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Genetic heterogeneity and physiological variation among seasonally separated clones of *Skeletonema marinoi* (Bacillariophyceae) in the Gullmar Fjord, Sweden

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Water samples and plankton net hauls were collected 24 times from Gullmar Fjord on the Swedish west coast from February 2004 to March 2005. The abundance of *Skeletonema marinoi* was estimated and individual clones isolated. Abundance was highest during the spring blooms in February to March. Subsequently, *S. marinoi* was detected in all samples but at lower abundances. At the end of September a second peak was recorded. All clones were pre-adapted to the same culturing conditions for more than 1.5 years. Large subunit (LSU) rDNA (D1–D3) was sequenced from 23 clones isolated from three different seasons, February, June and September. Six microsatellite loci were genotyped for 19 clones to estimate within-season genetic diversity. Three clones from each season were selected for physiological experiments at different salinity and temperature combinations and monitored for average number of divisions per day, maximum cell densities, biovolume, and total RNA concentration per cell. Differentiation of physiological response among the clones was partly attributed to the month of isolation. The February isolates had a significantly higher division rate and larger biovolumes, while the September clones attained higher cell densities. The June clones were isolated during the time of the year when the natural abundance is lowest, and exhibited the smallest genetic and physiological variation, which suggests that they were under strong selection pressure. The differential physiological responses and degree of genetic heterogeneity among seasonally separated clones could indicate that different populations succeed each other in the fjord.

Key words: differentiation, genetic, Gullmar Fjord, heterogeneity, LSU rDNA, physiological response, *Skeletonema marinoi*

Introduction

Many diatom species are known to have global distributions and some of these are able to bloom under a wide variety of environmental conditions. In order for diatoms with extensive geographic ranges to successfully survive selective pressure in a variety of ecological niches, it is hypothesized that they display high phenotypic and genetic diversity (Rynearson & Armbrust, 2000). Genetic and physiological variation in a given environment should allow them to thrive over much broader ranges of hydrographic conditions than is possible for a homogeneous population, and this undoubtedly contributes to their ecological success throughout the marine environment.

Several studies have shown high levels of genetic diversity within a phytoplankton species on local or regional scales (e.g. Gallagher, 1980; Orsini *et al.*, 2004; Shankle *et al.*, 2004; Nagai *et al.*, 2007). Results indicate that, despite the potential for widespread dispersal by water currents, distinct populations exist on both large (Evans *et al.*, 2005), and small geographic scales (Rynearson *et al.*, 2006), and that they are maintained in a specific area for long periods (Rynearson & Armbrust, 2004). Several experiments have revealed that clonal cultures kept under identical conditions display a large variety of physiological responses (e.g. Brand, 1982; Liang *et al.*, 2005). In a few studies, the differentiated physiological characteristics of particular clones have also been linked to genetically distinct, and geographically or temporally restricted populations (Moore & Chisholm, 1999; Rynearson & Armbrust, 2004), and repeated monitoring of particular clones after years in culture has identified the response as endogenous and independent of external conditions (Gallagher, 1982). Thus, genetically differentiated populations express particular physiological

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characteristics that are suited to their indigenous environment (Ridley, 1996), and it is therefore suggested that advection from a particular environment is a loss for the species, as advected cells are probably less well adapted to the new environment (Brand, 1982; Moore & Chisholm, 1999).

Skeletonema marinoi Sarno & Zingone is a species that contributes significantly to phytoplankton blooms in temperate waters, including the Skagerrak-Kattegat and Baltic Sea (e.g. Tallberg & Heiskanen, 1998, therein reported as *S. costatum*; Godhe *et al*., 2006; Ellegaard *et al*., 2008). It is found throughout the year in these waters (Lange *et al*., 1992), and displays pronounced density peaks, especially during the spring bloom. It has a benthic resting stage, which is very abundant in Scandinavian sediments (McQuoid, 2002). Examination of the *S. marinoi* seed bank estimates up to 3.5 million propagules g^{-1} sediment, and these cells are capable of surviving for several decades in the sediments (McQuoid *et al*., 2002). In addition, *S. marinoi* is an important primary producer and constitutes a valuable food source for higher trophic levels. Thus, the study of this organism is of high ecological relevance. *Skeletonema marinoi* forms long clonal chains, is easy to isolate, and to maintain in culture (Godhe *et al*., 2006). This is an important factor, because bias due to a strain's ability to survive in culture is eliminated. Consequently, it is well suited as a model phytoplankton species for studies on intraspecific genetic and physiological variation and differentiation.

Significant genetic heterogeneity among local and regional populations of *Skeletonema* has previously been demonstrated with several molecular methods such as allozyme studies (Gallagher, 1980), sequencing of ribosomal DNA (Alverson & Kolnick, 2005; Godhe *et al*., 2006; Ellegaard *et al*., 2008), Random Amplified Polymorphic DNA, RAPD (Godhe *et al*., 2006), and genotyping of microsatellite loci (Almany *et al*., 2009). In previous microcosm experiments conducted over many seasons in the Gullmar Fjord, vegetative populations of *Skeletonema* were established from both planktonic and benthic propagules. In one of these experiments, *S. marinoi* cells were larger when seeded by benthic propagules and smaller when arising from planktonic cells (McQuoid & Godhe, 2004). Because repeated vegetative division reduces diatom cell size, *S. marinoi* seeded from the plankton may have been dividing for many more generations than the benthic resting stages. However, size variation between treatments and seasons may also reflect the presence of genetically distinct clones at different times of the year in the same fjord. In fact, clones of *S. marinoi* isolated from different seasons in Gullmar Fjord showed heterogeneity within the LSU rDNA sequence, with differences even among clones from the same net sample (Ellegaard *et al*., 2008). These size differences and the heterogeneity of a conserved part of the genome could indicate the presence of several populations possibly with distinct temporal distributions.

In this study we report on the seasonal distribution of *Skeletonema marinoi* in Gullmar Fjord, on the Swedish west coast. Monoclonal cultures were established throughout the year and pre-adapted to identical culturing conditions. The purpose was to investigate: (i) if isolates from different seasons (February, June and September) exhibited physiological and morphological differentiation, (ii) how the seasonally separated isolates responded under particular environmental regimes, (iii) if alleged physiological variation among the seasonally separated isolates was stable when two selected environmental parameters were changed, (iv) if observed physiological differentiation could be coupled to genetic heterogeneity. The genotypic and phenotypic parameters examined were LSU rDNA sequences, six microsatellite loci, average number of divisions per day, maximum cell densities, biovolumes, and RNA concentration per cell.

