Bloom dynamics and population genetics of marine phytoplankton — Community, species and population aspects

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Abstract

Phytoplankton are the most important primary producers in the world's oceans and coastal waters, accounting for nearly half of the global net primary production. Although they are such important organisms, little is known about the ecology and dynamics of phytoplankton. The importance of phytoplankton resting stages, the coupling between cells in the sediment and watermass and how environmental changes affect the population structure is uncertain. The record of a specific species in a given area is foregone by either advection of the species from adjacent areas, or by growth of a few cells present in the water. Many groups of phytoplankton have the ability to form resting stages to provide short- or long-term survival, and these stages can be resuspended and subsequently germinate and thereby be re-established in the water mass.

Diatoms constitute the single largest group of microalgae and they are mainly marine but found in all aquatic environments. Diatom blooms can develop fast, and they can grow at low levels of light, which gives the cells an advantage during spring blooms in temperate areas where light is a limiting factor. To successfully meet selective pressure in a variety of ecological niches, it is hypothesized that diatoms display high phenotypic and genetic diversity. *Skeletonema marinoi* (Sarno et Zingone) is a marine diatom, often dominating temperate coastal waters during spring bloom. The mechanisms for dispersal and expansion of populations of this species are, as for most diatoms, complex and difficult to predict. Possibly the presence of different populations at different seasons is caused by strong directional selection in a continuously growing population, or by a complete replacement of one population by another.

The general aim of this thesis was to study marine phytoplankton dynamics at community, species and population level, and we used *S. marinoi* as model organism for the population studies. In order to perform some investigations, appropriate methods have been developed. I have focused on the interaction between water mass and sediment, both in temperate waters and in a tropical area, investigated the importance of resting stages and small-scale hydrographical changes for the phytoplankton community structure as well as population genetics and microevolutional processes of population dynamics.

The results from a tropical area show that benthic resting stages contribute to blooms by resuspension, germination, and proliferation as planktonic cells in the water column, and thus, the cells can influence the phytoplankton community in the water column. There can be an alternation of the species composition if a plankton community is seeded by resting stages or by planktonic cells, and geographically the strategies of seeding can differ within the same species. The composition of the phytoplankton community is exceedingly affected by small-scale hydrographic changes and several of these factors are potentially tightly coupled. These changes have implications on the sampling, and therefore frequent sampling is important.

When clones of *S. marinoi* were examined, the morphological character defining another species of the same genus–*Skeletonema dohrnii* (Sarno et Kooistra)–was found in most of the clones. The phylogenetic variation in LSU rDNA in the *S. marinoi* clade were of the same magnitude or greater than differences between *S. dohrnii* and *S. marinoi.* The two species are not suggested to be merged since there may be a separation in the biogeographical distribution of the two species.

A series of molecular methods were used to study various aspects of phytoplankton ecology. For estimating the proportion of dinoflagellate versus diatom biovolume or biomass and the absolute diatom biovolume or biomass, real-time PCR technique constitutes a quick and accurate method. Another useful tool is microsatellite markers, and the characterisation and development of primers enabled the study of population genetics of *S. marinoi.* Resting stages from undisturbed and dated sediment cores from a fjord with anoxic bottom conditions, were germinated and cultures established. The fjord has during a few decades been hypereutrophicated and populations found during this time were significantly different from populations found before and after. The post- and pre-eutrophication populations showed no significant genetic difference. Environmental changes may favour only some populations from a pool of several different populations at a specific location, and maybe other changes would favour different populations.

Keywords: Phytoplankton, dynamics, *Skeletonema marinoi*, population genetics, resting stages

"The least movement is of importance to all nature. The entire ocean is affected by a pebble."

- Blaise Pascal

List of papers

This thesis is based on the following papers, referred to in the text by their roman numerals.

I **Härnström, K.**, Godhe, A., Saravanan, V., Karunasagar, I., Karunasagar, I., Rehnstam-Holm, A.-S. (2007) Tropic phytoplankton community development - a study of mesocosms inoculated with different life stages. Marine Ecology Progress Series. 346: 75-88.

II **Härnström, K.**, Karunasagar, I., Godhe, A. (2009) Phytoplankton species assemblages and their relationship to hydrographic factors—a study at the old port in Mangalore, coastal Arabian Sea. Indian Journal of Marine Sciences. 38(2): 224-235.

III Godhe, A., Asplund, M., **Härnström, K.**, Saravanan, V., Tyagi, A., Karunasagar, I. (2008) Quantification of diatom and dinoflagellate biomasses in coastal marine seawater samples by Real-time PCR. Applied and Environmental Microbiology. 74(23): 7174-7182

IV Ellegaard, M., Godhe, A., **Härnström, K.**, McQuoid, M.R. (2008) The species concept in a marine diatom: LSU rDNA–based phylogenetic differentiation in *Skeletonema marinoi/dohrnii* (Bacillariophyceae) is not reflected in morphology. Phycologia. 47(2): 156-167.

V Godhe, A, **Härnström, K.**, Saravanan, V., Halldén, C., Karunasagar, I., Karunasagar, I., Microsatellite markers for the marine diatom *Skeletonema marinoi* (Bacillariophyceae). Molecular Ecology Resources. Accepted.

VI **Härnström, K.**, Ellegaard, M., Andersen, T.J., Godhe, A. Changes in genetic structure through time—the sediment archive of *Skeletonema*. Manuscript

A doctoral thesis at a university in Sweden is produced as a monograph or as a collection of papers. In the latter case, the introductory part constitutes the formal thesis, which summarizes the accompanying papers. These have already been published or are manuscripts at various stages (in press, submitted or in manuscript).

Paper I, III and IV are reprinted with kind permission from Inter-Research (I), American Society for Microbiology (III) and Allen Press Publishing Services (IV).

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1. Introduction

General

In the world's oceans and coastal waters, phytoplankton are the most important primary producers, and they account for nearly half of the world's net primary production (e.g. Field et al. 1998). Twenty years back, about 5000 marine phytoplankton species were described (Sournia et al. 1991), but this is most likely an underestimation. Species diversity in marine phytoplankton appears extremely low (Simon et al. 2008) and probably many species remain yet undiscovered or unidentified. Different groups of microalgae constitute the phytoplankton community and exist in wide ranges of sizes, shapes and functions. Several microalgal species are considered cosmopolitan, but this assumption might be revised since species are divided into a number of new species (e.g. Sarno et al. 2005) and large genetic and morphologic variation, even within species, is found (e.g. Amato et al. 2007, paper IV).

