestrup, Ø. (2006) The biogeography of harmful algae. In: Granelli, E., Turner, J.T. [Eds.] *Ecology of Harmful Algae*, pp. 23–35.

Lundholm, N., Moestrup, Ø., Kotaki, Y., Hoef-Emden, K., Scholin, C., Miller, P. (2006) Inter- and intraspecific variation of the *Pseudo-nitzschia delicatissima* complex (Bacillariophyceae) illustrated by rRNA probes, morphological data and phylogenetic analyses. *Journal of Phycology*, 42, 464–481.

Lynch, M., Gabriel, W., Wood, A.M. (1991) Adaptive and demographic responses of plankton populations to environmental change. *Limnology and Oceanography*, 36, 1301–1312.

MacIntyre, J.G., Cullen, J.J., Cembella, A.D. (1997) Vertical migration, nutrition and toxicity in the dinoflagellate *Alexandrium tamarense*. *Marine Ecology Progress Series*, 148, 201–216.

MacKenzie, L., de Salas, M., Adamson, J., Beuzenberg, V. (2004) The dinoflagellate genus *Alexandrium* (Halim) in New Zealand coastal waters: comparative morphology, toxicity and molecular genetics. *Harmful Algae*, 3, 71–92.

MacKinnon, S.L., Walter, J.A., Quilliam, M.A., Cembella, A.D., LeBlanc, P., Burton, I.W., Hardstaff, W.R., Lewis, N.I. (2006) Spirolides isolated from Danish strains of the toxigenic dinoflagellate *Alexandrium ostenfeldii*. *Journal of Natural Products*, 69, 983–987.

Manhart, J.R., McCourt, R.M., Wood, A.M., Leatham, T. (1992) Molecular data and species concepts in the algae. *Journal of Phycology*, 28, 730–737.

Mann, D.G. (1999) The species concept in diatoms. *Phycologia*, 38, 437–495.

Mann, D.G. (2001) Freshwater algae: Taxonomy, biogeography and conservation. *Phycologia*, 40, 387–389.

Mantel, N. (1967) The detection of disease clustering and a generalized regression approach. *Cancer Research*, 27, 209–220.

Maranda, L., Anderson, D.M., Shimizu, Y. (1985) Comparison of toxicity between populations of *Gonyaulax tamarensis* of eastern North American waters. *Estuarine, Coastal and Shelf Science*, 21, 401–410.

Margalef, R. (1958) Temporal succession and spatial heterogeneity in phytoplankton. In: Buzzati-Traverso, A.A. [Ed.] *Perspectives in Marine Biology*. University of California Press, Los Angeles, pp. 145–158.

Margalef, R. (1978) Life-forms of phytoplankton as survival alternatives in an unstable environment. *Oceanologica Acta*, 1, 493–509.

Margalef, R., Estrada, M. (1987) Synoptic distribution of summer microplankton (algae and protozoa) across the principal front in the Western Mediterranean. *Investigaciones Pesqueria*, 51, 121–140.

Margalef, R., Estrada, M., Blasco, D. (1979) Functional morphology of organisms involved in red tides, as adapted to decaying turbulence. In: Tylor, D.L., Seliger, H.H. [Eds.] *Toxin dinoflagellate blooms*. Elsevier, Amsterdam, pp. 89–94.

Mariette, S., Le Corre, V., Austerlitz, F., Kremer, A. (2002) Sampling within the genome for measuring within-population diversity: Trade-offs between markers. *Molecular Ecology*, 11, 1145–1156.

Martínez, R., Añíbarro, C., Fernández, S. (2006) Genetic variability among *Alexandrium tamarense* and *Alexandrium minutum* strains studied by RAPD banding pattern analysis. *Harmful Algae*, 5, 599–607.

Martins, C.A., Kulis, D., Franca, S., Anderson, D.M. (2004) The loss of PSP toxin production in a formerly toxic *Alexandrium lusitanicum* clone. *Toxicon*, 43, 195–205.

Masseret, E., Grzebyk, D., Nagai, S., Genovesi, B., Lasserre, B., Laabir, M., Collos, Y., Vaquer, A., Berrebi, P. (2009) Unexpected genetic diversity among and within populations of the toxic dinoflagellate *Alexandrium catenella* as revealed by nuclear microsatellite markers. *Applied and Environmental Microbiology*, 75, 2037–2045.

Matsuoka, K., Cho, H.J., Jacobsen, D.M. (2000) Observation of the feeding behaviour and growth rates of the heterotrophic dinoflagellate *Polykrikos kofoidii* (Polykrikaceae, Dinophyceae). *Phycologia*, 39, 82–86.

Maynard Smith, J., Smith, N.H., O'Rourke, M., Spratt, B.G. (1993) How clonal are bacteria? *Proceedings of the National Academy of Sciences of the United States of America*, 90, 4384–4388.

McClintock, J.B., Baker, B.J. (2001) *Marine Chemical Ecology*. CRC Press, Boca Raton, Florida, 610 pp.

McManus, G.B., Katz, L.A. (2009) Molecular and morphological methods for identifying plankton: What makes a successful marriage? *Journal of Plankton Research*, 31, 1119–1129.

McMinn, A., Scott, F.J. (2005) Dinoflagellates. In: Scott, F.J., Marchant, H.J. [Eds.] *Antarctic marine protists*. Australian Biological Resources Study; Australian Antarctic Division., Canberra & Hobart, pp. 202–250.

Medlin, L., Lange, M., Wellbrock, U., Donner, G., Elbrächter, M., Hummert, C., Luckas, B. (1998) Sequence comparisons link toxic European isolates of *Alexandrium tamarense* from the Orkney Islands to toxic North American stocks. *European Journal of Protistology*, 34, 329–335.

Medlin, L.K. (2003) Application of molecular techniques for genetic differentiation of microalgae. In: Norton, T., A. [Ed.] *Out of the Past: Collected Reviews to celebrate the Jubilee of the British Phycological Society*. British Phycological Society, Belfast, pp. 31–48.

Medlin, L.K., Barker, G.L.A., Campbell, L., Green, J.C., Hayes, P.K., Marie, D., Wrieden, S., Vaulot, D. (1996) Genetic characterisation of *Emiliania huxley* (Haptophyta). *Journal of Marine Systems*, 9, 13–31.