Materials and methods

Sampling site, field samples and clones

The sampling site was located in the Gullmar Fjord (58°16′N; 11°26′E) on the Swedish west coast (Fig. 1). Two major current systems affect the Swedish west coast: the low salinity surface Baltic current running parallel to the coast, and the central Skagerrak water

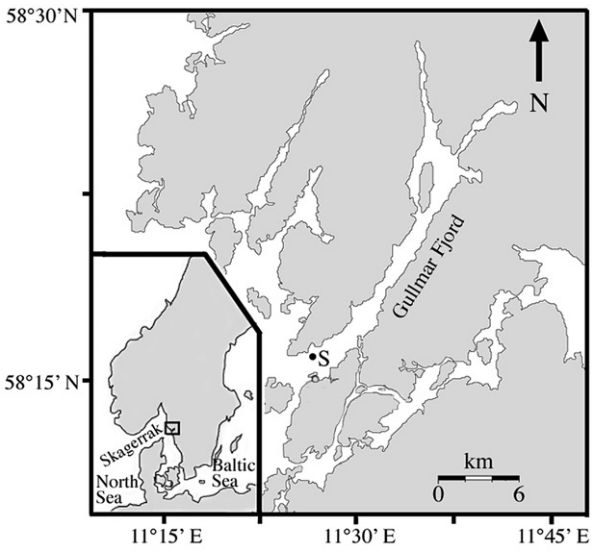

Fig. 1. Location of sampling site in the Gullmar Fjord, Sweden. Water and net samples were collected at Släggö (S).

circulation pattern resulting in an inflow of more saline North Atlantic water. Thus, the water is permanently stratified in terms of salinity and a pronounced halocline is present (Lindahl, 1995). Gullmar Fjord has an entrance sill at 43 m depth and a maximum depth of 120 m. The density fluctuation of the coastal water outside the fjord is most important for water exchange above sill level in the Gullmar Fjord, whereas the tidal force in the fjord is of minor importance. The average turnover time for the water above the halocline is 16–28 days. The horizontal exchange of surface water is characterized by incoming water that is only partially mixed with the resident water before it is flushed out of the fjord again. The position of the halocline is most stable during May to August (10–15 m) when intermediate water (deeper than 20 m) is very seldom in contact with the surface, and consequently, the exchange rate with the coastal water outside the fjord is greater. Upwelling and downwelling events have larger amplitudes during the rest of the year, and therefore the position of the halocline is more variable (5–20 m) and there is less exchange with the coastal water. The salinity of the surface water is 24–27 PSU throughout the year but temperature varies, with the lowest recorded temperatures in February to March (2–4°C), and the highest in August to September (15–17°C) (Arneborg, 2004).

Field sampling was conducted 24 times from 18 January 2004, to 17 March 2005, during the expected growth season of *S. marinoi*. Samples were collected more frequently during expected bloom periods (i.e. February–March and September–October), and not at all during the summer (June 3–August 11) and winter (October 28–February 10), when *S. marinoi* is known to be absent or present in very low numbers (McQuoid & Godhe, 2004; SMHI 2008). Water samples were collected from the surface (0–3 m depth) using a plastic 1.6-l Ruttner water bottle. Plankton was simultaneously obtained from the surface water by several net hauls (>10 μm). Lugol's-fixed water samples were settled in sedimentation chambers overnight (Utermöhl, 1958), and densities of *S. marinoi* were estimated at 200 or 400× using a Zeiss Axiovert 135 inverted microscope. On each sampling occasion approximately 10 individual chains of cells were isolated by micropipetting from the fresh plankton net samples. The chains were transferred to a drop of sterile f/2 medium (Guillard, 1975, 26 PSU). This was repeated several times to clean the cells. Each clone was then transferred to a small (5-cm diameter) Petri dish with 8 ml of f/2 medium and incubated at 10°C. When growth was established, cells were transferred to 50-ml Nunc flasks with f/2 medium (26 PSU), and reinoculated every third week. The clones were kept at 10°C and 50 μmol photons $m^{-2} s^{-1}$, with a 12-h:12-h light–dark photoperiod. The xenic monoclones were grown and adapted to identical culture conditions, as described above, for 20–28 months prior to the experiment. In this study, clones isolated at three different times of the year (February, June and September) were selected (Table 1). The clones are available at University of Gothenburg Marine Algal Culture Collection (GUMACC) (www.marecol.gu.se/gumacc.html).

Table 1. Monoclonal strains of *Skeletonema marinoi*.

Strain	Isolation date	LSU rDNA GenBank accession number	Microsatellite genotyping	Physiological experiment
GF04 1A	20-Feb-04	EU285593	x	
GF04 1B	20-Feb-04		x	
GF04 1F	20-Feb-04	DQ438874[a]	x	x
GF04 1G	20-Feb-04	DQ438873[a]	x	x
GF04 1H	20-Feb-04	EU285594		
GF04 1I	20-Feb-04	EU285595		
GF04 1J	20-Feb-04	DQ438872[a]		x
GF04 7A	02-Jun-04	EU285596	x	
GF04 7B	02-Jun-04		x	
GF04 7C	02-Jun-04	EF655655	x	x
GF04 7D	02-Jun-04	EU285597	x	
GF04 7E	02-Jun-04	EU285598	x	
GF04 7F	02-Jun-04	EF655656	x	x
GF04 7H	02-Jun-04		x	
GF04 7I	02-Jun-04	EU285599	x	
GF04 7J	02-Jun-04	EF655657	x	x
GF04 9A	23-Sep-04	DQ438871[a]	x	x
GF04 9B	23-Sep-04	DQ438870[a]	x	x
GF04 9C	23-Sep-04	EU285600	x	
GF04 9D	23-Sep-04	DQ438869[a]	x	x
GF04 9E	23-Sep-04	EU285601	x	
GF04 9F	23-Sep-04	EU285602		
GF04 9G	23-Sep-04		x	
GF05 2S	21-Feb-05	DQ438868[a]		
GF05 2T	21-Feb-05	DQ438866[a]		
GF05 2U	21-Feb-05	DQ438867[a]		
GF05 2W	21-Feb-05	EU285603		

[a]From Ellegaard *et al.* 2008.

DNA extraction, PCR, sequencing, and determination of microsatellite allele sizes

DNA was extracted from clonal cultures 1–2 weeks after transfer to 50-ml Nunc flasks. Prior to DNA extraction, the exponential phase monoclonal cultures were scanned microscopically to ensure that the cells were healthy, and that no contaminating protists were present. The cultures were transferred to 50-ml centrifuge tubes and centrifuged at 5000 *g* for 7 min. Pellets were resuspended in 420 µl milli-Q water and transferred to microfuge tubes. Cells were lysed and DNA extracted as described in Godhe *et al.* (2001). DNA concentration and purity were measured with a spectrophotometer. If sample purity was low, i.e. 260/280 ratio <1.3, the extract was further purified with a Flexi Prep Kit (Amersham Pharmacia) following the manufacturer's instructions. Extracted DNA was stored at −80°C.

The LSU rDNA (D1–D3) of 23 clones isolated from the three seasons (Table 1) was amplified by PCR. To eliminate potential Taq errors associated with the original PCR, the region was amplified more than once. Reactions were run in total volumes of 50 µl, consisting of approximately 100 ng template DNA, Taq buffer containing 1.5 mM $MgCl_2$, 0.5 µM of each primer, 200 µM deoxyribonucleotide triphosphate (dNTP), 2.5 U Taq polymerase (QIAGEN, Valencia, CA, USA) and sterile milli-Q water. LSU rDNA sequences were amplified using the primers DIR and D3Ca (Lenaers *et al.*, 1989; Scholin *et al.*, 1994). Amplifications were carried out in a thermal cycler (Perkin Elmer GeneAmp PCR System 2400, Wellesley, MA, USA) as follows: initially, 94°C for 5 min; followed by 30 cycles of denaturation at 94°C for 1 min; annealing at 50°C for 1 min; extension at 72°C for 1 min. After the cycles, extension was completed at 72°C for 5 min. The PCR product was loaded onto a 0.8% agarose gel in 1 × TBE, and ethidium bromide stained gels were studied under UV-transillumination. PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN) following the manufacturer's instructions. Final concentration of the PCR product was measured using a spectrophotometer. For each clone, two or three PCR products were sequenced. Each 10-µl sequencing reaction contained approximately 50 ng of PCR product together with the primers. LSU rDNA (D1–D3) fragments were sequenced using the primers DIR, D2C, D2CF (Scholin *et al.*, 1994), and D3Ca (Lenaers *et al.*, 1989). LSU rDNA sequences were determined using the CEQ Dye Terminator Cycle Sequencing Kit following the manufacturer's instructions. Reaction products were run on a CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA). Sequences were deposited in GenBank with accession numbers given in Table 1.

