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# Effects of Grazer Presence on Genetic Structure of a Phenotypically Diverse Diatom Population

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**Abstract** Studies of predator–prey systems in both aquatic and terrestrial environments have shown that grazers structure the intraspecific diversity of prey species, given that the prey populations are phenotypically variable. Populations of phytoplankton have traditionally considered comprising only low intraspecific variation, hence selective grazing as a potentially structuring factor of both genetic and phenotypic diversity has not been comprehensively studied. In this study, we compared strain specific growth rates, production of polyunsaturated aldehydes, and chain length of the marine diatom *Skeletonema marinoi* in both grazer and non-grazer conditions by conducting monoclonal experiments. Additionally, a mesocosm experiment was performed with multiclonal experimental *S. marinoi* populations exposed to grazers at different levels of copepod concentration to test effects of grazer presence on diatom diversity in close to natural conditions. Our results show that distinct genotypes of a geographically restricted population exhibit variable phenotypic traits relevant to grazing interactions such as chain length and growth rates. Grazer presence affected clonal richness and evenness of multiclonal *Skeletonema* populations in the mesocosms, likely in conjunction with intrinsic interactions among the diatom strains. Only the production of polyunsaturated aldehydes was not affected by grazer presence. Our findings suggest that grazing can be an important factor structuring diatom population diversity in the sea and emphasize the importance of considering clonal differences when characterizing species and their role in nature.

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## Introduction

Ecological dynamics are driven by rapid evolution in predator–prey systems, insinuating that ecological and evolutionary processes occur on similar time scales [1]. Therefore, both phenotypic characteristics (e.g., size of prey) and genetic variation (e.g., population structure) may be configured in interacting predator and prey species. Populations of marine microalgae are genetically structured over geographical scales [2, 3]. Also, within a restricted geographic site, one population may consist of numerous distinct genotypes [4]. There is also evidence for significant intraspecific variability in ecologically important physiological traits (e.g., growth rate, toxin production) in single populations from geographically restricted locations [5].

It is well known that grazers may structure communities through direct and indirect interactions [6, 7]. Copepod grazers may, e.g., suppress larger phytoplankton, ciliates,

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and heterotrophic dinoflagellates, thereby liberating smaller (<5 μm) phytoplankton from predation and competition [8]. Similarly there is some evidence for differential grazing within a population. Microzooplankton and copepods graze on different size classes of *Phaeocystis* sp., and *Phaeocystis* in turn respond to chemical cues from grazers by adjusting colony size to minimize losses to grazers [9]. In terrestrial ecosystems, grazers may maintain intraspecific diversity by suppressing successful genotypes. A classical example is the predation of thrushes on garden snails. The thrush adjusts its search image to the most abundant snail morphotype [10]. The most abundant morph will consequently be suppressed and a stable polymorphism maintained. Similarly, dominating bacteria will be suppressed by phages for physical reason. The encounter rate between pathogen and bacteria will increase with increasing densities, resulting in "kill the winner" dynamics favoring genetic diversity also in bacterial communities [11]. Relevance of similar processes in phytoplankton is, however, largely unknown.

Genetic diversity of natural populations (not only phytoplankton) is regulated by selection, migration (gene flow), random genetic drift, mutation, and inbreeding. This theoretical framework is well established [12]. However, ecological mechanisms, such as grazing (top–down mechanism), that may be important in configuring genetic diversity deserves more attention within marine phytoplankton populations. It has been demonstrated in both field and experimental studies that increased grazing of planktonic bacteria leads to shifts in the phenotypic and genotypic composition of bacterial assemblages [13]. Studies on plants indicate that allelopathy, autotoxicity, and resource competition [14, 15] configure intraspecific diversity. In addition, genotypes of the same species may facilitate each other and show niche complementarity [16, 17]. Furthermore, bottom–up mechanisms may configure intraspecific diversity. For instance, temperature or salinity tolerance ranges may result in dominance of certain genotypes in a population [18].

*Skeletonema marinoi* is a common diatom in temperate estuaries and coastal waters where it often forms dense blooms [19]. The species occurs frequently in the Baltic Sea and often dominates the phytoplankton community during the productively important spring bloom period [20]. It is likely to be a common prey species for copepods in the Baltic Sea [21]. The diatom–copepod food web link is considered essential for trophic energy transfer in marine ecosystems [22, 23]. The species is also a known producer of fatty acid-derived secondary metabolites, such as polyunsaturated aldehydes (PUAs) [24], proposed to have negative effects on copepod reproduction [25], although this is still debated [26]. Within this species, a high variability of PUA production has been observed [27, 28]. Whether copepods selectively avoid PUA producing strains, or not, is not known.