Even though phytoplankton are such important organisms, little is known about their ecology and dynamics such as distribution and genetic variation. Their small size, physiological differences, complex life cycles and three-dimensional fluctuating habitat might explain parts of the lack of knowledge. Many groups of phytoplankton have benthic resting stages as a part of their life cycle, and these are formed asexually or sexually (e.g. McQuoid & Hobson 1996). These stages are commonly known to provide short- or long-term survival, and may also contribute to natural dispersal by currents or when transported in ballast water tanks (Hallegraeff & Bolch 1992). A record of a specific species in a given area is foregone by either advection of the species from adjacent areas, growth of a few cells present in the water, or resuspension and subsequent germination of resting stages. Exponentially growing cells could possibly be transported into the surface waters of an area and immediately result in a large population of a species (Godhe et al. 2002b). Some of the vegetative cells in a given area may survive stress, and these cells can grow and accumulate once favourable conditions are re-established (Backhaus et al. 1999, 2003). Benthic resting stages may also act as an important inoculation source for initiating planktonic blooms, and this phenomenon is known from neritic regions (Tommasa et al. 2000). Here, accumulation areas of resting stages are present, and the cells can be brought up to the surface water by currents or upwelling.

A series of molecular methods, usually developed for other areas of interest, have recently been applied on phytoplankton population ecology, often with satisfying results, and one widely used tool is microsatellite markers (Rynearson & Armburst 2000, Evans et al. 2004, Nagai et al. 2004, Nagai et al. 2006). Several authors have by this means caught a glimpse of phytoplankton population ecology, but still many questions remain to be answered. I have tried to contribute to this knowledge by studying phytoplankton from community to population level, and integrated molecular methods in many of my experiments (paper III, IV, V and VI), in order to study relative biomass, interspecies variation and population dynamics.

Diatoms constitute the single largest group of microalgae. They are mainly marine but found in all aquatic environments. Diatoms are, with only a few exceptions, autotrophic organisms, and assimilate nutrients like phosphate, nitrate and silica from their surroundings. They are often associated with turbulent cold water, but can be found in any kind of temperature and under any conditions. Blooms can develop fast, and they are good at utilizing low levels of light, which gives them an advantage in temperate areas during spring blooms. All diatoms have a protecting silica wall (frustule). Most are unicellular, but many species have the ability to form chains. Many diatom species are known to form benthic resting stages (McQuoid & Hobson 1996), but the importance of these stages as seeding propagules for planktonic blooms is speculative. There are different kinds of diatom resting stages, such as spores (hypnospores), resting cells, and winter stages. Hereafter I refer to all these various types as resting stages. The process of forming resting stages is asexual, but with some exceptions were spores are originating from auxospores formed sexually (McQuoid & Hobson 1996).

An increasing number of scientists have emphasized the importance of diatom benthic life stages in terms of function and influence on water column dynamics (e.g. Raffaelli et al. 2003). As some diatom

species are able to bloom under a wide variety of environmental conditions, and many are known to have vast geographical distributions, it is hypothesized that they display high phenotypic and genetic diversity to successfully meet selective pressure in a variety of ecological niches. Although some studies have shown high levels of genetic diversity in diatoms (e.g. Rynearson et al. 2006), it is necessary to gain more knowledge about the amount and structure of genetic variation to further understand phytoplankton bloom composition and diversity, the role of resting stages or fugitive cells as propagules of genetic variation, and the temporal coupling between populations.

In paper I and II, I have mainly focused on community- and species structure of diatoms, but some parts also include dinoflagellates and other microalgae. Paper III includes both diatoms and dinoflagellates as groups, and paper IV, V and VI is particularly focusing on the diatom *Skeletonema marinoi* at species and population level.

Community structure

Phytoplankton communities in marine environments usually include several taxonomic groups, and these contribute to primary production and interaction between trophic levels (Roy et al. 2006). There are seasonal patterns in phytoplankton diversity and composition, but also in the magnitude of primary production. Changes in species composition and diversity might regulate the photosynthetic response to adjust to limiting factors (Duarte et al. 2006). It is crucial to accurately quantify phytoplankton biomass and to determine community composition, in order to better understand the dynamics of marine ecosystems. Compared to other marine systems, tropical waters have small seasonal changes, but despite this, brief increases in growth, different from the annual average, are frequent (Quasim et al. 1972). Freshwater influence is also known to have a profound effect on phytoplankton biomass, productivity, and community composition, in a variety of geographical areas and in different habitat types (Cloern et al. 1985). Short-term phytoplankton blooms are often triggered by differences in salinity, or due to water column stratification. Some areas are very dynamic because of short-term tidal variability, causing temporal change of the phytoplankton community (Almeida et al. 2002), but other factors, like exchange between sediment and water column, and zooplankton grazing, affect the species diversity as well (Carstensen et al. 2007).

Species

Most species of microalgae have high growth rates with dense abundance and high capacity of dispersal (Simon et al. 2008). The species composition of microalgae is known to change frequently, due to different photosynthetic and metabolic characteristics for each species (Jouenne et al. 2007). The species succession is to a large extent influenced by short-term changes, but these have often been neglected (Pannard et al. 2008, paper II). The microalgae are for example strongly affected by changes in pH (Hansen 2002), and nutrients (e.g. Ou et al. 2006) on community level as well as species level.

Genetic studies on microalgae have during the last decades revealed a wide cryptic diversity, but the ecological and evolutionary processes forming new species and how new species are maintained, are not yet understood (Simon et al. 2008). The species concept in diatoms is debated, and one suggested approach to species delimitation includes evidence of a suite of characters within morphology, genetic data, ecology, mating systems, physiology and mating behaviour (Mann 1999). The question of species delimitation and cryptic species within protists have recently being addressed in several studies (e.g. Beszteri et al. 2005, Lundholm et al. 2006). Usually these studies include data from both morphological and DNA sequence analysis, and often morphologically identical strains have differences in rDNA sequence (Beszteri et al. 2005, Lilly et al. 2005). In some cases, the sequence-based clades are defined as cryptic species (Beszteri et al. 2005), while this variation is accepted within a species in others (Sarno et al. 2005). The opposite case, where identical strains with regard to rDNA sequence were morphologically different, is rare but has been observed (Logares et al. 2007). It is difficult to weight morphological variation relative to variation in DNA sequence, and the question is how much

DNA sequence variation should be accepted within a species when describing new species or emending species descriptions. Among dinoflagellates, many genetic variants have been described as clades, ribotypes or as species complex, instead of species (e.g. Scholin et al. 1995, Santos et al. 2004). The diatom species *Skeletonema costatum* (Greville) Cleve has recently been emended, with several new species described (Sarno et al. 2005). One of the new species is *Skeletonema marinoi* (Sarno et Zingone), which contributes significantly to phytoplankton blooms in temperate waters. Species of the genus *Skeletonema* are occurring worldwide, and are wide-spread in both plankton and benthos (McQuoid 2002).