FAM-labelled forward primers (Almany *et al.*, 2009) were used to amplify six microsatellite loci of 19 selected clones isolated from the three seasons (Table 1). Final reaction volume was 24 µl, containing 1.6 ng µl^{-1} template DNA, 1× PCR-buffer from Perkin Elmer, 0.33 µM of each primer, 250 µM deoxyribonucleotide triphosphate (dNTP), 0.04 U Taq polymerase (AmpliTaq Gold, Applied Biosystems) and 2.5 mM $MgCl_2$. Amplification conditions were identical for all microsatellite loci and were carried out in an ABI 9700 (Applied Biosystems) machine as follows: initially, 95°C for 10 min; followed by 35 cycles of denaturation at 95°C for 30 s; annealing at 63°C for 30 s; extension at 72°C for 30 s. Final extension was at 72°C for 10 min. One µl PCR product was added to 0.2 µl 500 ROX™ (GeneScan™) and 9 µl Hi-Di formamide (Applied Biosystems), and thereafter analysed on an ABI 3730 capillary electrophoresis machine (Applied Biosystems). Allele sizes were determined using GeneMapper software version 3.0 (Applied Biosystems).

Selection of clones for experimentation

Nine genetically different clones representing the three seasons were randomly selected for the physiological experiment (Table 1). Growth rates under standard culturing conditions (f/2, 26 PSU, 10°C, 50 µmol photons m^{-2} s^{-1}, 12-h:12-h light–dark photoperiod) for these clones were determined as described earlier (Tayler *et al.*, 2009). Briefly, three replicate 1-l conical flasks containing f/2 media with silicate were inoculated with exponentially growing cells to give starting densities of approximately 50 cells ml^{-1}. The resulting cultures (27) were maintained at 10°C with gentle aeration and a 12-h:12-h photoperiod. Cell densities were calculated every 1 to 3 days (on day 6, 7, 9, 12, 15, 17, 19, and 20) using a haemocytometer and the average specific growth rates estimated during the exponential growth phase.

Physiological experiment

The effects on the response variables (average number of divisions per day, maximum cell densities, biovolumes, and RNA concentration per cell) of different seasons, temperatures and salinities were investigated. The experiment was conducted over 3 weeks in June–July 2006, and commenced 5 days after reinoculation to ensure that the clonal isolates (three for each season) were in exponential phase. The experiment was designed to expose the clones to typical summer (17°C, 26 PSU) and winter (3°C, 26 PSU) conditions, but also to more extreme salinities over a range of temperatures. Using a factorial design (3 × 4 × 3), each season (three clonal isolates as replicates) was subjected to four different salinities (5, 15, 26 and 35 PSU), and three different temperature regimes (3, 10 and 17°C), yielding 36 different combinations (Fig. 2).

Natural seawater (35 PSU) collected from 35 m depth in the Gullmar Fjord was diluted to salinities of 5, 15, and 26 PSU with deionized water. These were autoclaved and enriched with f/2 medium (Guillard, 1975) to produce the reduced salinity media. During the experiment clones were kept at a constant light intensity (50 µmol photons m^{-2} s^{-1}) under a 12-h:12-h light–dark regime. Prior to the start of the experiment the cell density of each clonal isolate was estimated, so that similar start concentrations could be established for all investigated seasons under all salinity–temperature combinations. Two ml of each isolate (comparable densities) were taken from the standard culture condition and

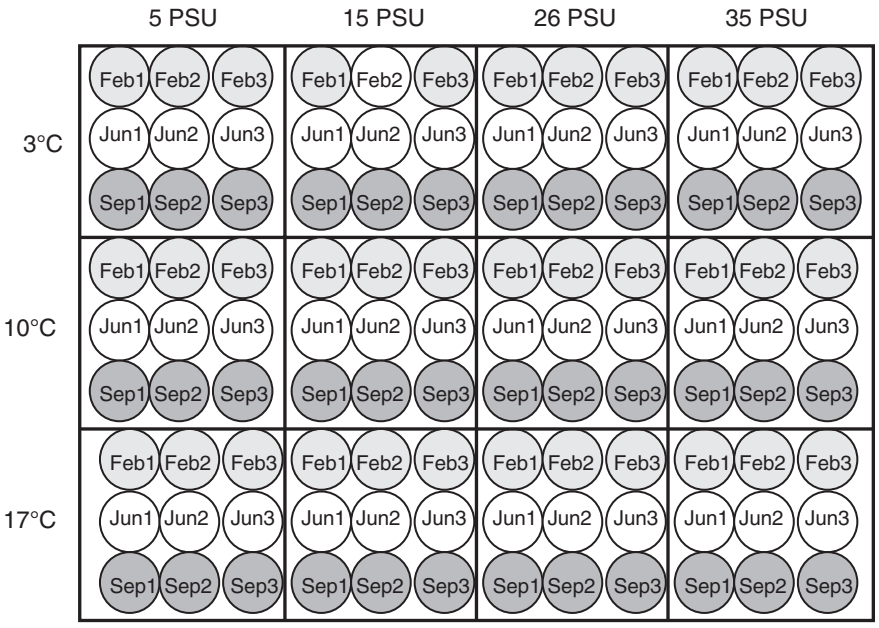

Fig. 2. Experimental design for physiological experiment. Each season (February, June and September) was replicated by three clones, and exposed to four different salinities (5, 15, 26 and 35 PSU) and three different temperature regimes (3, 10 and 17°C).

inoculated into 50-ml Nunc bottles in each salinity-temperature combination. Later the same day, a 1.5-ml aliquot from each bottle was fixed in 2% Lugol's solution to estimate initial cell densities. Subsequently, all seasonal replicates in all salinity–temperature combinations were sampled in the same manner once a week until the end of the experiment. Cell densities (cells ml^{-1}) were estimated by counting a minimum of 300 cells, settled overnight in 10-ml sedimentation chambers (Utermöhl, 1958), under an inverted microscope at 400× magnification. Length and width of 10 random cells from different filaments of each seasonal replicate from each treatment were measured when the culture was in exponential phase. Biovolumes were calculated following Sun & Liu (2003).

RNA extraction

Total RNA concentrations per cell were measured, in order to investigate whether overall intracellular activity was dependent on the season of isolation, and/or the temperature and salinity of the growth media. Extraction of RNA was performed once during the experiment, on day 7. Fifteen ml of each seasonal replicate from each salinity–temperature condition was transferred to a 50-ml centrifuge tube and pelleted as described for DNA extraction. The supernatant was decanted and the pelleted cells were transferred to clean 1.5-ml microfuge tubes. Total RNA was extracted using TRI-REAGENT (Sigma) following the manufacturer's instructions. After extraction, total RNA concentration (μg ml^{-1}) was immediately quantified using a spectrophotometer (Pharmacia Biotech GeneQuant II, Buckinghamshire, UK). Reproducibility of RNA extraction was estimated by repeating the extraction twice with three different isolates.