Medlin, L.K., Elwood, H.J., Stickel, S., Sogin, M.L. (1991) Genetic and morphological variation in the diatom *Skeletonema costatum* (Bacillariophyta): evidence for a new species, *Skeletonema pseudocostatum*. *Journal of Phycology*, 27, 514–524.

Medlin, L.K., Lange, M., Barker, G.L.A., Hayes, P.K. (1995) Can molecular techniques change our ideas about species concepts? In: Joint, I., Falkowski, P., Carr, N. [Eds.] *Molecular Ecology of Aquatic Mircrobes*, pp. 133–152.

Medlin, L.K., Lange, M., Nothig, E.M. (2000) Genetic diversity in the marine phytoplankton: A review and a consideration of Antarctic phytoplankton. *Antarctic Science*, 12, 325–333.

Medlin, L.K., Metfies, K., John, U., Olsen, J. (2007) Algal Molecular Systematics: A Review of the Past and Prospects for the Future. In: Brodie, J., Lewis, J. [Eds.] *Unravelling the algae. The past, present and future of algal systematics*. CRC Press, London, Uk, pp. 341–353.

Mee, L.D., Espinosa, M., Diaz, G. (1986) Paralytic shellfish poisoning with a *Gymnodinium catatenum* red tide on the Pacific coast of Mexico. *Marine Environmental Research*, 19, 77–92.

Miles, C.O., Samdal, I.A., Aasen, J.A.G., Jensen, D.J., Quilliam, M.A., Petersen, D., Briggs, L.M., Wilkins, A.L., Rise, F., Cooney, J.M., MacKenzie, L.A. (2005) Evidence for numerous analogs of yessotoxin in *Protoceratium reticulatum*. *Harmful Algae*, 4, 1075–1091.

Miller, M.P. (1997) Tools for population genetic analyses (TFPGA) 1.3: a Windows program for the analysis of allozyme and molecular population genetic data. Northern Arizona University, Flagstaff.

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.

Mold, J.D., Bowden, J.P., Stanger, D.W., Maurer, J.E., Lynch, J.M., Wyler, R.S., Schantz, E.J., Riegel, B. (1957) Paralytic shellfish poison. VII. Evidence for the purity of the poison from toxic clams and mussels. *Journal of the Americam Chemical Society*, 79, 5235–5238.

Molisch, H. (1937) *Der Einfluss einer Pflanze auf die Andere – Allelopathie.* Gustav Fisher, Jena, Germany, 106 pp.

Montresor, M., John, U., Beran, A., Medlin, L.K. (2004) *Alexandrium tamutum* sp nov. (Dinophyceae): A new nontoxic species in the genus *Alexandrium*. *Journal of Phycology*, 40, 398–411.

Montresor, M., Sgrosso, S., Procaccini, G., Kooistra, W.H.C.F. (2003) Intraspecific diversity in *Scrippsiella trochoidea* (Dinophyceae): evidence for cryptic species. *Phycologia*, 42, 56–70.

Moreira, D., von der Heyden, S., Bass, D., Lopez-Garcia, P., Chao, E., Cavalier-Smith, T. (2007) Global eukaryote phylogeny: Combined small- and large-subunit ribosomal DNA trees support monophyly of Rhizaria, Retaria and Excavata. *Molecular Phylogenetics and Evolution*, 44, 255–266.

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, Proceedings of the Third International Conference on Toxic Dinoflagellates*. Elsevier, New York, Amsterdam, pp. 165–170.

Mougel, C., Thioulouse, J., Perrière, G., Nesme, X. (2002) A mathematical method for determining genome divergence and species delineation using AFLP. *International Journal of Systematic and Evolutionary Microbiology*, 52, 573–586.

Mueller, U.G., Wolfenbarger, L.R. (1999) AFLP genotyping and fingerprinting. *Trends in Ecology and Evolution*, 14, 389–394.

Nagai, S., Lian, C., Hamaguchi, M., Matsuyama, Y., Itakaru, S., Hogetsu, T. (2004) Development of microsatellite markers in the toxic dinoflagellate *Alexandrium tamarense* (Dinophyceae). *Molecular Ecology Notes*, 4, 83–85.

Nagai, S., Lian, C., Yamaguchi, S., Hamaguchi, M., Matsuyama, Y., Itakura, S., Shimada, H., Kaga, S., Yamauchi, H., Sonda, Y., Nishikawa, T., Kim, C.H., Hogetsu, T. (2007a) Microsatellite markers reveal population genetic structure of the toxic dinoflagellate *Alexandrium tamarense* (Dinophyceae) in Japanese coastal waters. *Journal of Phycology*, 43, 43–54.

Nagai, S., McCauley, L., Yasuda, N., Erdner, D.L., Kulis, D.M., Matsuyama, Y., Itakura, S., Anderson, D.M. (2006a) Development of microsatellite markers in the toxic dinoflagellate *Alexandrium minutum* (Dinophyceae). *Molecular Ecology Notes*, 6, 756–758.

Nagai, S., Nishitani, G., Sakamoto, S., Sugaya, T., Lee, C.K., Kim, C.H., Itakura, S., Yamaguchi, M. (2009) Genetic structuring and transfer of marine dinoflagellate *Cochlodinium polykrikoides* in Japanese and Korean coastal waters revealed by microsatellites. *Molecular Ecology*, 18, 2337–2352.

Nagai, S., Nishitani, G., Yamaguchi, S., Yasuda, N., Lian, C.L., Itakura, S., Yamaguchi, M. (2007b) Development of microsatellite markers in the noxious red tide-causing dinoflagellate *Heterocapsa circularisquama* (Dinophyceae). *Molecular Ecology Notes*, 7, 993–995.

Nagai, S., Yamaguchi, S., Lian, C.L., Matsuyama, Y., Itakura, S. (2006b) Development of microsatellite markers in the noxious red tide-causing algae *Heterosigma akashiwo* (Raphidophyceae). *Molecular Ecology Notes*, 6, 477–479.

Narahashi, T., Moore, J.W. (1968) Neuroactive Agents and Nerve Membrane Conductances. *Journal of General Physiology*, 51, 93–101.

Needler, A.B. (1949) Paralytic shellfish poisoning and *Gonyaulax tamarensis*. *Journal of the Fisheries Research Board Canada*, 7, 490–504.

Nei, M. (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, 89, 583–590.

Nei, M. (1987) *Molecular evolutionary genetics*, 1st ed. Columbia University Press, New York, 512 pp.