During a mesocosm experiment, we offered a natural community of copepods eight genetically distinctive strains of *S. marinoi*, which varied in physiological properties, such as growth rate, PUA production, and chain length. The aim was to examine the potential effect of grazing pressure—reflected by different grazer levels—on the genetic and phenotypic diversity of the experimental populations. Multiple genotypes of *S. marinoi* and a natural copepod community were incubated at bloom densities in mesocosms in order to mimic natural conditions. The density of *Skeletonema*, the nutrient concentration, and the light availability were carefully monitored to ensure that the phytoplankton community was in exponential non-limited growth phase, in order to avoid confounding factors of genotype competition. Our hypothesis was that selective grazing by copepods alters the genetic diversity (clonal richness, evenness of clonal proportions) of the responding *S. marinoi* population, since copepods choose among strains with varying physiological traits and in doing so configure the genetic and phenotypic make-up of the population.

## Material and Methods

### Establishment and Maintenance of *S. marinoi* Strains

The *S. marinoi* strains used in this study originated from germinated resting stages. A sediment core was collected from monitoring station C14 (N 62°07.15′, E 18°33.14′) located in the central Bothnia Sea using a LIMNOS gravity corer. The core was retrieved from 83 m depth at the end of April 2011. The upper 10 cm of the core was recovered and stored in dark at 4 °C until processed. Individual chains were isolated and clonal cultures established essentially as described in Godhe and Härnström [2]. From the 40 isolates maintained routinely in f/2 enriched [29] local sterilized sea water at 4 °C with a 12:12 ligth/dark cycle at an irradiance of 40 μmol photons $m^{-2}$ $s^{-1}$, eight were randomly selected for the consecutive experiments and analyses. We amplified eight microsatellite loci (S.mar 1-8) [30] according to a protocol described in Godhe and Härnström [2] in order to genotype the strains.

### Characterization of Clonal Phenotypic Properties

In order to characterize the genetically distinct strains we conducted two different sets of monoclonal experiments. (a) A growth experiment aimed at estimating maximum growth rates of each strain. (b) A PUA induction experiment with monoclonal incubations with grazers and a control without grazers was conducted in order to study the potential effect of grazers on inducing production of PUAs in *S. marinoi*.

In the growth experiment, we estimated the maximum growth rate of each *S. marinoi* strain in single strain incubations (three replicates) for 5 days in f/2 medium with a L/D cycle of 16 h:8 h (light intensity, 75 μmol photons $m^{-2}$ $s^{-1}$) in

10 °C. Starting concentration was 5,000 cells $ml^{-1}$. A daily sample of 1.5 ml was taken from each bottle and fixed in Lugol's solution for cell enumeration. Cell abundance estimates were based on counts using a gridded 1 ml Sedgewick Rafter cell. Growth rates were calculated from the rate of increase in cell concentrations from ln transformed growth curves according to the method of Wood et al. [31].

In the PUA induction experiment, the purpose was to establish strain specific PUA production profiles for the individual experimental strains. Single strains were added to the same grazer community as used in the mesocosms (see below). In replicates of three, with and without copepods, the *S. marinoi* strains were incubated for four days on a slowly rotating plankton wheel at a temperature of 10 ° C and at a light intensity of approximately 40 μmol photons $m^{-2}$ $s^{-1}$. Six bottles filled with 250 ml of f/2 enriched filtered seawater containing each algal strain (start concentration 40, 000 cells $ml^{-1}$) were placed on the plankton wheel and five adult copepods were added to three of them, the other three were used as controls. The copepods were from the same natural community collected for the mesocosm experiment. After 3 days, 1.5 ml was sampled for cell counts and fixed with acid Lugol's solution. The cell abundance was estimated as above for the growth experiment. We also measured the chain length (number of cells per chain) of 25 randomly chosen *S. marinoi* chains from each replicate. The remaining volume was filtered onto GF/F filters and further processed for PUA analysis [32]. The start concentration of 40, 000 cells $ml^{-1}$ was calculated using a mean value of growth rate (0.8), estimated from the growth rate experiment, to ensure that enough biomass could be filtered for PUA analysis at the end of the experiment. The start concentration of 20 μg C copepods $l^{-1}$ was used to mimic the highest level of copepods subsequently used in the mesocosms.