Data analysis

Consensus sequences for each clone were produced using Sequencher 4.1.2 (Gene Codes Corporation, Ann Arbor, MI, USA). Alignment provided information on pair-wise base pair substitutions among all the investigated clones in this experiment. Several *Skeletonema* species have been described based on LSU rDNA sequences and morphological features revealed in electron microscopy (Sarno *et al*., 2005, 2007). Our clones were identified to species level by aligning the sequences with previously published *Skeletonema* LSU rDNA sequences (Sarno *et al*., 2005; Godhe *et al*., 2006; Sarno *et al*., 2007). *Thalassiosira rotula* (AJ633505) was used as the outgroup. In total, 785 characters at the 5′ end of the LSU rDNA were included in the alignment. According to Modeltest 3.7 (Posada & Crandall, 1998), the GTR + G model (General Time Reversible) with a Gamma distributed rate of variation across sites, was most appropriate for analysing the dataset. Maximum likelihood (ML) analysis was performed using the heuristic search in PAUP* 4.0 b10 (Swofford, 2002). Genepop version 4.0 (Raymond & Rousset, 1995) was used to calculate within season genetic diversity based on allele size (ρ_{IS}), i.e. mean squared allele size differences among clones isolated on the same day (Slatkin, 1995).

The growth rate under standard culture conditions and the average number of divisions per day in the physiological experiment were calculated using $N_t = R^t \times N_0$, where R is the number of divisions per day, N_0 is cell density (cells ml^{-1}) at the onset of the experiment, and N_t is cell abundance on day t. RNA concentration per cell was calculated by dividing the total RNA concentration (μg ml^{-1}), by cell density (cells ml^{-1}) on the same day.

The influence of temperature, salinity, and season of isolation on the average number of divisions per day,

maximum cell densities, and biovolumes was tested with univariate analysis of variance (ANOVA). Temperature, salinity and growth phase (exponential/stationary) were first tested with RNA concentration per cell as the response variable. The effect of temperature, salinity and season was then tested against RNA concentrations per cell from isolates in stationary phase ($n = 74$). Each season was replicated by three isolates. Variation of the response variables of individual clones was not tested, and clones were not included as a factor in any of the ANOVAs. Significance was defined as $p < 0.05$. Significant interactions were further tested with one-way ANOVA. All data were tested for heterogeneous variances with Levene's test of equality of error variances. Average numbers of doublings per day and RNA concentration per cell were log transformed prior to analyses. Post-hoc tests were performed with Student Newman-Keuls (SNK) tests, and Chi-Square tests were used to determine significant association between pairs of examined variables (i.e. average number of divisions per day, maximum cell densities, cell biovolumes, or RNA concentration per cell). All statistical analyses were made in SPSS 11.0.4 for Mac OS X (SPSS Inc., Chicago, IL, USA).

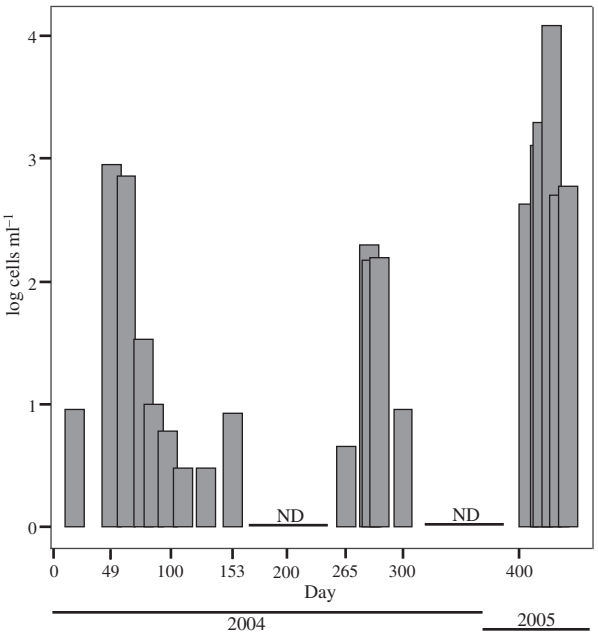

Fig. 3. Abundance of *Skeletonema marinoi* (log cells ml^{-1}) in 24 water samples collected in the Gullmar Fjord from 18 January 2004 to 17 March 2005. No *S. marinoi* was detected in four water samples. ND represents no data. The clones used in the physiological experiments originated from day 49, 153 and 265.

Results

Occurrence, isolation and genetic diversity

The abundance of *S. marinoi* in the Gullmar Fjord was highest (897 cells ml^{-1}, 2004, 12 000 cells ml^{-1}, 2005) during the spring bloom, i.e. in February and the beginning of March (Fig. 3). From March to June, it was detected in all samples but at very low densities (<10 cells ml^{-1}). At the end of September and beginning of October 2004, another peak was recorded, reaching a maximum of 200 *S. marinoi* cells ml^{-1}.

From 203 (80.5%) isolated chains of *Skeletonema marinoi*, monoclonal xenic cultures were successfully established, and grew to sufficiently high densities for DNA extraction. After 4 years in culture 123 (60.5%) strains had survived. The high proportion of isolate survival and random choice from a large pool of clones for each season minimized the possibility that the results were biased by the suite of clones selected.

LSU rDNA (D1–D3) was sequenced from 23 clones, and 19 clones were genotyped at six microsatellite loci. All clones were identified as *S. marinoi* based on the LSU rDNA (D1–D3) sequence alignment and ML analysis (data not shown). Sequence alignment of the 23 clones displayed a high degree of similarity, but five nucleotide positions were polymorphic, confirming the genetic heterogeneity of the clones. Clones isolated in February and September 2004 had five and four polymorphic sites, respectively. Four clones isolated in February 2005 had two polymorphic sites, and the seven clones isolated in June 2004 had a single polymorphic site (Table 2). Genetic diversity, estimated by allele size differences in six microsatellite loci among individuals isolated at different times of the year, was lowest for clones isolated in June 2004, followed by the September and then the February clones (Table 3).

Nine genetically distinct clones, isolated from different seasons (three February isolates from the spring bloom, three June 2004 isolates when cell densities were low, and three September 2004 isolates just prior to the autumn bloom), were selected for physiological experimentation (Fig. 3; Table 1). The growth rates of these nine clones under standard culture conditions varied significantly with isolation date (Fig. 4; ANOVA, $p < 0.01$); the February clones grew significantly faster than both June and September clones ($p < 0.05$, SNK). There were no significant differences between clones of the same isolation date (ANOVA $p > 0.05$). All clones were in mid- to late-stationary growth after 20 days.

Physiological experiment

There were no significant associations between any pairs of response variables examined in the physiological experiment, i.e. average number of divisions per day, maximum cell densities, cell biovolumes, or RNA concentration per cell.

The average number of divisions per day differed significantly (ANOVA) with respect to salinity

Table 2. Polymorphic nucleotide positions in LSU rDNA (D1–D3) within the different seasons.