Nishitani, G., Nagai, S., Lian, C.L., Sakiyama, S., Oohashi, A., Miyamura, K. (2009) Development of microsatellite markers in the marine phytoplankton *Karenia mikimotoi* (Dinophyceae). *Conservation Genetics*, 10, 713–715.

Nishitani, G., Nagai, S., Lian, C.L., Yamaguchi, H., Sakamoto, S., Yoshimatsu, S., Oyama, K., Itakura, S., Yamaguchi, M. (2007) Development of compound microsatellite markers in the harmful red tide species *Chattonella ovata* (Raphidophyceae). *Molecular Ecology Notes*, 7, 1251–1253.

Noguchi, T., Onoue, Y., Maruyama, J., Hashimoto, K., Nishio, S., Ikeda, T. (1983) The new paralytic shellfish poison from *Protogonyaulax catenella*. *Bulletin of the Japanese Society of Scientific Fisheries*, 49, 1931.

Nybom, H. (2004) Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. *Molecular Ecology*, 13, 1143–1155.

Nygaard, K., Tobiesen, A. (1993) Bacterivory in algae: A survival strategy during nutrient limitation. *Limnology and Oceanography*, 38, 273–279.

Ogata, T., Ishimaru, T., Kodama, M. (1987a) Effect of water temperature and light intensity on growth rate and toxicity change in *Protogonyaulax tamarensis*. *Marine Biology*, 95, 217–220.

Ogata, T., Kodama, M., Fukuyo, Y., Inoue, T., Kamiya, H., Matsuura, F., Sekiguchi, K., Watanabe, S. (1982) The occurrence of *Protogonyaulax* spp. in Ofunato Bay, in association with the toxification of the scallop *Patinopecten yessoensis*. *Bulletin of the Japanese Society of Scientific Fisheries*, 48, 563–566.

Ogata, T., Kodama, M., Ishimaru, T. (1987b) Toxin production in the dinoflagellate *Protogonyaulax tamarensis*. *Toxicon*, 25, 923–928.

Okolodkov, Y.B. (2005) The global distributional patterns of toxic, bloom dinoflagellates recorded from the Eurasian Arctic. *Harmful Algae*, 4, 351–369.

Orlova, T.Y., Selina, M.S., Lilly, E.L., Kulis, D.M., Anderson, D.M. (2007) Morphogenetic and toxin composition variability of *Alexandrium tamarense* (Dinophyceae) from the east coast of Russia. *Phycologia*, 46, 534–548.

Oshima, Y. (1995a) Post-column derivatization HPLC methods for paralytic shellfish poisons. In: Hallegraeff, G.M., Anderson, D.M., Cembella, A.D. [Eds.] *Manual on Harmful Marine Microalgae, IOC Manuals and Guides No. 33.* UNESCO, Paris, pp. 81–94.

Oshima, Y. (1995b) Post-column derivatization liquid chromatographic method for paralytic shellfish toxins. *Journal of AOAC International*, 78, 528–532.

Oshima, Y., Blackburn, S.I., Hallegraeff, G.M. (1993) Comparative study on paralytic shellfish toxin profiles of the dinoflagellate *Gymnodinium catenatum* from three different countries. *Marine Biology*, 116, 471–476.

Oshima, Y., Bolch, C.J., Hallegraeff, G.M. (1992) Toxin composition of resting cysts of *Alexandrium tamarense* (Dinophyceae). *Toxicon*, 30, 1539–1544.

Oshima, Y., Buckley, L.J., Alam, M., Shimizu, Y. (1977) Heterogeneity of paralytic shellfish poisons. Three new toxins from cultured *Gonyaulax tamarensis* cells, *Mya arenaria* and *Saxidomus giganteus*. *Comparative Biochemistry and Physiology C: Comparative Pharmacology*, 57, 31–34.

Oshima, Y., Hayakawa, T., Hashimoto, M., Kotaki, Y., Yasumoto, T. (1982a) Classification of *Protogonyaulax tamarensis* from Northern Japan into three strains by toxin composition. *Bulletin of the Japanese Society of Scientific Fisheries*, 48, 851–854.

Oshima, Y., Itakura, H., Lee, K.C., Yasumoto, T., Blackburn, S., Hallegraeff, G. (1991) Toxin production by the dinoflagellate *Gymnodinium catenatum*. In: Smayda, T.J., Shimizu, Y. [Eds.] *5. Intermational Conference on Toxic Marine Phytoplankton*. Elsevier, Newport, pp. 907–912.

Oshima, Y., Singh, H.T., Fukuyo, Y., Yasumoto, T. (1982b) Identification and toxicity of the resting cysts of *Protogonyaulax* found in Ofunato Bay. *Bulletin of the Japanese Society of Scientific Fisheries*, 48, 1303–1305.

Paerl, H.W. (1988) Nuisance phytoplankton blooms in coastal, estuarine and inlet waters. *Limnology and Oceanography*, 33, 823–847.

Parke, M., Dixon, P.S. (1976) Check-list of British marine algae – Third revision. *Journal of the Marine Biological Association of the United Kingdom*, 56, 527–594.

Parkhill, J.P., 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.

Peakall, R., Smouse, P.E. (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, 6, 288–295.

Pedersen, M.F., Hansen, P.J. (2003) Effects of high pH on the growth and survival of six marine heterotrophic protists. *Marine Ecology Progress Series*, 33, 33–41.

Pelser, P.B., Gravendeel, B., van der Meijden, R. (2003) Phylogeny reconstruction in the gap between too little and too much divergence: the closest relatives of *Senecio jacobaea* (Asteraceae) according to DNA sequences and AFLPs. *Molecular Phylogenetics and Evolution*, 29, 613–628.

Penna, A., Fraga, S., Maso, M., Giacobbe, M.G., Bravo, I., Garces, E., Vila, M., Bertozzini, E., Andreoni, F., Luglie, A., Vernesi, C. (2008) Phylogenetic relationships among the Mediterranean *Alexandrium* (Dinophyceae) species based on sequences of 5.8S gene and Internal Transcript Spacers of the rRNA operon. *European Journal of Phycology*, 43, 163–178.

Penna, A., Garcés, E., Vila, M., Giacobbe, M.G., Fraga, S., Lugliè, A., Bravo, I., Bertozzini, E., Vernesi, C. (2005) *Alexandrium catenella* (Dinophyceae), a toxic ribotype expanding in the NW Mediterranean Sea. *Marine Biology*, 148, 13–23.