Skeletonema marinoi was chosen as model organism for population genetic- and morphological studies (paper IV-VI) for different reasons. It is frequently found and is reported to cause blooms in many parts of the world (e.g. Kooistra et al. 2008), easy to sample, isolate and keep in culture. The survival rate in culture is high——in my studies on average about 80%——and this high rate is important to avoid biased results. Another crucial issue for my studies with this species is its ability to form resting stages, and the possibility to germinate resting stages from the sediment, even after decades of dormancy. An additional advantage is that there is many reference cultures available, which enable global studies.

The different plausible seeding strategies, i.e. by cells originating from adjacent areas carried with current, by a few cells in watermass or through germination of resting stages reintroduced to the watermass, are all possible for *S. marinoi*. I have focused on the latter; the importance of coupling between watermass and sediment for the recruitment of resting stages. *Skeletonema marinoi* is found all the year round in temperate coastal waters, and has pronounced density peaks, especially during early spring bloom (e.g. Tallberg & Heiskanen 1998, therein reported as *S. costatum*). It forms benthic resting stages, capable of surviving for several decades buried in the sediments, where they can be very abundant (up to 3.5 million propagules g^{-1}, McQuoid et al. 2002). The study of this organism is of high ecologic relevance since *S. marinoi* constitutes a valuable food source for higher trophic levels. To use the genus *Skeletonema* in ecological studies as a model organism, it was necessary to address the question of species identification because of the splitting of the species *S. costatum*. It was previously considered cosmopolitan, and is now divided into several new species whereof one is *S. marinoi* (Sarno et al. 2005).

Population level

Skeletonema marinoi is a chain-forming diatom, very abundant and during spring bloom often dominating temperate coastal waters. It reproduces vegetatively, but formation of auxospores has been observed (e.g. Sutherland et al. 2001), indicating sexual reproduction. Having a life cycle with alternation between vegetative and sexual reproduction, as in *S. marinoi*, can be an obstacle when studying population dynamics, and genetically, this is a crucial factor having implications at both individual level and population level (Balloux et al. 2003). Phytoplankton cells divide asexually, approximately once per day, and selection for individual clonal lineages could be quite rapid, resulting in population differentiation (paper VI, Godhe & Härnström in prep). Different populations present at different seasons could be explained by strong directional selection in a continuously growing population, or by a complete replacement of one population by another (Gallagher 1982). Mechanisms for dispersal and expansion in several dinoflagellates have been revealed by identification of local populations and individuals using population genetic methods (Nagai et al. 2007). This is important, especially since globalization and new introduction of harmful microorganisms via cargo-vessel ballast water or translocation of shellfish stocks, have been considered as problems of deep concern.

2. Aims of the thesis

General

The overall aim of this thesis was to study phytoplankton dynamics comprehensively, from community to population level, and to do population genetic studies using a common diatom as model organism. I have especially focused on the interaction between water mass and sediment. Other issues highlighted include method development, phytoplankton community structure (focusing on diatoms), and genetic variability and microevolution of populations. These findings will increase the understanding of dynamics and ecological and microevolutional processes of phytoplankton.

Paper I

This mesocosm study was carried out in Mangalore, India, and the aim was to observe and compare the growth and species composition of tropical phytoplankton assemblages seeded with different life stages, with focus on diatoms. It is documented that resting stages in temperate waters contribute to blooms in the watermass. One would expect resting stages to play a significant role in these areas, since conditions are varying during the year, with distinct seasons, both favourable and unfavourable for diatoms. Do we find the same contribution in tropical waters, where seasons are less pronounced, and external conditions are less variable throughout the year? Do microalgae in this area have the ability to form resting stages?

Paper II

The purpose of this study was to investigate the taxonomic composition of coastal phytoplankton assemblages and how these are influenced by small-scale hydrographical factors.

Paper III

Here we described two new primer sets, targeting the small-subunit (SSU) ribosomal DNA (rDNA), to be used in real-time PCR assays for the quantification of both dinoflagellates and diatoms in samples from the field. Initially, the primers were tested on cultures, and later also applied on numerous field samples. This method is useful for estimating the proportion of dinoflagellate versus diatom biovolume or biomass and the absolute diatom biovolume or biomass.

Paper IV

We tested in paper IV, if rDNA and morphological data actually support the description of two new species of *Skeletonema*; *Skeletonema dohrnii* (Sarno et Kooistra) and *S. marinoi*. We studied genetic variation of the LSU rDNA within *S. marinoi* and evaluated morphological characters used to distinguish between the two morphologically similar *S. marinoi* and *S. dohrnii*.

Paper V

This paper reports on the characterization of eight polymorphic microsatellite loci in *S. marinoi*, in order to investigate the population dynamics of diatoms. Monoclonal cultures, isolated from the Swedish west coast, were genotyped, and we expected these loci to be useful in gathering information on genetic structure and gene flow among populations of *S. marinoi*. We needed a tool, precise enough to discriminate between populations of the same species, and microsatellites are commonly used markers in studies of populations or individuals in other organisms.

Paper VI

In paper VI we compared the population genetics in *S. marinoi*, using the microsatellite markers developed in paper V, and how this was affected by the environmental conditions in Mariager fjord over the past 100 years. ^{210}Pb dated undisturbed, sediment cores from the anoxic Mariager Fjord were

analysed. This is a novel kind of study on diatoms, investigating interactions between selection and environmental change, especially microevolutionary adaptation in response to changes in phosphorus loading.