Season	N	27[a]	68	70	558	576
February 2004	6	:/A	C/T	A/C	A/T	G/T
June 2004	7	:	C/T	A	T	G
September 2004	6	:/A	C/T	A	A/T	G/T
February 2005	4	:	C/T	A/C	T	G

[a]Numbers correspond to the nucleotide on the rDNA sequences submitted to GenBank.

Table 3. Allele size-based gene diversity within different seasons.

Season	N	ρ_{IS}[a]
February 2004	4	0.73
June 2004	9	0.49
September 2004	6	0.59

[a]F_{IS} analogue (Slatkin, 1995), where ρ_{IS} approaching 0 means less differentiation, and ρ_{IS} approaching 1 means high differentiation.

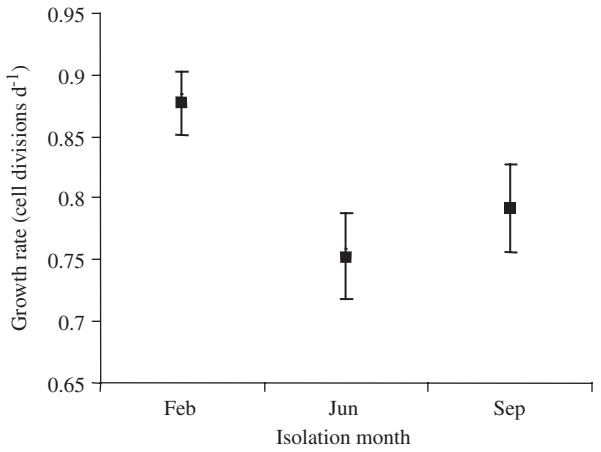

Fig. 4. Mean population growth rates under standard culturing conditions of the *Skeletonema marinoi* clones selected for the physiological experiment. Means ± SEs.

($p<0.01$), temperature ($p<0.01$), isolation month ($p<0.05$), and there was significant interaction between temperature and isolation month ($p<0.01$). Cultures grown in the lowest salinity (5 PSU) underwent fewer divisions per day than those at higher salinities, whereas no difference was found among the highest (15, 26 and 35 PSU) salinities ($p<0.05$, SNK, Fig. 5*A*). Cultures grown at 3°C divided less frequently than cultures grown at 10°C, and cultures grown at 17°C divided faster than at the lower temperatures ($p<0.05$, SNK, Fig. 5*B*). The February isolates divided more frequently than the June and September isolates ($p<0.05$, SNK, Fig. 5*C*). To investigate the effect of interaction between temperature and isolation month, one-way ANOVA tests were

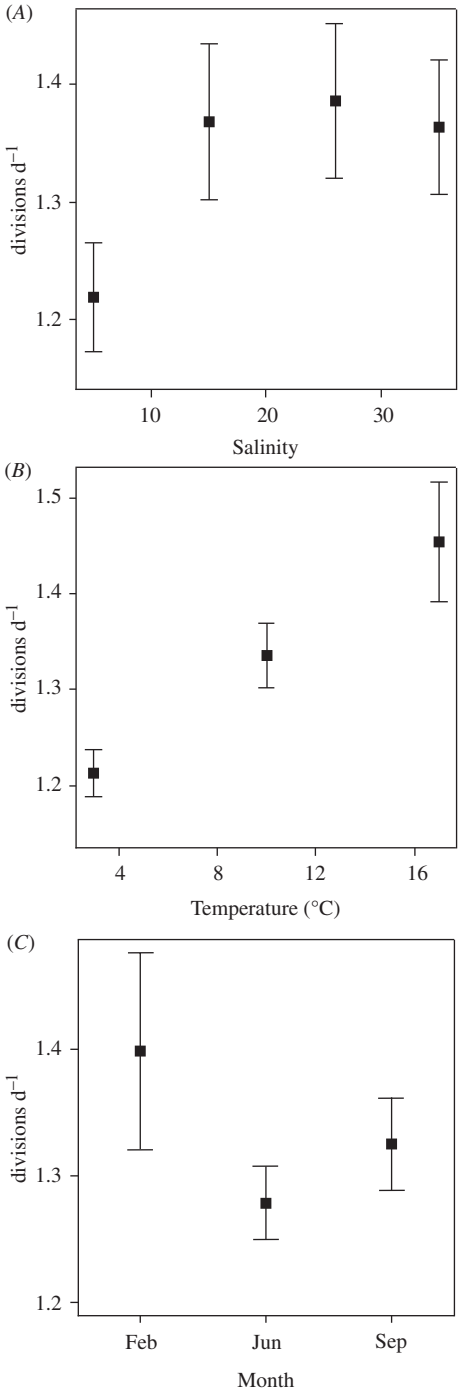

Fig. 5. Average number of divisions per day among the different salinities (*A*), temperatures (*B*), isolation month (*C*) (Month). Symbols are means (salinity: $n=27$; temperature: $n=36$; month: $n=36$). Error bars represent 95% confidence interval (CI).

conducted for each temperature. This indicated that the average number of divisions per day was significantly different ($p<0.01$) with season only at 17°C. Variation in the average division rate among the June isolates was less than that among the February and September isolates (Table 4).

Maximum cell densities for the different salinity–temperature combinations ranged from 6.4×10^4 (5 PSU, 3°C, GF04 1F) to 4.6×10^6 (26 PSU, 10°C,

Table 4. Variance (σ^2) in phenotypic parameters examined within different seasons.

Season	Average number of divisions per day (division d^{-1})	Maximum cell densities (cells ($\times 10^6$) ml^{-1})	Biovolume (μm^3)	RNA concentration per cell (log pg RNA $cell^{-1}$)
February 2004	0.052	1.22	10917	0.15
June 2004	0.007	0.89	2336	0.10
September 2004	0.012	1.45	1428	0.12

GF04 9A) cells ml^{-1}. There was no significant relationship between the initial and the maximum cell densities of respective clones. Maximum cell densities differed significantly (ANOVA) with regard to salinity ($p<0.01$), temperature ($p<0.01$), and sampling month ($p<0.05$). Cultures grown in the lowest salinity (5 PSU) had lower cell densities than in higher salinities, whereas no significant difference was found between the higher salinities ($p<0.05$, SNK, Fig. 6*A*). Cultures grown at 10°C had higher maximum cell densities than cultures grown at 3 and 17°C ($p<0.05$, SNK, Fig. 6*B*). The clones isolated in September displayed higher maximum cell densities than those isolated in June and February ($p<0.05$, SNK, Fig. 6*C*). The variation in maximum cell densities among the September isolates was larger than that among the February isolates, which in turn was larger than among the June isolates (Table 4).

Biovolumes for the different salinity–temperature combinations ranged from 280 (5 PSU, 10°C, GF04 1J) to 63 (35 PSU, 10°C, GF04 9A) μm^3. Biovolumes were significantly different (ANOVA) with regard to salinity ($p<0.01$), temperature ($p<0.01$), and sampling month ($p<0.05$). Cultures grown in 5 PSU had larger biovolumes, whereas no difference was found between the higher salinities ($p<0.05$, SNK, Fig. 7*A*). Cultures grown at 3°C had larger biovolumes than cultures grown in 10 and 17°C ($p<0.05$, SNK, Fig. 7*B*). The February clones were significantly larger than the September clones ($p<0.05$, SNK, Fig. 7*C*), and variation in cell size among February isolates was larger than at among June and September isolates (Table 4).