Persich, G.R., Kulis, D.M., Lilly, E.L., Anderson, D.M., Garcia, V.M.T. (2006) Probable origin and toxin profile of *Alexandrium tamarense* (Lebour) Balech from southern Brazil. *Harmful Algae*, 5, 36–44.

Persson, A., Godhe, A., Karlson, B. (2000) Dinoflagellate cysts in recent sediments from the West Coast of Sweden. *Botanica Marina*, 69–79.

Pettay, D.T., Lajeunesse, T.C. (2007) Microsatellites from clade B *Symbiodinium* spp. specialized for Caribbean corals in the genus *Madracis*. *Molecular Ecology Notes*, 7, 1271–1274.

Poulton, N.J. (2001) *Physiological and behavioral diagnostics of nitrogen limitation for the toxic dinoflagellate* Alexandrium fundyense. Ph.D. dissertation, Massachusetts Institute of Technology, Cambridge, 246 pp.

Prakash, A. (1963) Source of paralytic shellfish toxin in the Bay of Fundy. *Journal Fisheries Research Board of Canada*, 20, 983–996.

Prakash, A. (1967) Growth and toxicity of a marine dinoflagellate, *Gonyaulax tamarensis*. *Journal of the Fisheries Research Board of Canada*, 24, 1589–1606.

Prakash, A., Medcof, J.C., Tennant, A.D. (1971) Paralytic shellfish poisoning in eastern Canada. *Bulletin of the Fisheries Research Board of Canada*, 177, 1–87.

Prakash, A., Taylor, F.J.R. (1966) A "red water" bloom of *Gonyaulax acatenella* in the Strait of Georgia and its relation to paralytic shellfish toxicity. *Journal Fisheries Research Board of Canada*, 23, 1265–1270.

Prandle, D. (1984) A modelling study of the mixing of ^{137}Cs in the seas of the European continental shelf. *Philosophical Transaction of the Royal Society of London A*, 310, 407–436.

Pritchard, J.K., Stephens, M., Donnelly, P. (2000a) Inference of population structure using multilocus genotype data. *Genetics*, 155, 945–959.

Pritchard, J.K., Stephens, M., Rosenberg, N.A., Donnelly, P. (2000b) Association mapping in structured populations. *American Journal of Human Genetics*, 67, 170–181.

Qi, Y., Qian, F. (1994) Taxonomic studies on red tide causative dinoflagellates in Dapeng Bay, South China Sea. *Oceanologia et Limnologia Sinica*, 25, 206–210.

R Development Core Team (2007) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Reigosa, M.J., Sanchez-Moreiras, A., Gonzales, L. (1999) Ecophysiological approach in allelopathy. *Critical Reviews in Plant Sciences*, 18, 577–608.

Rensel, J.E., Whyte, J.N.C. (2003) Finfish mariculture and harmful algal blooms. In: Hallegraeff, G.M., Anderson, D.M., Cembella, A.D. [Eds.] *Manual on Harmful Marine Microalgae*. UNESCO Publishing, Paris, pp. 693–722.

Renshaw, M.A., Soltysiak, K., Arreola, D., Loret, P., Patton, J.C., Gold, J.R., Campbell, L. (2006) Microsatellite DNA markers for population genetic studies in the dinoflagellate *Karenia brevis*. *Molecular Ecology Notes*, 6, 1157–1159.

Reyes-Vásquez, G., Férraz-Reyes, E., Vásquez, E. (1979) Toxic dinoflagellate blooms in northeastern Venezuela during 1977. In: Taylor, D.L., Seliger, H.H. [Eds.] *Toxic Dinoflagellate Blooms, Proceedings of the Second International Conference on Toxic Dinoflagellate Blooms*. Elsevier, New York, pp. 191–194.

Rice, E.L. (1974) *Allelopathy*. Academic Press, New York, 353 pp.

Rice, E.L. (1984) *Allelopathy*, 2nd ed. Academic Press, New York, NY, 442 pp.

Riegel, B., Stanger, D.W., Wikholm, D.M., Mold, J.D., Sommer, H. (1949) Paralytic shellfish poison. V. The primary source of the poison, the marine plankton organism *Gonyaulax catenella*. *Journal of Biological Chemistry*, 177, 7–11.

Rieseberg, L.H. (1996) Homology among RAPD fragments in interspecific comparisons. *Molecular Ecology*, 5, 99–105.

Rieseberg, L.H., Widmer, A., Arntz, A.M., Burke, J.M. (2002) Directional selection is the primary cause of phenotypic diversification. *Proceedings of the National Academy of Sciences of the United States of America*, 99, 12242–12245.

Rosenberg, N.A., Nordborg, M. (2006) A general population-genetic model for the production by population structure of spurious genotype-phenotype associations in discrete, admixed or spatially distributed populations. *Genetics*, 173, 1665–1678.

Röttgers, S. (2002) *Phylogenie und Genexpression der Glutamat-Synthase (GOGAT) in verschiedenen Algengruppen*. Ph.D. dissertation, Justus-Liebig-Universität Gießen, 132 pp.

Round, F.E., Crawford, R.M., Mann, D.G. (1990) *The diatoms: Biology and morphology of the genera*. Cambridge University Press, Cambridge, 758 pp.

Roy, S., Chattopadhyay, J. (2007) Towards a resolution of 'the paradox of the plankton': A brief overview of the proposed mechanisms. *Ecological Complexity*, 4, 26–33.

Rozen, S., Skaletsky, H.J. (2000) PRIMER3 on the WWW for general users and for biologist programmers. In: Krawetz, S., Misener, S. [Eds.] *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Humana Press, Totowa, NJ, pp. 365–386.

Ruiz Sebastián, C., Etheridge, S.M., Cook, P.A., O'Ryan, C., Pitcher, G.C. (2005) Phylogenetic analysis of toxic *Alexandrium* (Dinophyceae) isolates from South Africa: Implications for the global phylogeography of the *Alexandrium tamarense* species complex. *Phycologia*, 44, 49–60.

Rynearson, T.A., Armbrust, E.V. (2000) DNA fingerprinting reveals extensive genetic diversity in a field population of the centric diatom *Ditylum brightwellii*. *Limnology and Oceanography*, 45, 1329–1340.

Rynearson, T.A., Armbrust, E.V. (2004) Genetic differentiation among populations of the planktonic marine diatom *Ditylum brightwellii* (Bacillariophyceae). *Journal of Phycology*, 40, 34–43.