3. Study areas and hydrography

India

Sampling and analyses in paper I and II were carried out in Mangalore, India (Fig. 1). The coastal waters off Mangalore provide for a multi-species fishery, and the area is known as one of the largest upwelling systems in the world, subsequently with high productivity (Krishnakumar & Bhat 2008). Phytoplankton productivity increases on a large scale during the southwest monsoon (June–September), when upwelling occurs, and during the cooling northeast monsoon (November–February)(Levy et al. 2007). The area has a variable abundance of both diatoms and dinoflagellates, often replaced by cyanobacteria (Krishnamurthy 1967, Parab et al. 2006) and changes in phytoplankton growth are seasonally driven primarily by the monsoons (Marra & Barber 2005). On annual basis, phytoplankton communities in marine tropical areas are known to be less dynamic than in temperate waters. The seasonal changes in net phytoplankton growth are smaller (Quasim et al. 1972) whereas the shorter scale variability occurs with brief pulses of increased growth, which are quite common. The Netravathi River enters the Arabian Sea approximately 2 km from Mangalore, and this area was the sampling site in paper II (Fig. 1).
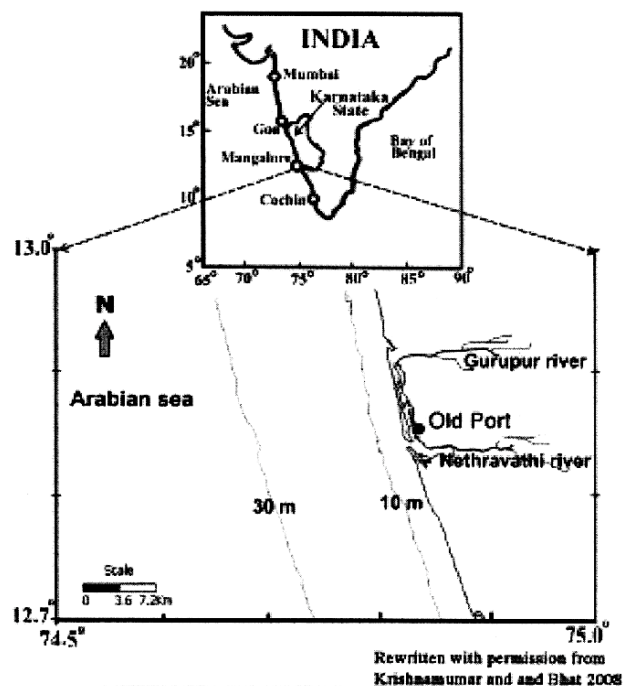

Fig. 1. Area of investigation and sampling site (Old Port), Mangalore, India.

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Sweden

Field samples for paper III and most samples in paper IV and V were collected on the Swedish west coast, in- or in connection with Gullmar Fjord (Fig. 2). The maximum depth of this fjord is more than 100 metres, it has an entrance sill of 43 metres and is known to have a rich bank of diatom propagules in the sediment (McQuoid 2002).The area is part of a fjord system, has strong thermohaline stratification and low tidal activity. The Gullmar Fjord area is partly anoxic and this preserves and laminates the sediment.
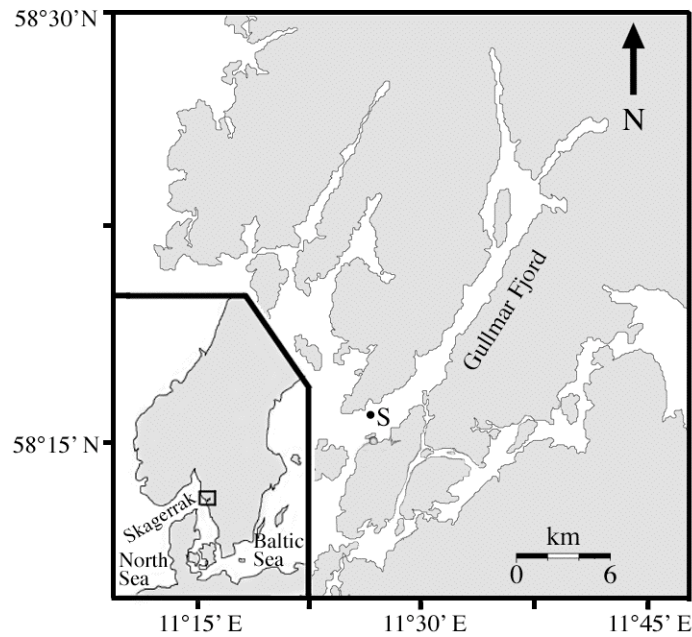
Fig. 2. Map of sampling site (S), Gullmar Fjord, Sweden.

Denmark

Mariager Fjord situated on the east coast of Jutland, Denmark (Fig. 3), is about 40 km long and a narrow sill-fjord, with high primary production and a permanently anoxic basin (Ellegaard et al. 2006). The fjord is temperate, highly eutrophic, and the eastern part is shallow with a maximum depth of 30 m. The mesozooplankton community in Mariager Fjord is sparse, but intense blooms of the marine diatom *S. marinoi*, with subsequent increases in chlorophyll *a* (Chl *a*) concentrations are frequent (Tiselius et al. 2008). It has previously been reported that the changes in species composition of diatoms and dinoflagellates are associable with an increased eutrophication in the area (Ellegaard et al. 2006). Sediment cores used in paper VI were collected from this site.
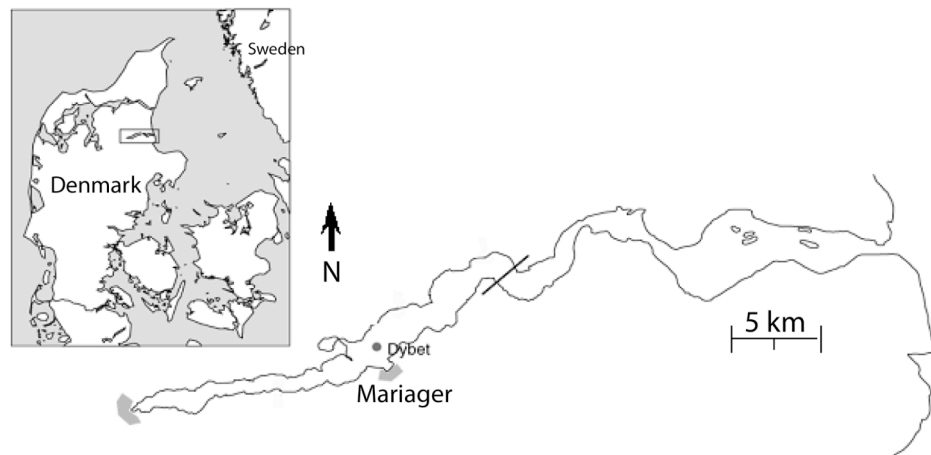

Fig. 3. Map of sampling site (Dybet), MariagerFjord, Denmark.

4. General methods

The methods are mainly based on mesocosm experiments, field samplings, isolation and cultivation of microalgae, real-time PCR, morphological studies, LSU sequence analyses and microsatellite marker analyses.

Sampling

Plankton

In paper I, IV and V, plankton nets (10-25 μm mesh size) were used to collect qualitative field samples of phytoplankton. This method provides high-density samples for e.g. inocula in mesocosms (paper I) and individual cell isolation (paper IV-V), but do not estimate the cell abundance in the water mass. At the Indian field station (paper I), phytoplankton at the sea surface were sampled from several net tows and pooled into bottles and later added to mesocosms. In paper IV and V phytoplankton samples were collected from the surface (0- to 3-m depth) with plankton net or water bottles at Kristineberg Marine Research Station, and thereafter, single cells were isolated by micropipetting and monoclonal cultures were established.