The reproducibility of the RNA extraction was 95% (SD 4.5%, $n=3$). RNA concentration per cell for the different salinity-temperature combinations ranged from 24 (26 PSU, 17°C, GF04 1J) to 4444 (5 PSU, 3°C, GF04 9B) pg $cell^{-1}$, and was significantly different (ANOVA) with respect to salinity ($p<0.01$), temperature ($p<0.01$), and growth phase ($p<0.01$). Cultures grown in the lowest salinity had significantly more RNA than cultures grown in more saline media ($p<0.05$, SNK, Fig. 8*A*). Cultures grown at 3°C had higher RNA concentration per cell than those at 10°C, and cultures grown at 17°C had less RNA than at either

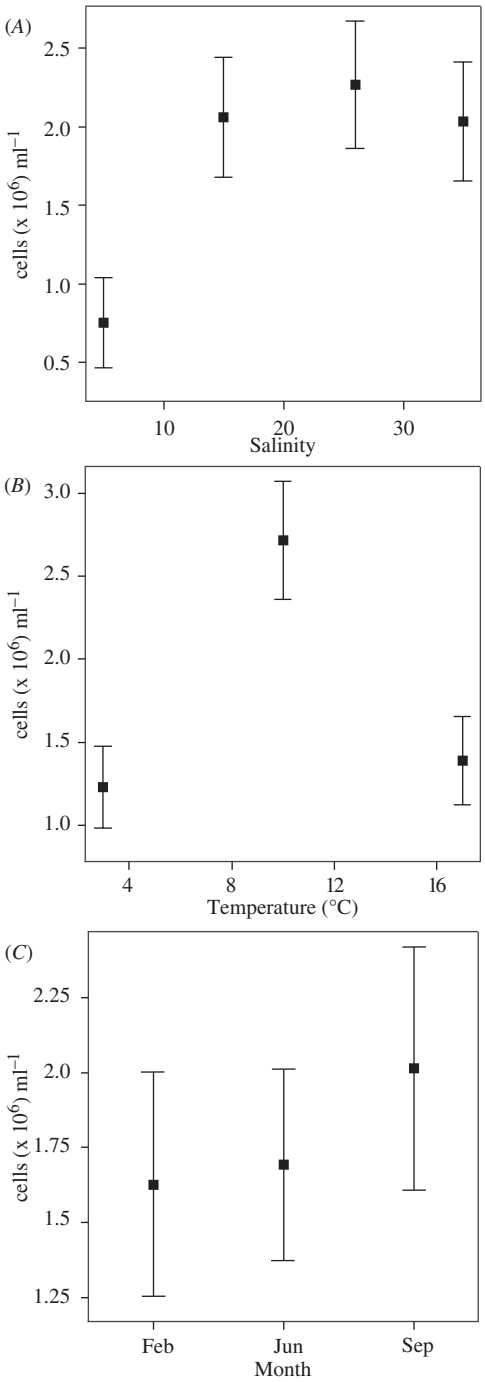

Fig. 6. Maximum cell densities (cells ml^{-1}) among the different salinities (*A*), temperatures (*B*), isolation months (*C*) (Month). Symbols are means (salinity: $n=27$; temperature: $n=36$; month: $n=36$). Error bars represent 95% confidence interval (CI).

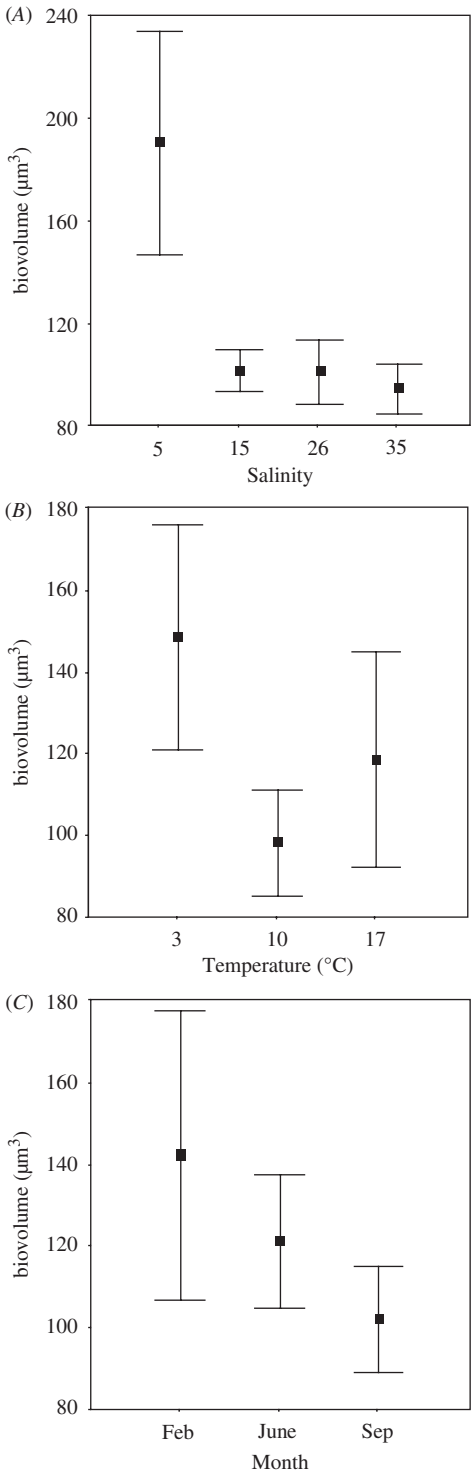

Fig. 7. Biovolumes (μm^3) recorded among the different salinities (*A*), temperatures (*B*), isolation months (*C*) (Month). Symbols are means (salinity: $n = 27$; temperature: $n = 36$, month; $n = 36$). Error bars represent 95% confidence interval (CI).

higher temperature ($p < 0.05$, SNK, Fig. 8*B*). Cultures in exponential phase had higher RNA concentration per cell than cultures in stationary phase (Fig. 8*C*). RNA concentrations per cell from the cultures in stationary phase ($n = 74$) were tested with ANOVA, whereas cultures in exponential phase could not be tested due to low sample size ($n = 34$). RNA concentrations in cultures that had reached stationary phase were significantly different with respect to salinity ($p < 0.01$), temperature ($p < 0.01$), and isolation month ($p < 0.05$). The June clones had significantly more RNA than the February and September clones ($p < 0.05$, SNK, Fig. 8*D*). Variation in log pg RNA concentration per cell among June isolates was less than that among the September and February isolates (Table 4).

Discussion

In this study, we have demonstrated that clones of *S. marinoi* isolated at different times of the year from the same location display different degrees of genetic diversity based on allele-size differences in six microsatellite loci, heterogeneity within the LSU rDNA sequence (D1–D3) and significantly different physiological characteristics. Differentiation of physiological response among the different clones was partly attributed to the month of isolation. The relative importance of the isolation month was dependent on physiological characteristics and experimental conditions. We have also shown that the magnitude of genetic diversity and the variation in physiological response is different for populations isolated at different times of the year.