Rynearson, T.A., Armbrust, E.V. (2005) Maintenance of clonal diversity during a spring bloom of the centric diatom *Ditylum brightwellii*. *Molecular Ecology*, 14, 1631–1640.

Sáez, A.G., Probert, I., Geisen, M., Quinn, P., Young, J.R., Medlin, L.K. (2003) Pseudo-cryptic speciation in coccolithophores. *Proceedings of the National Academy of Sciences of the United States of America*, 100, 7163–7168.

Sako, Y., Adachi, M., Ishida, Y. (1993) Preparation and characterization of monoclonal antibodies to *Alexandrium* species. In: Smayda, T.J., Shimizu, Y. [Eds.] *Toxic Phytoplankton Blooms in the Sea*. Elsevier, Amsterdam, pp. 87–93.

Sako, Y., Kim, C.H., Ishida, Y. (1992) Mendelian inheritance of paralytic shellfish poisoning toxin in the marine dinoflagellate *Alexandrium catenella*. *Bioscience, Biotechnology and Biochemistry*, 56, 692–694.

Sako, Y., Kim, C.H., Ninomiya, H., Adachi, M., 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.

Sako, Y., Naya, N., Yoshida, T., Kim, C.H., Uchida, A., Ishida, Y. (1995) Studies on stability and heredity of PSP toxin composition in the toxic dinoflagellate *Alexandrium*. In: Sako, Y., et al. [Eds.] *Harmful Marine Algal Blooms*. Lavoisier, Paris, pp. 345–350.

Saldarriaga, J.F., Taylor, F., Cavalier-Smith, T., Menden-Deuer, S., Keeling, P.J. (2004) Molecular data and the evolutionary history of dinoflagellates. *European Journal of Protistology*, 40, 85–111.

Santos, S.R., Coffroth, M.A. (2003) Molecular genetic evidence that dinoflagellates belonging to the genus *Symbiodinium* Freudenthal are haploid. *Biological Bulletin*, 204, 10–20.

Sarno, D., Kooistra, W.H.C.F., Balzano, S., Hargraves, P.E., Zingone, A. (2007) Diversity in the genus *Skeletonema* (Bacillariophyceae): III. Phylogenetic position and morphological variability of *Skeletonema costatum* and *Skeletonema grevillei*, with the description of *Skeletonema ardens* sp. nov. *Journal of Phycology*, 43, 156–170.

Sarno, D., Kooistra, W.H.C.F., Medlin, L.K., Percopo, I., Zingone, A. (2005) Diversity in the genus *Skeletonema* (Bacillariophyceae). II. An assessment of the taxonomy of *S. costatum*-like species with the description of four new species. *Journal of Phycology*, 41, 151–176.

Schantz, E.J., Ghazarossian, V.E., Schnoes, H.K., Strong, F.M., Springer, J.P., Pezzanite, J.O., Clardy, J. (1975a) Paralytic poisons from marine dinoflagellates. In: LoCicero, V.R. [Ed.] *Proceedings of the First International Conference on Toxic Dinoflagellate Blooms*. Massachusetts Science and Technology Foundation, Wakefield, MA, pp. 267–274.

Schantz, E.J., Ghazarossian, V.E., Schnoes, H.K., Strong, F.M., Springer, J.P., Pezzanite, J.O., Clardy, J. (1975b) The structure of saxitoxin. *Journal of the American Chemical Society*, 97, 1238–1239.

Schantz, E.J., Lynch, J.M., Vayvada, G., Matsumoto, K., Rapoport, H. (1966) The purification and characterization of the poison produced by *Gonyaulax catenella* in axenic culture. *Biochemistry*, 5, 1191–1195.

Schantz, E.J., Mold, J.D., Stanger, D.W., Shavel, J., Riel, F.J., Bowden, J.P., Lynch, J.M., Wyler, R.S., Riegel, B., Sommer, H. (1957) Paralytic shellfish poison. VI. A procedure for the isolation and purification of the poison from toxic clam and mussel tissues. *Journal of the American Chemical Society*, 79, 5230–5235.

Schmidt, L.E., 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.

Schmidt, R.J., Loeblich III, A.R. (1979a) A discussion of the systematics of toxic *Gonyaulax* species containing paralytic shellfish poison. In: Taylor, D.L., Seliger, H.H. [Eds.] *Toxic Dinoflagellate Blooms, Proceedings of the Second Conference on Toxic Dinoflagellate Bloooms*. Elsevier, New York, pp. 83–88.

Schmidt, R.J., Loeblich III, A.R. (1979b) Distribution of paralytic shellfish poison among Pyrrhophyta. *Journal of the Marine Biological Association of the United Kingdom*, 59, 479-487.

Schneider, S., Roessli, D., Excoffier, L. (2000) *Arlequin: A software for population genetics data analysis. Ver 2.000*. Genetics and Biometry Laboratory, Department of Anthropology, University of Geneva, 111 pp.

Schnepf, E., Elbrächter, M. (1999) Dinophyte chloroplasts and phylogeny – A review. *Grana*, 38, 81–97.

Scholin, C., Herzog, A.M., Sogin, M., Anderson, D.M. (1994) Identification of group- and strain-specific genetic markers for globally distributed *Alexandrium* (Dinophyceae). II. Sequence analysis of a fragment of the LSU rDNA gene. *Journal of Phycology*, 30, 999–1011.

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, Berlin, Heidelberg, New York, pp. 13–27.

Scholin, C.A., Anderson, D.M. (1994) Identification of group- and strain-specific genetic markers for globally distributed *Alexandrium* (Dinophyceae). I. RFLP analysis of SSU rRNA genes. *Journal of Phycology*, 30, 744–754.

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., 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., 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.

Selander, E., Cervin, G., Pavia, H. (2008) Effects of nitrate and phosphate on grazer-induced toxin production in *Alexandrium minutum*. *Limnology and Oceanography*, 53, 523–530.

Selander, E., Thor, P., Toth, G., Pavia, H. (2006) Copepods induce paralytic shellfish toxin production in marine dinoflagellates. *Proceedings of the Royal Society of London, Series B: Biological Sciences*, 273, 1673–1680.

Sellner, K.G., Doucette, G.J., Kirkpatrick, G.J. (2003) Harmful algal blooms: Causes, impacts and detection. *Journal of Industrial Microbiology and Biotechnology*, 30, 383–406.

Sham, P. (2001) Shifting paradigms in gene-mapping methodology for complex traits. *Pharmacogenomics*, 2, 195–202.