## Preparation of Large-Scale Experimental Cultures

The eight experimental strains of *S. marinoi* (C1402, C1404, C1405, C1406, C1407, C1416, C1417, and C1419) were transferred to the experimental temperature of 10 °C and the culture volume was successively increased from 50 ml to approximately 40 l. Larger volumes were grown in aerated culture bags. On every alternate day, the *S. marinoi* strains were diluted with 1/3 of their volume to obtain nutrient replete semi-continuous cultures.

## Mesocosm Experimental Setup

The experiment was run in twelve cylindrical mesocosm tanks 4.86 m high and 0.74 m wide (total volume ~2,000 l) at the MesoAqua facility, Umeå Marine Sciences Centre, Sweden. Seawater collected outside the station at 8 m depth was passed through a filter set consisting of a 10-μm pre-filter (Fsi Filter Spec. Int.) and three parallel 1 μm patron filters (Roki Techno Co. Ltd). Before any experimental organisms were added to the mesocosms, three 50 ml subsamples from one tank were fixed in Lugol's solution and microscopically examined to confirm the absence of any contaminating organisms. Hydrographic conditions and continuous circulation of the water column by thermal convection were controlled from a computer. Convection was obtained by warming the lower section of the mesocosms to 10.5 °C and simultaneously cooling the upper section to 9.5 °C. The water column was completely mixed within 24 h. The irradiance directly below the water surface was 500±30 μmol $m^{-2}$ $s^{-1}$ with a light/dark cycle of 12 h:12 h. Nutrients were added in excess, corresponding to five times winter conditions in the Bothnian Bay [33]. Each tank received additions of ammonia, nitrate, phosphate and silica to the final concentrations of 28 μg $l^{-1}$ $NH_4Cl$, 300 μg $l^{-1}$ $NaNO_3$, 46 μg $l^{-1}$ $NaH_2PO_4 \times H_2O$, and 590 μg $l^{-1}$ $Na_2SiO_3 \times 5H_2O$. Nutrients were added again at day 9 and measured at day 10 to ensure that nutrient supplies were continuous and in excess throughout the experiment. Inorganic nutrient analyses were performed using a QuAAtro Autoanalyzer. The mesocosm experiment was run for 12 days.

Copepods were collected with net tows (mesh size 90 μm and sieved through a 500-μm net to remove larger predators) from the sea outside the station and kept for up to two weeks in 200 l tanks containing filtered seawater (FSW, 0.2 μm). They were fed with *Rhodomonas salina* and *Isochrysis galbana* from batch cultures maintained at the same conditions as described for *S. marinoi*. Prior to the experiment, the copepods were carefully pooled together in one 200 l tank and six subsamples of 8–10 copepods were analyzed for particulate organic carbon (POC). The animals were filtered onto precombusted (450 °C, 2 h) Whatman GF/F glass fiber filters and dried in 60 °C for 24 h. Particulate organic C was analyzed using an elemental analyzer (Carlo Ebra 1108). Before these were added to the mesocosm tanks, the copepods were rinsed with FSW on 100 μm net in order to remove remaining *R. salina* and *I. galbana* cells. Copepods were starved by adding them to the tanks 24 h before the diatoms. We used three treatments with different initial copepod densities (low 1 μg C $l^{-1}$, medium 10 μg C $l^{-1}$, high 20 μg C $l^{-1}$; Fig. 1) within the natural range of the Baltic Sea [34, 35] and one control treatment without copepods. Incidentally, two replicates (instead of three) were without grazers (control) and the low grazer level included four replicates. The medium and high grazer treatments had three replicates. A mixture of all eight *Skeletonema* strains was added in identical proportions to each mesocosm. The total initial concentration of *S. marinoi* was ~100 μg C $l^{-1}$ (*S. marinoi* contained on average 26.2 pg C per cell). The diatom density corresponded to natural levels of a developing population in a spring bloom in NW Baltic Sea [36].