In diatoms, the nuclear-encoded LSU rRNA gene clearly contains phylogenetic information (Van der Auwera & De Wachter, 1998) and intraspecific variation in LSU has been shown for several diatom species (e.g. Lundholm *et al.*, 2002). Recent work also highlighted the importance of this gene for taxonomic assignments within the genus *Skeletonema* (Sarno *et al.*, 2005, 2007). By sequencing the LSU rDNA (D1–D3) in this study, we could verify that all the clones belonged to one species, and we could confirm different degrees of heterogeneity within the LSU sequences of clones isolated in February, June and September. The ribosomal loci are part of a multigene family formed by up to thousands of tandem copies of ribosomal units. In *S. marinoi* the number of rRNA gene copies per cell is estimated at 61 (Godhe *et al.*, 2008). Intragenomic rDNA polymorphism has been reported from monoclonal strains belonging to *Skeletonema* (Alverson & Kolnick, 2005). Thus, the heterogeneity reported here could theoretically be a consequence of intra-clonal variation. However, the base-pair substitutions observed were consistent in the sequencing reads from multiple PCR-products with several primers, and there was a clear difference in the degree of polymorphism between the seasons.

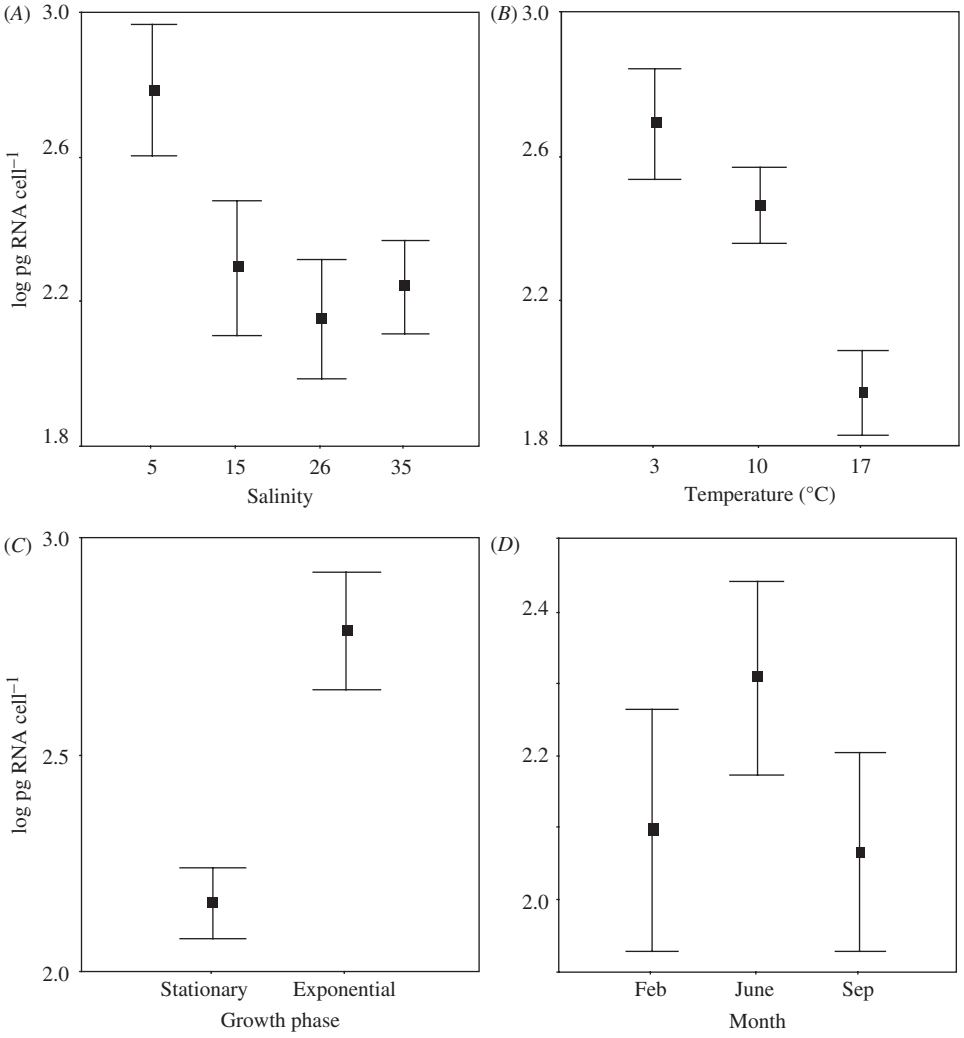

Fig. 8. RNA concentration per cell (log pg RNA cell^{-1}) among different salinities (*A*; $n = 27$), temperatures (*B*; $n = 36$), growth phases (*C*; stationary $n = 74$, exponential $n = 34$), isolation months (*D*; February: $n = 23$, June: $n = 25$, September: $n = 26$). Symbols are means. Error bars represent 95% confidence interval (CI).

All studies designed to investigate the presence or absence of small scale intraspecific heterogeneity in natural samples within any phytoplankton species reveal high levels of genetic diversity and low probability of isolating multiple lineages from the same clone, even within a single water sample (e.g. Orsini *et al.*, 2004; Evans *et al.*, 2005; Iglesias-Rodríguez *et al.*, 2006; Nagai *et al.*, 2007). None of our isolates belonged to the same clonal lineage, as revealed by genotyping the six microsatellite loci. Thus, the differentiated physiological responses in the investigated clones could indicate genetic origin. Moreover, the greater degree of genetic diversity based on allele size differences and the larger LSU rDNA polymorphism among the February and September clones compared to the June clones suggests that the overall genetic diversity among June isolates is less than the February and September isolates.

The observed division rates were comparable to previous reports for *Skeletonema* (Shi *et al.*, 2004; Takabayashi *et al.*, 2006). The estimated growth rates of the same isolates from the same seasons under standard culture conditions were based on triplicate cell counts of each isolate every 1–3 days when the cultures were in exponential growth phase, whereas the average numbers of division per day from the physiological experiment for each season were estimated based on weekly cell counts of three different isolates. Therefore there is a discrepancy between the mean observed growth rates under standard culture conditions (0.88, 0.79, 0.75 cell divisions d^{-1}) and physiological experiments (1.4, 1.32, 1.28 divisions d^{-1}) for February, June and September respectively. The growth rates under standard culture conditions are probably more realistic. However, irrespective of the method, the February isolates displayed significantly faster growth than the September and June isolates. This suggests that observed growth rate is partly controlled by environmental cues, but also that the February clones possess an

endogenous ability to grow considerably faster if the conditions are favourable. *Skeletonema marinoi* is an important species that contributes substantially to the spring bloom in the Fjord and coastal water. When the light and hydrographic conditions become favourable, algal biomass can increase several-fold within a matter of days. Generally Chl *a* concentrations increase from less than 1, to more than $10\,\mu g\,l^{-1}$, over a period of 2 weeks (Andersson *et al*., 2006). Provided that populations possess endogenous characteristics that are advantageous for the seasonal niches they inhabit, the fast response of the February clones appears realistic. In contrast, hydrography and light conditions are more stable during late spring and early autumn, and Chl *a* concentrations rarely reach more than $2–3\,\mu g\,l^{-1}$ (Andersson *et al*., 2006). Changes in algal biomass are gradual and populations adapted for these conditions may not have the same advantage of a very fast growth rate. Intraspecific variation of maximum growth rates in genetically separated clones pre-adapted to identical conditions has previously been reported for different marine phytoplankton species. Observed growth rates were higher for the coccolithophorid *Gephyrocapsa oceanica* isolated from neritic waters and lower for clones originating from oceanic water (Brand, 1982). *Ditylum brightwelli* isolated from the same location but separate water masses, defined by salinity and temperature gradients during different tidal amplitudes, also displayed significantly different growth rates (Rynearson & Armbrust, 2004).