In paper I (samples taken from the mesosocsms) paper II, and paper III quantitative sampling was carried out in order to quantify and identify phytoplankton. Samples were collected by plastic ladles (paper I) or by a water sampler (paper II and III), fixed in 1% Lugol's solution, and settled in sediment

chambers with known volumes. In paper III, IV and V, pre-established monoclonal cultures were also used in the experiments.

Sediment
Sediment samples for paper I was collected using a gravity corer at a water depth of 30 m, and sediment inocula was added to the mesocosms to simulate resuspension of top sediment from a seafloor area of 1 m^2. In paper IV, sediment cores were collected and the top centimetre of sediment was sampled and kept dark and cool until processed. For paper VI, sediment cores was taken in Mariager Fjord, using a modified HAPS corer, kept cold, and sliced at the time of analyse.

Analyses

Environment
In paper I and II, various environmental factors were analysed. On frequent basis we monitored temperature, salinity, pH, nitrate, silica, ammonia, phosphate, oxygen, Chl *a*, estimations of bacteria, tidal stage (paper II) and phytoplankton were identified and quantified. In both papers the analyses were a fundament in order to compare the environmental parameters with the phytoplankton community.

PCR and sequencing, fragment analysis
In paper III-VI, extracted DNA from monoclonal cultures of *Skeletonema* (and the dinoflagellate *Prorocentrum lima*, paper III) was used. In paper III primers were developed to target diatoms and dinoflagellates, and specificity of these primers was tested. We amplified the small subunit (SSU) rDNA in *S. marinoi* and *P. lima* in order to prepare plasmid standards to be used in the real-time PCR. SSU rDNA is an important marker for the understanding of species origin and diversity. The conventional PCR identifies if the target DNA is present, but the real-time PCR detect the target DNA *and* quantify it. After the protocol was designed using monoclonal cultures, we preformed real-time analysis on field samples. In paper IV, large subunit (LSU) rDNA domains D1-D3 were amplified by PCR with subsequent sequencing. LSU is, just like SSU, a marker used for phylogenetical analyses.

To gain information in the population structure in *S. marinoi*, we developed microsatellite marker for this species (paper V). Genomic DNA from cultures were processed following a protocol based on Bloor et al. (2001). Primers were designed to flanking regions of the microsatellite repeat. After the primers successfully had been tested, they were used on monoclonal cultures, and the sizes of the microsatellite fragments were determined (paper V and VI). After extraction of DNA from the cultures established from undisturbed sediment cores, and subsequent measure of concentration and amplifying PCR, the fragment analysis was carried out for totally eight microsatellite loci (paper VI).

Statistics
Comparisons of phytoplankton development among the different inocula in paper I were made with single-factor ANOVA. To trace changes in the community of the different treatments, Canonical Correspondence Analysis (CCA) was used. The software, CANOCO, is an acronym for CANOnical Community Ordination, and this method relates the species composition in communities to the environment (ter Braak 1990) . The ordination plots given after analysis displays in an optimal way the community composition variation in relation to the environment. This method was also used to analyse the community ecology in paper II. Additionally in this paper, the diversity of phytoplankton community was estimated by calculation of Shannon's diversity index, and correlations were tested with Spearman and Parson correlation coefficients. Calculation in paper III included estimation number of DNA copies, and the gene copy number per cell was analysed against length and volume of the cells. Proportion of SSU rDNA copies per litre seawater was compared to the proportion of dinoflagellate and diatom biomass and biovolume. In paper IV, consensus sequences for each clone were produced and aligned. A Maximum Likelihood (ML) tree was done using PAUP software (Swofford 2002). Statistical analyses

for paper V and VI were carried out using GENEPOP (Raymond & Rousset 1995). Linkage disequilibrium between loci, heterozygosity (observed and expected) and deviation from Hardy-Weinberg equilibrium (HWE) at the different loci, were calculated. MICROCHECKER (Van Oosterhout et al. 2004), was used to trace stuttering, allele drop-out and null alleles in these two studies.

5. Results and Discussion

Community structure

Cell abundance

Diatom resting stages have no dormancy period, but they sometimes show a lag period of growth for about a week. Vegetative cells can on the other hand begin to grow immediately (Itakura et al. 1997, Kuwata & Takahashi 1999), and thus the type of inoculum can influence the timing of bloom development. This has previously been demonstrated in a microcosm experiment in Scandinavia, which showed that phytoplankton bloom development was faster in plankton-inoculated microcosms, compared to sediment inoculated (McQuoid & Godhe 2004). The low dinoflagellate cyst abundances found in the tropical study area (Fig. 1) (Godhe et al. 2000, Godhe et al. 2002a) could indicate that benthic resting stages in general are less abundant compared to temperate waters, but despite this, they can influence the phytoplankton community in the water column.

Results in paper I indicate that species composition can change if a population is seeded by resting stages or by planktonic cells. One important finding in this study was that in the sediment-inoculated mesocosms, phytoplankton growth lagged behind compared to the mesocosms inoculated with plankton (paper I, Fig. 1C), which shows that the timing of the bloom was significantly affected by inoculum type. This was a consequence of two different seeding strategies, where the first is proliferation of vegetative cells originating from the plankton. These cells attain exponential growth very soon after inoculation. The second strategy is delayed germination of resting stages and subsequent growth of vegetative cells, and this is what we observed in the sediment-inoculated mesocosms. A peak in diatom abundances (paper I, Fig. 1C) was observed six days after inoculation of planktonic cells, and after 13 days in the sediment mesocosms. Mesocosms inoculated with plankton-plus-sediment, showed both these distinct abundance maxima. The sediment mesocosms initially displayed low diatom abundances, and reached one density peak with maximum cell abundance on day 13, coinciding with the second peak in the mixed treatment. The contribution of resting stages to phytoplankton blooms in the tropics is poorly investigated but this experiment demonstrated that resuspended resting stages are important for phytoplankton bloom dynamics in the area.