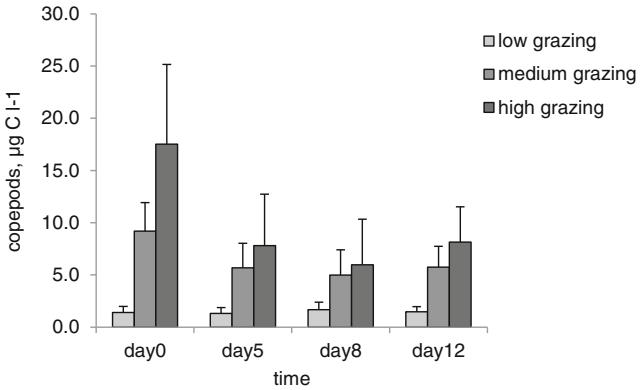


**Fig. 1** Copepod biomass (µg C $l^{-1}$) in the mesocosm experiment. Three experimental conditions with rising copepod density and a control ($N=2$) without copepods were used in the mesocosm experiment. Low grazing density ($N=4$), medium, and the high grazing density ($N=3$). *Error bars* represent standard error (SE)

## Mesocosm Sampling and Analyses

The mix of eight *S. marinoi* strains was added 24 h before the first sampling performed on day 0, to ensure complete turnover of the water column before sampling. Samples for PUA and phytoplankton were collected four times during the experiment (days 0, 5, 8, and 12) as described in Paul et al. [37]. Copepod abundance was sampled the same days by taking 15 l of water from each mesocosm tank. Copepods were collected gently by using a 90 µm net to a final of 50 ml, which was fixed (4 % formalin) and stored in 8 °C for later analysis. Temperature and pH were measured daily by a Seaguard and Hanna sond. Initial samples for POC of *S. marinoi* cultures were filtered (20 ml) onto Whatman GF/F glass fiber filters and analyzed as described for copepods above. Depending on cell concentration, 50 to 100 ml samples were filtered for analysis of chlorophyll *a* (chl *a*) onto Whatman GF/F glassfiber filters extracted in ethanol and analyzed according to the method described in Paul et al. [37].

## Determining Clonal Composition of *S. marinoi*

At the end of the experiment, on day 12, 50 chains of *S. marinoi* were isolated from each tank by micropipetting. Each chain was transferred to a series of f/2 medium drops in order to ensure that only one single chain was isolated. The single chain was transferred to sterile 48 well NUNC plates with 1.5 ml f/2 medium and put into the growth conditions as described above for the establishment of monoclonal cultures. When the growth was confirmed, the strains were transferred into 50 ml NUNC culturing flasks. DNA was extracted from exponentially growing strains using a CTAB based protocol previously described by Kooistra et al. [38]. Genotyping of the re-isolated strains by microsatellite loci to assess the clonal proportions per mesocosm was performed as described above for the experimental strains. In average, 72 % of the initially isolated strains yielded genetic data.

## Diatom Analyses

Abundances of *S. marinoi* were estimated using an inverted microscope (Leica DMI3000 B). Depending on the cell density in the sample, it was analyzed in a 2, 10, or 50-ml sedimentation chamber [39]. Samples settled for 24 h and subsequently the whole chamber bottom area was analyzed. Cell abundance estimates for the individual growth rate and PUA production experiments were done using a gridded 1 ml Sedgewick Rafter cell.

## Zooplankton Analysis

Samples were counted according to standard methods [40–42] using a light microscope (Zeiss Discovery V8). Species assignments were done according to a series of determinative keys [43–47]. Grazing was quantified individually for each mesocosm as in [48].

$$F = V\left(\mu - \left(\frac{lnC_t - lnC_0}{\Delta t}\right)\right)/N \quad (1)$$

where $F$ (clearance) is the volume swept clear (ml $ind^{-1}$ $day^{-1}$), $V$ is the volume, $\mu$ is the growth rate in controls ($day^{-1}$), $C_t$ the concentration at time $t$, and $N$ the number of grazers. The rate of ingestion ($I$) is calculated by multiplying the volume swept clear with the average cell concentration during the same time. Apparent growth rate ($day^{-1}$) is calculated as:

$$(lnC_t - lnC_0)/\Delta t \quad (2)$$

and consequently shows the combined effect of growth and grazing.

## Data Analyses

Data obtained from the monoclonal experiments were studied with analysis of variance (ANOVA). The growth experiment and chain length reduction in the PUA induction experiment were analyzed with one-way ANOVAs. Differences in PUA production were analyzed with a two-way ANOVA in GraphPad Prism 6 using Tukey's multiple comparisons to test for significant differences between individuals and treatments. The confidence interval (CI) was set to 95 %. The normality of variances was tested with Kolmogorov–Smirnov and the homogeneity of variances was tested with Levene's test in SPSS v. 16. The mesocosm data, i.e., differences in PUA content, chain length, clonal proportions, clonal richness, and evenness of clonal proportions were analyzed using permutational

multivariate analysis of variance (PERMANOVA) in the software Primer 6 with the PERMANOVA + add-on package which can handle unbalanced experimental design. These analyses were based on data converted into Bray Curtis similarity indexes. In order to investigate significance level the residuals were permutated under a reduced model (9,999 permutations). All analyses were two-factored with time, strain or grazer density as fixed factors. Pair wise comparisons were conducted to observe differences between individual treatments or strains. Correlation analysis performed in GraphPad Prism 6 was used to compare grazer density with apparent growth rates. This was also done for comparing the final clonal proportions in mesocosms with respective PUA content per cell. Best-fit curves were calculated for observing the level of correlation between clonal richness and evenness of clonal proportions and concentration of grazers. Comparison of curves in GraphPad Prism 6 resulted in the use of non-linear second order polynomial (quadratic) correlation curves.