Diverse records of maximum cell densities among different isolates with respect to salinity, temperature and isolation month, suggest environmental control and genetic differentiation as determining factors. All clones, irrespective of salinity-temperature combination, reached stationary phase before the experiment was terminated. If the maximum cell densities observed were simply a function of the environment, all clones exposed to identical salinity-temperature conditions should eventually reach the same cell concentration since the limiting factors were the same for all clones. Extremely low salinity resulted in low maximum cell densities in all clones, with 5 PSU possibly representing the lowest salinity the populations can tolerate. However, in accordance with previous studies (Rijstenbil *et al*., 1989; McQuoid, 2005), a rapid shift to a new condition, from approximately 10 PSU to 15 or 35 PSU, did not have any significant effect. Considerably higher maximum cell densities were attained at 10°C, which probably reflects the adaptation of all clones to their growth conditions prior to the experiment, i.e. the shift to a new temperature will invariably negatively affect the maximum cell density. The September isolates reached significantly higher cell densities than the February or June isolates. In September the phytoplankton community composition in the Gullmar Fjord is a mix of different phyla. The flora is frequently dominated by dinoflagellates (SMHI, 2008), which are generally less competitive than diatoms during nutrient replete conditions (Graham, 2000). Therefore, it might be suggested that this seasonal niche provides an opportunity for the *S. marinoi* population to grow and attain a large population, which is perhaps not possible during the spring bloom when competitors include other quickly growing diatom species.

Shifts in environmental parameters are known to influence cell morphology in diatoms. For instance, *Skeletonema* responds to elevated nutrient concentrations by increasing its biovolume (Carter *et al*., 2005; Eker-Develi *et al*., 2006). In this study low salinity and temperature implied larger cell size and lower maximum cell density, which might be interpreted as a culture effect, with larger cells at low densities However, cells from the June isolates were small and displayed low cell densities while the February isolates were significantly larger but had equally low maximum cell densities. The natural phosphate and nitrate concentrations in the surface water of the Gullmar Fjord were highest in February (Lindahl, 1995), when the temperature is relatively low. This could explain the larger size of cells isolated from a spring bloom in February. However, given the long period of acclimation to identical growth conditions prior to this study, it suggests that size differentiation is maintained in culture among seasonally separated populations.

Total RNA concentration per cell reflects overall intracellular activity (Hillis *et al*., 1996). Consequently, cultures in exponential growth phase and isolates subjected to adverse conditions will display high RNA concentration per cell, which was confirmed here. These isolates will engage the cell machinery at different levels to provide fast-growing cells with essential components, or to handle stress and withstand the situation. The high RNA concentrations of isolates subjected to low salinity (5 PSU) are most probably a consequence of the adverse conditions. The effects of temperature on RNA concentration were inversely related to the effect of temperature on the number of doublings per day. This was due to the experimental design, in which RNA concentration per cell was measured only once during the experiment, i.e. after 7 days. After 1 week, all clones subjected to 17°C, and many of the clones subjected to 10°C, had already reached stationary growth phase, whereas the clonal cultures grown at 3°C had barely reached, or were midway

through, the exponential growth phase. Interestingly, RNA concentration per cell among the isolates that had reached stationary phase was significantly affected by isolation month. The June isolates had significantly higher RNA concentration per cell than the February and September isolates. The natural abundance of *S. marinoi* in Gullmar Fjord during the summer months is low, or undetectable, most probably due to adverse environmental conditions or predation. The impact of grazers on the overall phytoplankton community is most notable after the spring bloom (Vargas *et al*., 2002). However, in a microcosm study previously conducted in the Gullmar Fjord at the end of May, we did not see much effect of grazing on *S. marinoi*. On the contrary, *Skeletonema* generally outgrows the grazers (McQuoid & Godhe, 2004). It is therefore suggested that the environmental conditions in June are non-optimal for *Skeletonema* growth, but that the population exploiting this seasonal niche possesses physiological characteristics to handle stressful conditions.

Analogous studies on the genetic diversity and physiological differentiation of marine phytoplankton are rare in the literature. However, two ecotypes of *Prochlorococcus* provide an example of niche divergence. Based on small subunit (SSU) rDNA sequences, surface *Prochlorococcus* ecotypes formed a distinct cluster, and a physiological response (measured as growth rates and Chl b/a_2 ratio) at sub-saturated light intensities was uniform for these clones. This contrasted with a deep water ecotype, which branched from the surface clones but did not form a distinct cluster in the SSU phylogeny inferred, and displayed a non-uniform physiological response to the same irradiances (Moore & Chisholm, 1999; Rocap *et al*., 2003). In the present study, the June *S. marinoi* clones had homogeneous LSU rDNA sequences, displayed less allele diversity in the genotyped microsatellite loci and less variation in all investigated variables, i.e. average number of doublings per day, maximum cell density, cell size, and RNA concentration per cell.

However, February and September isolates displayed higher allele diversities, and had five and four base-pair substitutions, respectively. In addition, these isolates displayed larger variation in their physiological response. The February and September clones were isolated from water samples with higher *S. marinoi* abundances and hypothetically larger numbers of clonal lineages. The larger variation in physiological response of the February and September isolates may be a consequence of a larger pool of genetic diversity compared to the June isolates.

All isolates in this study may constitute members of a single population, of which the June isolates represent remnant clonal lineages from the February bloom. In this study three clones were treated as replicates for each season. To determine whether our findings are inherent to the different seasons, the same experiment should be replicated with additional clones, representing the same seasons, from a different year. However, the differentiated physiological responses of the temporally separated isolates indicate that different populations may succeed each other in the Fjord.

Conclusion

Temporal and spatial, intraspecific genetic and physiological differentiation is commonly observed at small and large scales in the marine environment. As a result, total biomass and production may be greater and extend over a longer time period than if the species is represented by a single undifferentiated population (Duffy & Stachowicz, 2006). Our data suggest that genetic and physiological differentiation is seen at very small scales, which is also supported by earlier studies (Gallagher, 1982). The plausible causes of the differentiated physiological characteristics are environmental conditions and genetic structure. Our data imply that both affect the responses observed.

All clones were maintained under exactly the same conditions for approximately 2 years prior to the experiment, but despite this they retained their physiological characteristics. Clonal cultures kept in nutrient-replete media are reported to propagate mainly asexually and to restore cell size through vegetative enlargement rather than auxosporulation and sexual reproduction (Hargraves & French, 1983). This is in accordance with regular observations of the clonal cultures isolated from the Gullmar Fjord, in which swarming cells are extremely rare. Asexual propagation minimizes the risk of genetic drift and random mutations through unequal crossing over, and thus the physiological response linked to particular isolation months shown in this experiment constitutes an intrinsic genetic structure of the clones exploiting the particular seasonal niche. Based on our observations we would like to warn against using only one or a few cultures to draw ecological conclusions on a species in general, since the particular isolate might exhibit different traits from another clone isolated at another time of year.

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