Sham, P.C., Zhao, J.H., Curtis, D. (2000) The effect of marker characteristics on the power to detect linkage disequilibrium due to single or multiple ancestral mutations. *Annals of Human Genetics*, 64, 161–169.

Shankle, A.M. (2001) *Population variability in the red tide forming dinoflagellate, Prorocentrum micans.* Ph.D. dissertation, University of California, San Diego, 130 pp.

Shankle, A.M., Mayali, X., Franks, P.J.S. (2004) Temporal patterns in population genetic diversity of *Prorocentrum micans* (Dinophyceae). *Journal of Phycology*, 40, 239–247.

Shimizu, Y. (1979) Developments in the study of paralytic shellfish toxins. In: Taylor, D.L., Seliger, H.H. [Eds.] *Toxic Dinoflagellate Blooms, Proceedings of the Second International Conference on Toxic Dinoflagellate Blooms*. Elsevier, New York, pp. 321–326.

Shimizu, Y. (1996) Microalgal metabolites: A new perspective. *Annual Review of Microbiology*, 50, 431–465.

Shimizu, Y. (2000) Chemistry and mechanism of action. In: Botana, L.M. [Ed.] *Seafood and Freshwater Toxins: Pharmacology, Physiology and Detection*. Marcel Dekker, New York, pp. 151–172.

Shimizu, Y., Alam, M., Fallon, W.E. (1975a) Purification and partial characterization of toxins from poisonous clams. In: LoCicero, V.R. [Ed.] *Proceedings of the First International Conference on Toxic Dinoflagellate Blooms*. Massachusetts Science and Technology Foundation, Wakefield, Mass., pp. 275–285.

Shimizu, Y., Alam, M., Oshima, Y., Buckley, L.J., Fallon, W.E., Kasai, H., Miura, I., Gullo, V.P., Nakanishi, K. (1977) Chemistry and distribution of deleterious dinoflagellate toxins. In: Faulkner, D.J., Fenical, W.H. [Eds.] *Marine Natural Products Chemistry*. Plenum Press, New York, pp. 261–269.

Shimizu, Y., Alam, M., Oshima, Y., Fallon, W.E. (1975b) Presence of four toxins in red tide infested clams and cultured *Gonyaulax tamarensis* cells. *Biochemical and Biophysical Research Communications*, 66, 731–737.

Shimizu, Y., Hsu, C.P., Fallon, W.E., Oshima, Y., Miura, I., Nakanishi, K. (1978) Structure of neosaxitoxin. *Journal of the American Chemical Society*, 100, 6791–6793.

Simon, N., Cras, A.L., Foulon, E., Lemee, R. (2009) Diversity and evolution of marine phytoplankton. *Comptes Rendus Biologies*, 332, 159–170.

Skovgaard, A. (1996) Engulfment of *Ceratium* spp. (Dinophyceae) by the thecate photosynthetic dinoflagellate *Fragilidium subglobosum*. *Phycologia*, 35, 490–499.

Skovgaard, A., Hansen, P.J. (2003) Food uptake in the harmful alga *Prymnesium parvum* mediated by excreted toxins. *Limnology and Oceanography*, 48, 1161–1166.

Slate, J. (2005) Quantitative trait locus mapping in natural populations: progress, caveats and future directions. *Molecular Ecology*, 14, 363–379.

Slatkin, M. (1994) Linkage disequilibrium in growing and stable populations. *Genetics*, 137, 331–336.

Smayda, T.J. (1980) Phytoplankton species succession. In: Morris, I. [Ed.] *The Physiological Ecology of Phytoplankton*. Blackwell Scientific Publications, Oxford, pp. 493–570.

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

Smayda, T.J. (1997) Harmful algal blooms: Their ecophysiology and general relevance to phytoplankton blooms in the sea. *Limnology and Oceanography*, 42, 1137–1153.

Smayda, T.J. (2002) Adaptive ecology, growth strategies and the global bloom expansion of dinoflagellates. *Journal of Oceanography*, 58, 281–294.

Smayda, T.J. (2006) *Harmful Algal Bloom Communities in Scottish Coastal Waters: Relationship to Fish Farming and Regional Comparisons – A Review*. Scottish Executive Environment Group, Edinburgh, UK, 219 pp.

Smayda, T.J. (2007) Reflections on the ballast water dispersal – harmful algal bloom paradigm. *Harmful Algae*, 6, 601–622.

Smayda, T.J., Reynolds, C.S. (2001) Community assembly in marine phytoplankton: Application of recent models to harmful dinoflagellate blooms. *Journal of Plankton Research*, 23, 447–461.

Smayda, T.J., Reynolds, C.S. (2003) Strategies of marine dinoflagellate survival and some rules of assembly. *Journal of Sea Research*, 49, 95–106.

Smetacek, V. (2001) A watery arms race. *Nature*, 411, 745.

Smith, E.A., Mackintosh, F.H., Grant, F., Gallacher, S. (2002) Sodium channel blocking (SCB) activity and transformation of paralytic shellfish toxins (PST) by dinoflagellate-associated bacteria. *Aquatic Microbial Ecology*, 29, 1–9.

Smith, H. (2008) Development of microsatellite markers in the noxious red tide-causing dinoflagellate *Heterocapsa circularisquama* (Dinophyceae). *Molecular Ecology Resources*, 8, 469–469.

Sokal, R.R., Rohlf, F.J. (1995) *Biometry: the principles and practice of statistics in biological research*, 3rd ed. Freeman, New York, NY, 880 pp.

Sommer, H., Meyer, K.F. (1937) Paralytic shellfish poisoning. *Archives of Pathology*, 24, 560–598.

Sorokin, Y.I., Sorokin, P.Y., Ravagnan, G. (1996) On an extremely dense bloom of the dinoflagellate *Alexandrium tamarense* in lagoons of the PO river delta: Impact on the environment. *Journal of Sea Research*, 35, 251–255.

Soudek Jr., D., Robinson, G.G.C. (1983) Electrophoretic analysis of the species and population structure of the diatom *Asterionella formosa*. *Canadian Journal of Botany*, 61, 418–433.

Sournia, A., Chretiennot-Dinet, M.J., Ricard, M. (1991) Marine phytoplankton: How many species in the world ocean? *Journal of Plankton Research*, 13, 1093–1099.