## Results

### Phenotypic Properties of Experimental Strains

*Growth Experiment* The maximum growth rates of *S. marinoi* strains were significantly different when grown in optimal conditions without grazers (one-way ANOVA $p < 0.0001$). Multiple comparison tests (Tukey's test) identified a significantly higher growth rate of strain C1402 compared to strains C1406, C1407, C1416, and C1419. Strain C1404 had a significantly higher growth rate than strains C1407 and C1416. Strains C1405 and C1417 grew faster than C1419 (Fig. 2a).

*PUA Induction Experiment* The range of PUA production (heptadienal and octadienal) was 0.0–0.15 fmol per cell. Strain C1416 did not show any production of PUA compounds. We observed significant differences in respect of PUA production among many of the monoclonal cultures (one-way ANOVA strain $p < 0.0001$, Fig. 2b). Multiple comparison tests showed that strain C1407 produced significantly more PUA than C1402, C1404, C1405, C1416, and C1417 and that C1419 had significantly higher production compared to other strains. Similar differences were valid in grazed treatment, i.e., the observed individual PUA production was unrelated to the presence of grazers (two-way ANOVA strain $p < 0.0001$, grazer $p = 0.15$, strain × grazer $p = 0.53$).

Chain length (defined as number of cells per chain) was significantly different among the strains in absence of grazers. Strains C1402 and C1407 formed the longest chains, on average four cells per chain. When the grazers were present, the chain length was significantly reduced in all strains (two-way ANOVA, strain $p < 0.0001$, grazer $p < 0.0001$, strain × grazer $p < 0.0001$, Fig. 2c). The strains responded differently to presence of grazers (one-way ANOVA, chain length reduction $p < 0.05$), strains C1402 and C1407 formed the longest chains also when grazers were present but the amount of reduction was not significantly different from the other strains. The chain length reduction of strain C1417 was significantly higher compared to the other strains (Tukey's test CI 95 %), and C1419 formed the shortest chains when grazers were present.

### Mesocsom Experiment

*Envrironmental Conditions* Temperature varied between 9.9 °C and 10.7 °C in the water column. pH varied between 7.6 at day 0 and 8.9 at day 12 (Table 1) indicating increase of phytoplankton density towards the end of the experiment. Nutrients, P, N, and Si, were not limiting growth of *S. marinoi* as 80–90 % of the nutrients remained on day 10.

*Copepod Population* The added mix of copepods was similar in all treatments. The number of copepods decreased during the experiment in all treatments except in the low grazing treatment (Supplementary Table 1).

*Diatom Population* The *S. marinoi* population showed positive growth during the mesocosm experimental period in the control and all the treatments (Table 1). During the first 5 days, increasing grazer density significantly decreased the apparent growth rates of *S. marinoi*. Later in the experiment, grazing did not reduce apparent growth rate in any treatment (Fig. 3). Grazing resulted in significantly lower apparent growth rate in the medium grazer treatment during the first 5 days of the experiment (PERMANOVA, pair wise comparison $p < 0.05$, Supplementary Fig. 1). The suppressing effect of grazing in the beginning of the experiment is confirmed by lower clearance rates towards the end of the experiment (Supplementary Table 2). The start of exponential growth phase was observed at day 8. The small differences in population size were not related to grazer level (PERMANOVA, time $p < 0.0001$, grazer $p = 0.41$, time × grazer $p = 0.87$). Chl *a* displayed a similar pattern, i.e., a significant increase from initial conditions, but the effect of grazing was non-significant (PERMANOVA, time $p < 0.0001$, grazer $p = 0.50$, time × grazer $p = 0.60$, Table 1). PUA (heptadienal and octadienal) content per cell increased significantly with time (PERMANOVA, time $p < 0.001$) from non-detectable levels on day 0 to 3.15–3.98 fmol per cell in the grazed treatments compared to 1.64 fmol per cell in the control (Table 1). However, we found no significant differences in PUA content per cell between the control and grazed treatments (PERMANOVA, grazer $p = 0.52$, time × grazer $p = 0.52$). The level of dissolved PUA increased towards the end of the experiment and was highest on day 12 in all treatments. The increase of dissolved PUA compounds was not significantly affected by grazing (PERMANOVA, time $p < 0.05$, grazer $p = 0.57$, time × grazer $p = 0.83$; Table 1). We found no correlation between