Species diversity

The study in paper II was conducted in the same area and season as the experiment in paper I. It demonstrated that species composition in a phytoplankton community changes over very short time periods. The relative abundance of each species might be dependent on different environmental variables. Rapid fluctuations in the diversity of the phytoplankton community were observed. To trace species diversity, Shannon's index was used. The index varied between 1.62 and 3.42 (paper II, Fig. 2F), where the first value indicates low species diversity, and the second a much higher diversity. When the diversity index was high, the proportion of diatoms was also significantly higher (paper II, Fig. 2E). In converse, at low species diversity index (paper II, Fig. 2F), significantly higher relative abundances for phytoplankton groups other than diatoms and dinoflagellates were found (paper II, Fig. 2E). There was a tendency (although statistically non-significant) toward correspondence between high Shannon's index, high diatom abundance, high Chl a concentration and high tide. Thus, the tidal stage may have implication on the sampling. Differences in tide could influence species diversity and the proportion of specific microalgal groups, and in this study, at low tide, the relative proportion of diatoms and dinoflagellates was low (paper II, Fig. 2D-E). Sometimes phytoplankton blooms in estuaries are spatially

patchy, and dominated by one or just a few species (Gallegos 1992), and variation in hydrographic variables can cause changes in species composition over short times and distances. In this study we found a significant correlation between Chl *a* concentration, and the relative abundance of dinoflagellates, and the overall results indicated that tides might have an influence on the composition of the phytoplankton community at a higher taxonomic level, but also at the species level. This finding shows how the results of e.g. weekly or monthly sampling may be affected, and it is especially important to consider this when working in tidal influenced areas.

In the mesocosms (paper I), high species diversity was noticed during the entire experiment, and the most frequent taxa were present in all the mesocosms. However, species proportion changed over time, depending on the type of inoculum, just as the environmental variables affected proportions of higher taxa in paper II. Mesocosms inoculated with plankton only (paper I, Fig. 2A), had initially similar proportions of the most common taxa. Mesocosms with plankton and sediment were initially showing high diversity of species, but the diversity declined towards the end of the experiment (paper I, Fig. 2B). Mesocosms inoculated with sediment only, displayed a different community structure (paper I, Fig. 2C). Dominating taxa had few representatives, with large initial relative abundance. Several species showed strong association with a particular type of inoculum, and this finding elucidates the importance of the inoculum in influencing the taxonomic composition of the plankton community. Generally, a natural phytoplankton community in the tropics displays high species diversity, and single-species phytoplankton blooms occur quite rarely (Subrahmanyan 1960, Subrahmanyan & Sarma 1960, Irigoien et al. 2004). Formation of resting stages is often seasonal; the annual variation in hydrographic parameters and phytoplankton biomass of the investigated area could affect this formation. Post-bloom conditions in late summer, with reduced inorganic nutrients of the water column (Subrahmanyan & Sarma 1960) would constitute the most plausible time for diatom resting stage formation in this part of the world. In summary we concluded that inoculum type significantly affected the development, the timing of maximum cell abundance, and taxonomic composition of the phytoplankton community.

The aim of paper III was to develop a protocol for real-time PCR, which is a fast and accurate method, in order to quantify two important phytoplankton groups, i.e. dinoflagellates and diatoms. Here we wanted to adapt a method for estimating the number of SSU rDNA copies of respective group. We demonstrated a significant relationship between the proportion of rDNA copies and biovolumes of the two dominant classes of phytoplankton in field samples, and hence, the real-time PCR-based technique presented here can be used to quickly assess proportional biomass. The reproducibility of the quantification of the SSU rDNA copies using this method was 78%, which is comparable to previous studies (Refardt & Ebert 2006). Field samples were subjected to two real-time PCR assays, one for diatoms and one for dinoflagellates. The field samples were highly variable with respect to the number of SSU rDNA copies, cell abundance, biovolume, and cellular carbon per litre of seawater for both targets. Different phytoplankton groups dominated the water column during the time period when samples were collected. A community of various small heterotrophic flagellates (e.g. *Telonema antarcticum*), ciliates, and small dinoflagellates (e.g. *Prorocentrum minimum*) and diatoms mainly of benthic origin, shifted to a community with less heterotrophic flagellates, but with dinoflagellates (e.g. *Ceratium tripos*, *Dinophysis norvegica*, *Prorocentrum minimum*) and the planktonic diatoms. The abundance of planktonic diatoms further increased due to a bloom of *Dactyliosolen fragilissimus* and *Proboscia alata,* and this revealed strong increases in the number of SSU rDNA copies of diatom origin. Using our method we produced a significant linear regression of the proportion of SSU rDNA copies of dinoflagellate and diatom origin versus the proportion of dinoflagellate and diatom biovolumes per litre seawater. Additionally, the number of diatom SSU rDNA copies displayed a significant linear relation with diatom biovolumes per litre seawater.

Species

It seems like diatom species from a global perspective may have different seeding strategies for

initiation of planktonic growth. The affinity of the major taxa in relation to inoculum and environmental variables differed in paper I. Some of the species are know to seed blooms from resting stages in temperate areas, and the results from this study also shows a strong association of specific species to inocula type in the CCA (paper I, Fig. 4). We found that a species may use the same (e.g. *Thalassiosira pseudonana*), or different (e.g. *Chaetoceros socialis* and *Skeletonema* sp.), strategies in different parts of the world. The various treatments had initially different abundances of some of the most common species, and *Thalassiosira* sp. was very abundant in the plankton mesocosms, whereas the abundances were much lower in the other treatments. Over the entire experimental time, *T. pseudonana* and *Thalassiosira* sp. were abundant in both inocula, and while *Thalassiosira* declined in the plankton mesocosms, two cell density peaks were observed in the plankton plus- sediment mesocosms. This is also reflected in the biplot, were they are located near the centre (paper I, Fig. 4). It is known that species within the genus *Thalassiosira* easily adapt to varying environments, and they are abundant in both the water column and in surface sediments (Pedersen et al. 2005, Kasim & Mukai 2006). Resting stages from this genus seed the plankton in temperate waters (Itakura et al. 1997, Ishikawa & Furuya 2004), and vegetative *Thalassiosira* cells have previously been successfully established from sediment slurries sampled in Indian waters (Mitbavkar & Anil 2002). *Skeletonema tropicum* in the plankton and plankton-plus-sediment mesocosms had the highest abundances at the start of the experiment, and there was a high affinity of *S. tropicum* and *Skeletonema* sp. (later identified as *Skeletonema grevillei*, Egardt 2009) to the plankton-inoculated mesocosms (paper I, Fig. 4). Resting stages of *Skeletonema* are reported to be successful in seeding blooms in temperate water (Itakura et al. 1997, McQuoid & Godhe 2004), but in our experiment in coastal SW India, the propagation by resting stages was not important. The plankton mesocosms were the only ones with good growth of *Chaetoceros socialis,* and in the other treatments the growth was poor. All *Chaetoceros* species were associated with the plankton inoculated mesocosms (paper I, Fig. 4), even though many species of this genus have resting stages, and continuous germination of spores from the same genus have been recorded from temperate waters (Itakura et al. 1997). Some of these resting stages were recorded in the sediment inoculum (*C. brevis*, *C. didymus* and *Chaetoceros* sp.), but did not contribute to the vegetative community in the sediment-inoculated mesocosms. A possible explanation for this is that germination of *Chaetoceros* spp. spores in the tropics might be seasonally triggered. Many of the species described above were also found in the study on phytoplankton assemblages in the field, and how they are linked to hydrographic parameters (paper II). In the genus *Chaetoceros*, most species were significantly correlated to salinity, tide and Chl *a*, but *Chaetoceros tenuissimus* was an exception. This species was recorded at low tide, when hydrographic conditions changed to more brackish-like. Elsewhere *C. tenuissimus* is often associated with brackish water environments (http://www.smhi.se). The abundance of each species in paper II seems to be dependent on particular environmental variables (similar to the results in paper I). For instance, high Chl *a* concentration, high tide, high salinity and high temperature were correlated to many planktonic diatoms.