Stolte, W., Panosso, R., Gisselson, L.A., Graneli, E. (2002) Utilization efficiency of nitrogen associated with riverine dissolved organic carbon (>1 kDa) by two toxin-producing phytoplankton species. *Aquatic Microbial Ecology*, 29, 97–105.

Strom, S. (2002) Novel interactions between phytoplankton and microzooplankton: Their influence on the coupling between growth and grazing rates in the sea. *Hydrobiologia*, 480, 41–54.

Sugg, L.M., VanDolah, F.M. (1999) No evidence for an allelopathic role of okadaic acid among ciguatera-associated dinoflagellates. *Journal of Phycology*, 35, 93–103.

Sullivan, J.J., Wekell, M.M., Kentala, L.L. (1985) Application of HPLC for the determination of PSP toxins in shellfish. *Journal of Food Science*, 50, 26–30.

Sullivan, J.M., Donaghay, P.L., Rines, J.E.B. (in press) Coastal thin layer dynamics: Consequences to biology and optics *Continental Shelf Research*.

Sunda, W.G., Granéli, E., Gobler, C.J. (2006) Positive feedback and the development and persistence of ecosystem disruptive algal blooms. *Journal of Phycology*, 42, 963–974.

Swofford, D.L., Olsen, G.J., Waddell, P.J., Hillis, D.M. (1996) Phylogenic inference. In: Hillis, D.M., Moritz, C., Mable, B.K. [Eds.] *Molecular Systematics*. Sinauer, Sunderland, MA, pp. 407–514.

Tautz, D., Renz, M. (1984) Simple sequences are ubiquitous repetitive components of eukaryotic genomes. *Nucleic Acids Research*, 12, 4127–4138.

Taylor, F.J.R. (1975) Taxonomic difficulties in red tide and paralytic shellfish poison studies: The "tamarensis complex" of *Gonyaulax*. *Environmental Letters*, 9, 103–119.

Taylor, F.J.R. (1976) Dinoflagellates from the International Indian Ocean Expedition. A report on material collected by the R.V. "Anton Bruun" 1963–1964. *Bibliotheca Botanica* 132, 1–234.

Taylor, F.J.R. (1979) The toxigenic gonyaulacoid dinoflagellates. In: Taylor, D.L., Seliger, H.H. [Eds.] *Toxic Dinoflagellate Blooms, Proceedings of the Second International Conference on Toxic Dinoflagellate Blooms*. Elsevier, New York, pp. 47–56.

Taylor, F.J.R. (1984) Toxic dinoflagellates: Taxonomic and biogeographic aspects with emphasis on *Protogonyaulax*. In: Ragelis, E.P. [Ed.] *Seafood Toxins*. American Chemical Society, Washington, D.C. (Proc. Symp. at 186th ACS Conf.), pp. 77–97.

Taylor, F.J.R. (1993) The species problem and its impact on harmful phytoplankton studies. In: Smayda, T.J., Shimizu, Y. [Eds.] *Toxic Phytoplankton Blooms in the Sea*. Elsevier, Amsterdam, pp. 81–86.

Taylor, F.J.R., Pollingher, U. (1987) Ecology of dinoflagellates. In: Taylor, F.J.R. [Ed.] *The biology of dinoflagellates*. Blackwell Scientific Publications, Oxford, pp. 399–529.

Taylor, J.R. (1997) *An introduction to error analysis: the study of uncertainties in physical measurements*. University Science Books, Sausalito, CA, 327 pp.

Thielert, G., Kaiser, I., Luckas, B. (1991) HPLC determination of PSP toxins. In: Fremy, J.M. [Ed.] *Proceedings of symposium on marine biotoxins*. Editions CNEVA, Maisons-Alfort, pp. 121–125.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G. (1997) The ClustalX windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, 25, 4876–4882.

Thornton, D.C.O. (2002) Individuals, clones or groups? Phytoplankton behaviour and units of selection. *Ethology Ecology & Evolution*, 14, 165–173.

Tillmann, U. (2003) Kill and eat your predator: A winning strategy of the planktonic flagellate *Prymnesium parvum*. *Aquatic Microbial Ecology*, 32, 73–84.

Tillmann, U. (2004) Interactions between planktonic microalgae and protozoan grazers. *Journal of Eukaryotic Microbiology*, 51, 156–168.

Tillmann, U., Alpermann, T., da Purificação, R.C., Krock, B., Cembella, A. (2009) Intra-population clonal variability in allelochemical potency of the toxigenic dinoflagellate *Alexandrium tamarense*. *Harmful Algae*, 8, 759–769.

Tillmann, U., Alpermann, T., John, U., Cembella, A. (2008a) Allelochemical interactions and short-term effects of the dinoflagellate *Alexandrium* on selected photoautotrophic and heterotrophic protists. *Harmful Algae*, 7, 52–64.

Tillmann, U., John, U. (2002) Toxic effects of *Alexandrium* spp. on heterotrophic dinoflagellates: An allelochemical defence mechanism independent of PSP-toxin content. *Marine Ecology Progress Series*, 230, 47–58.

Tillmann, U., John, U., Cembella, A.D. (2007) On the allelochemical potency of the marine dinoflagellate *Alexandrium ostenfeldii* against heterotrophic and autotrophic protists. *Journal of Plankton Research*, 29, 527–543.

Tillmann, U., John, U., Krock, B., Cembella, A.D. (2008b) Allelopathic effects of bioactive compounds produced by harmful algae. In: Moestrup, Ø., et al. [Eds.] *Proceedings of the 12th International Conference on Harmful Algae*. International Society for the Study of Harmful Algae and Intergovernmental Oceanographic Commission of UNESCO, Copenhagen, pp. 12–18.

Tivang, J.G., Nienhuis, J., Smith, O.S. (1994) Estimation of sampling variance of molecular marker data using the bootstrap procedure. *Theoretical and Applied Genetics*, 89, 259–264.

Toriumi, S., Takano, H. (1979) Some species of *Gonyaulax* of the "catenella group" occurring in Japanese coastal waters. In: Taylor, D.L., Seliger, H.H. [Eds.] *Toxic Dinoflagellate Blooms, Proceedings of the Second International Conference on Toxic Dinoflagellate Blooms*. Elsevier, New York, pp. 57–60.

Townsend, D.W., Bennett, S.L., Thomas, M.A. (2005a) Diel vertical distributions of the red tide dinoflagellate *Alexandrium fundyense* in the Gulf of Maine. *Deep-Sea Research Part II: Topical Studies in Oceanography*, 52, 2593–2602.