Papers III-VI in this thesis are mainly molecular studies. At species level, the real-time PCR of monoclonal cultures in paper III revealed a correlation between the number of gene copies per cell and the biovolume of the cell (paper III, Fig. 1). It has previously been confirmed that in marine protists there is a relationship between rDNA copy number and cell length (Zhu et al. 2005). The relationship was verified with our data set, and the number of SSU rDNA copies per cell in diatoms ranged from 61 (*S. marinoi*, smaller diatom) to 36896 (*Ditylum brightwelli*, larger diatom) and in dinoflagellates from 1057 (*Pentapharsodinium faeroense*, smaller dinoflagellate) to 12812 (*Lingulodinium polyedrum*, larger dinoflagellate). We could also determine the ecologically relevant linear relationship between cell copy number and biovolume, which we utilized to develop this method of quantification.

All *S. marinoi* clones examined in paper IV were sequenced (LSU rDNA) and examined in transmission electron microscopy (TEM). Morphologically, the clones showed characteristics of both *S. marinoi* and *S. dohrnii*. We concluded that these two species show overlapping ranges with regard to morphometrics and cannot be distinguished by features of the frustule or girdle bands. In the original

description (Sarno et al. 2005), the morphological character separating the two species was the structure of the girdle bands. Our study showed that the *S. marinoi* clones fell into the range of both species with regard to most morphological features (paper IV, e.g. Fig. 20). The morphological criteria for discriminating between the two species are not adequate, and we could not identify any other morphological characters in *S. marinoi* that were different from those published for *S. dohrnii*. We can of course not exclude that future studies will define morphological characters, which make it possible to distinguish between *S. dohrnii* and *S. marinoi*.

All analysed *S. marinoi* clones fell into the same clade with 89% bootstrap support in the phylogenetic analyses of the LSU rDNA sequences, and the LSU-based cladogram correspond to the separation of the two taxa as individual species according to the original descriptions (Sarno et al. 2005). The same descriptions of *S. dohrnii* and *S. marinoi* did not reveal any intraspecific genetic variation within the SSU or the LSU rDNA, and the genetic distance between the two species consisted of three substitutions within the LSU rDNA (D1-D3). However, the number of clones in the study was small. This indicates that in order to accurately study the representation of the genetic diversity within a morphospecies, many clones may be necessary. We found that even clones isolated from the same plankton net sample displayed different levels of heterogeneity within the LSU rDNA, and that heterogeneity exists on very small spatial and temporal scales, in accordance with other studies (paper VI, Montresor et al. 2003). *Skeletonema marinoi* is divided into several clades with as great or greater base-pair divergence as that between *S. marinoi* and *S. dohrnii*, and subsequently the differences in LSU sequences between the two species are mirrored by intraspecific variation in the same sequence. It is possible that resting stages contribute to the heterogeneity observed within the planktonic community at a particular time, since resting stages of different cohorts could be resuspended simultaneously (McQuoid & Godhe 2004). One explanation to the variation within the LSU detected on a small spatial and temporal scale could be that these cohorts can originate from transported cells or from resuspension of years of sedimentation. At present, there is less support for separating the species in the *S. marinoi–dohrnii* complex. Since large intra-specific variation is found within these species (Kooistra et al. 2008, paper IV). LSU rDNA alone does not necessarily contain enough information to generate any distinct information on speciation, nor does the morphology. It is still possible though, that there are differences in their biogeographical distribution which could support the separation into two distinct species, but recent biogeographical studies of *S. dohrnii* has shown a large geographical range (Zingone et al. 2006) and at present we do not suggest that the two species are merged.

Population level

The knowledge of population structure among eukaryotic phytoplankton is limited, but in the diatom *Pseudo-nitzschia pungens,* there is evidence for a largely unstructured population that spans across a 200 km region of the North Sea (Evans et al. 2005). Contradictive, there is also evidence that populations in other diatoms—*D. brightwellii* and *S. marinoi*—less than 100 km apart are genetically different (Rynearson et al. 2006, paper VI). We have indications that genetic variation in *S. marinoi* might be due to temporal successions of different populations (Godhe & Härnström in prep). The mechanisms of population genetic structuring and perhaps also local adaptation are probably complex since there is maintenance of both spatial and temporal genetic structure over compressed scales (kilometres and months). A few earlier studies have described intraspecific genetic heterogeneity within and among phytoplankton populations (Iglesias-Rodriguez et al. 2006, Nagai et al. 2007) Genetic differentiation among populations can either be a consequence of genetic drift, and hence indicative of dispersal barriers. Another possible explanation is that in presence of gene flow among populations, local selection pressure gives rise to significantly differentiated populations. To increase the awareness of dispersal rates and spatial patterns of populations, molecular techniques are useful tools (Hastings & Harrison 1994), and it is possible with appropriate techniques to reveal populations and metapopulations.

Microsatellite markers for other marine diatom*s* have successfully been used in population studies (e.g. Rynearson et al. 2006), and in order to gather information on the genetic structure and gene flow in populations of *S. marinoi*, we characterized species-specific microsatellite markers (paper V). During the development of the primers, 45 monoclonal cultures from Gullmar Fjord (Fig. 2) were genotyped, and the observed number of alleles per locus ranged from 5 to 22. Of eight loci, none were in significant linkage disequilibrium, and the heterozygosities ranged from 0.38-0.90 (observed) and 0.42-0.93 (expected). Departure from Hardy Weinberg equilibrium (HWE) was found and could be due to the reproduction mode of diatoms. Reproduction in centric diatoms is normally asexual, by division, resulting in smaller and smaller cells. The ability to reproduce sexually can be triggered when the cell size drops below a critical level (Round et al. 1990). The two different ways to reproduce can somewhat complicate the work on species like *S. marinoi,* since the reproduction strategy has a significant effect on the genetics. However, only a very high rate of clonal reproduction will have an impact on the heterozygosity (Balloux et al. 2003). Clonal reproduction in diploids, like *S. marinoi*, causes a larger genetic diversity in each single locus, with decreased number of genotypes compared to sexual reproduction.

We applied the microsatellite markers on populations established from a sediment core, sampled in an anoxic fjord (paper VI). The sediment layers 1-7 in that study represents a time span of more than 100 years spread in time, where layer 1 is the oldest and 7 the most recent. The survival rate of the clonal cultures had large variations, but were in general high, on average 66%, with reduced survival in the older parts of the sediment. This is in accordance with another study on the viability of resting stages (McQuoid et al. 2002). Some loci showed heterozygote deficiency, but most of the layers were significantly separated in genic pair wise comparison (paper VI, Table 5). Samples from the different sediment layers were overall genetically differentiated, and the genetic differentiation within and among samples was also substantial. The generally high level of differentiation among populations of *S. marinoi* is not surprising. The analysed sediment cores have a large time span (>100 years) compared to the short generation time of *S. marinoi* (approximately one cell division day^{-1}, Taylor et al. 2009). All cells represented in the water column, potentially have the capacity to form resting stages. It is possible that resting stage formation is more dependent on external environmental factors than internal, genetic factors. We hypothesize that if a large pool of resting stages are resuspended into the water column, many of these will germinate and resume vegetative growth due to illumination, but depending on the existing environmental and hydrographic conditions, clones most fit for the particular microhabitat might rapidly out-compete less fit clones. The environment will thus also function as a selection gradient affecting the population dynamics in the water column and subsequently what is accumulated in the sediment. Over all loci, the lowest diversity and the mean squared allele size difference among individuals within populations were found in layer originating from around 1980 (layer 4) when loadings of nitrogen (N) and phosphorus (P) were the highest recorded in the area (Andersen et al. 1998). Results from the PCA showed that sediment layer 3 (1920) and 4 (1980) were different from each other and all the others (paper VI, Fig. 3), and at the time these layers were accumulated the sediment layers were probably most influenced by eutrophication, since the loadings of N and P was reported to be very high (Ellegaard et al. 2006). The nutrient concentrations in Mariager Fjord were not extreme when layer 1 and 2 were accumulated (before 1920), and during the accumulation of layer 6 (2000) and 7 (2006) the nutrient loadings had started to decrease drastically (Ellegaard et al. 2006). The decrease was occasioned by legislative actions in the late 1980s to reduce the nutrient loads (Conley et al. 2002). Perhaps similar environmental conditions, taking place more than 100 years apart in the same geographical area where different clones are represented and specific genotypes shared in layer 1, 2, 6 and 7 are advantageous in an environment without excess of nutrients. It is possible that the eutrophic conditions made Mariager Fjord a more marginalized environment, and ecological periphery or bottle-necks may promote genetic perturbation and isolation (Johannesson & Andre 2006). The decreased genetic diversity within the cells in the watermass (and afterwards also in the germinated resting stages in the sediment) in the layer from around 1980 (layer 4) might be due to a potential marginalization. The

extremely high Chl *a* concentrations (18-63µg I^{-1}), during the late 70s and the beginning of the 80s, could at the same time have caused an increase in *Skeletonema* abundance, since eutrophication is reported to have this effect in different geographical areas (Kiss et al. 1993, Ramaiah & Nair 1998). Environmental changes affect phytoplankton at community level, and a study on phytoplankton species diversity showed a significant reduction of species during hypereutrophication (Crossetti et al. 2008), and our study suggests that this also is reflected at population level.

6. Conclusions

Some of the main findings at phytoplankton community level were the importance of resting stages seeding in tropical waters. Our study is the first of its kind in the tropics, while there are many reports on resting stages seeding the water column in temperate areas (e.g. McQuoid & Godhe 2004). This is in accordance with our results from SW India where benthic resting stages had the ability to seed the water column. This seeding influenced the phytoplankton community in the water column despite a presumed low density of resting stages in tropical sediments, and the species composition was altered if a population was seeded by resting stages or by planktonic cells. The seeding strategies among species may differ at different sites, and some species have the same strategy in tropical waters as documented in temperate waters, whereas others have different strategies in tropical waters compared to temperate waters.

 At the study site in the coastal Arabian Sea, small-scale hydrographic changes turned out to be important factors affecting the composition of the natural phytoplankton community, and several of these factors are potentially tightly coupled. Moreover, the changes of patterns in the community structure seem to be more apparent at higher taxonomic levels (e.g. diatoms and dinoflagellates).

 In paper IV, the differences in LSU-based phylogeny between the species *Skeletonema dohrnii* and *S. marinoi* were mirrored by intraspecific variation within the *S. marinoi* clade in the same sequence, and the variation was found even between clones isolated from a single plankton sample. The morphological character defining for *S. dohrnii* was encountered in the examined clones of *S. marinoi* in paper and the species concept in these two species is not reflected in the morphology.

 Population studies, using microsatellite markers revealed that populations of *S. marinoi* found during the time of hypereutrophication were significantly different from populations found before and after this period. There were no significant genetic differences between post- and pre-eutrophication populations though. Therefore we suggest it is very likely that environmental changes will favour only some populations from a pool of many different populations at a specific location, and that different changes could favour particular populations.

7. Future outlook

There are many new ways to gain more insight into the field of phytoplankton dynamics, at both community- and population level, and in different marine ecosystems. The access to microsatellite markers for *S. marinoi* makes it possible to do further population studies on this species. A comparison of resting stages from recent sediment and vegetative cells in the water mass could reveal potential seasonal fluxes in the dynamics. Further investigations including identification and monitoring of different species of *Skeletonema*, using molecular techniques in combination with scanning electron microscopic (SEM) and transmission electron microscopic (TEM) could be informative. *Skeletonema* can cause serious economic losses (e.g. Nishikawa et al. 2009) and identification of the species diversity and what species in the genus are causing the problem can be of importance. After confirming what dominant species that causes problems, it is possible to develop microsatellite markers for these, and to do additional population genetic studies. It is

elementary to investigate genetic structures and connectivity among local populations and how they affect a potential gene flow. If gene flows are detected, which are the controlling factors? How does natural dispersion by ocean/tidal currents and those of human activity, impact on the gene flow of populations? What is the cue for initiation of phytoplankton blooms, and what is the role of resting stages in seeding planktonic populations?

In a broader perspective, future comparisons of the biodiversity of phytoplankton, not only locally, but also regionally and globally, would give a wider understanding of e.g. phytoplankton dispersal, since dynamics might vary at these different levels. It is possible to see how populations are connected or isolated at regional and worldwide scales, and to study the genetic similarities/differences and gene flow among and between regional populations and the dispersal routes. Generally, studies on community dynamics at large scale would make a great contribution to what we know at this point.

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