Townsend, D.W., Pettigrew, N.R., Thomas, A.C. (2005b) On the nature of *Alexandrium fundyense* blooms in the Gulf of Maine. *Deep-Sea Research Part II: Topical Studies in Oceanography*, 52, 2603-2630.

Turner, J.T., Tester, P.A., Hansen, P.J. (1998) Interactions between toxic marine phytoplankton and metazoan and protistan grazers. In: Anderson, D.M., Cembella, A.D., Hallegraeff, G.M. [Eds.] *Physiological Ecology of Harmful Algae Blooms*. Springer, Berlin, Heidelberg, New York, pp. 453–474.

van Oppen, M.J.H., Klerk, H., Olsen, J.L., Stam, W.T. (1996) Hidden diversity in marine algae: Some examples of genetic variation below the species level. *Journal of the Marine Biological Association of the United Kingdom*, 76, 239–242.

Vanormelingen, P., Chepurnov, V.A., Mann, D.G., Sabbe, K., Vyverman, W. (2008) Genetic divergence and reproductive barriers among morphologically heterogeneous sympatric clones of *Eunotia bilunaris* sensu lato (Bacillariophyta). *Protist*, 159, 73–90.

Vekemans, X., Beauwens, T., Lemaire, M., Roldan-Ruiz, I. (2002) Data from amplified fragment length polymorphism (AFLP) markers show indication of size homoplasy and of a relationship between degree of homoplasy and fragment size. *Molecular Ecology*, 11, 139–151.

Vila, M., Camp, J., Garcés, E., Maso, M., Delgado, M. (2001a) High resolution spatio-temporal detection of potentially harmful dinoflagellates in confined waters of the NW Mediterranean. *Journal of Plankton Research*, 23, 497–514.

Vila, M., Garcés, E., Maso, M., Camp, J. (2001b) Is the distribution of the toxic dinoflagellate *Alexandrium catenella* expanding along the NW Mediterranean coast? *Marine Ecology Progress Series*, 222, 73–83.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., Zabeau, M. (1995) AFLP: A new technique for DNA fingerprinting. *Nucleic Acids Research*, 23, 4407–4414.

Wang, C.H., Ho, A.Y.T., Qian, P.Y., Wong, P.K., Hsieh, D.P.H. (2004) Antibiotic treatment enhances C2 toxin production by *Alexandrium tamarense* in batch cultures. *Harmful Algae*, 3, 21–28.

Watras, C.J., Chisholm, S.W., Anderson, D.M. (1982) Regulation of growth in an estuarine clone of *Gonyaulax tamarensis* Lebour: Salinity-dependent temperature responses. *Journal of Experimental Marine Biology and Ecology*, 62, 25–37.

Whedon, W.F., Kofoid, C.A. (1936) Dinoflagellata of the San Francisco Region. I. On the skeletal morphology of two new species, *Gonyaulax catenella* and *G. acatenella*. *University of California Publications in Zoology*, 41, 25–34.

Wichmann, C.F., Niemczura, W.P., Schnoes, H.K., Hall, S., Reichardt, P.B., Darling, S.D. (1981) Structures of two novel toxins from *Protogonyaulax*. *Journal of the American Chemical Society*, 103, 6977–6978.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A., Tingey, S.V. (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research*, 18, 6531–6535.

Windust, A.J., Wright, J.L.C., McLachlan, J.L. (1996) The effects of the diarrhetic shellfish poisoning toxins, okadaic acid and dinophysistoxin-1, on the growth of microalgae. *Marine Biology*, 126, 19–25.

Wood, A.M., Leatham, T. (1992) The species concept in phytoplankton ecology. *Journal of Phycology*, 28, 723–729.

Work, T.M., Beale, A.M., Fritz, L., Quilliam, M.A., Silver, M., Buck, K., Wright, J.L.C. (1993) Domoic acid intoxication of brown pelicans and cormorants in Santa Cruz, California. In: Smayda, T.J., Shimizu, Y. [Eds.] *Toxic Phytoplankton Blooms in the Sea*. Elsevier, Amsterdam, pp. 643–649.

Wright, J.L.C., Cembella, A.D. (1998) Ecophysiology and biosynthesis of polyether marine biotoxins. In: Anderson, D.M., Cembella, A.D., Hallegraeff, G.M. [Eds.] *Physiological Ecology of Harmful Algal Blooms*. Springer, Berlin, Heidelberg, New York, pp. 427–451.

Wright, S. (1978) *Variability within and among natural populations, Vol.4. Evolution and the genetics of populations: A treatise in four volumes*. Univ. Chicago Press, Chicago, 580 pp.

Wyatt, T. (1990) Do algal blooms play homeostatic roles? In: Granéli, E., Sundström, B., Edler, L., Anderson, D.M. [Eds.] *Toxic Marine Phytoplankton*. Elsevier, New York, pp. 249–252.

Wyatt, T. (1995) Global spreading, time series, models and monitoring. In: Lassus, P., Arzul, G., Erard-Le Denn, E., Gentien, P., Marcaillou LeBaut, C. [Eds.] *Harmful Marine Algal Blooms*. Lavoisier, Paris, pp. 755–764.

Wyatt, T., Jenkinson, I.R. (1997) Notes on *Alexandrium* population dynamics. *Journal of Plankton Research*, 19, 551–575.

Xu, F.X., Sun, M. (2001) Comparative analysis of phylogenetic relationships of grain amaranths and their wild relatives (Amaranthus; Amaranthaceae) using internal transcribed spacer, amplified fragment length polymorphism, and double-primer fluorescent intersimple sequence repeat markers. *Molecular Phylogenetics and Evolution*, 21, 372–387.

Yamamoto, T., Tarutani, K. (1999) Growth and phosphate uptake kinetics of the toxic dinoflagellate *Alexandrium tamarense* from Hiroshima Bay in the Seto Inland Sea, Japan. *Phycological Research*, 47, 27–32.

Yoshida, T., Jones, L.E., Ellner, S.P., Fussmann, G.F., Hairston, N.G. (2003) Rapid evolution drives ecological dynamics in a predator-prey system. *Nature*, 424, 303–306.

Yoshida, T., Sako, Y., Uchida, A. (2001) Geographic differences in paralytic shellfish poisoning toxin profiles among Japanese populations of *Alexandrium tamarense* and *A. catenella* (Dinophyceae). *Phycological Research*, 49, 13–21.

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.