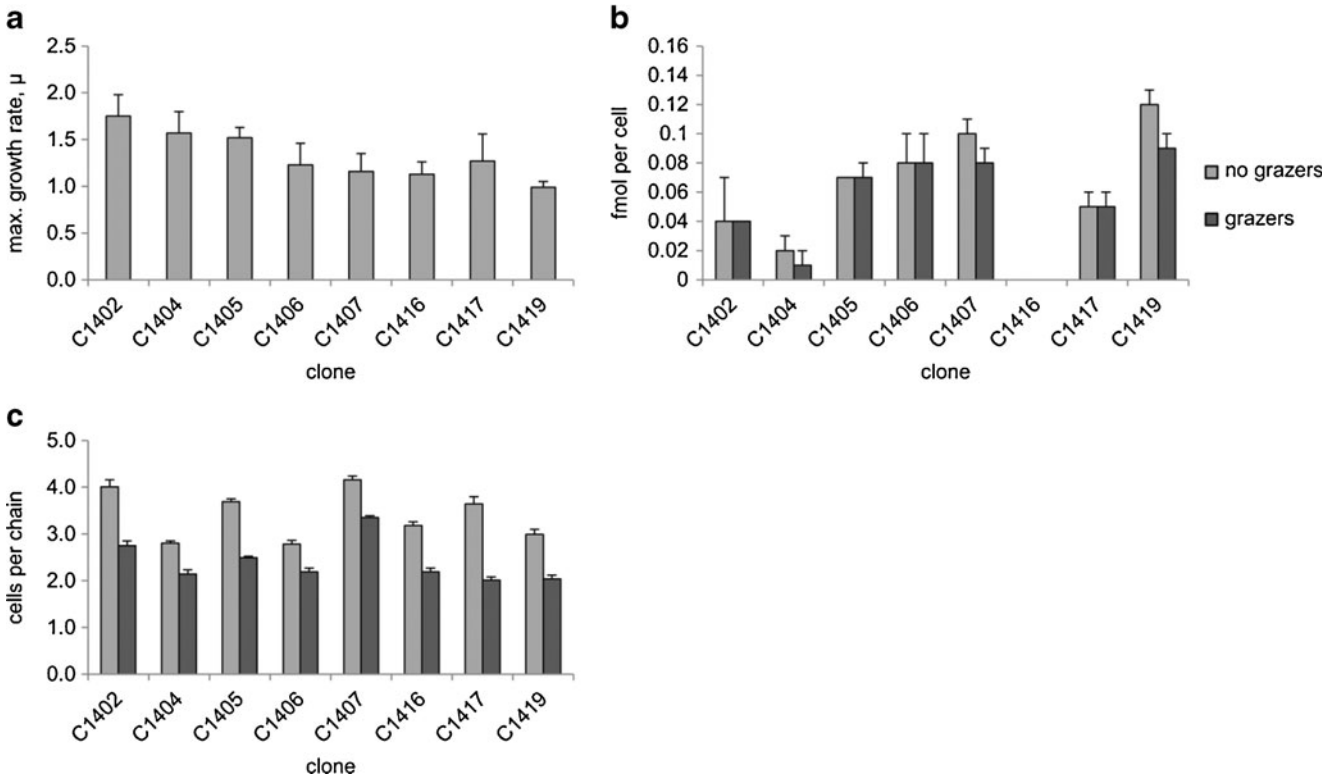


**Fig. 2** Experiments for phenotypic characterization of individual strains of *S. marinoi* used in the mesocosm experiment. **a** Maximum growth rates (μ) of monocultures incubated without grazers at optimal growth conditions. **b** PUA content per cell (fmol cell$^{-1}$) in monocultures of *S. marinoi* incubated with and without grazers. **c** Chain length (cells per chain) in monoclonal cultures with and without grazers (labeling as in **b**). *Error bars* represent SE of three replicates

PUA content in strains and the final proportions of respective strains in the mesocosms ($R^2$=0.05).

## Genetic Structure of *S. marinoi* in Mesocosms

Initially each strain made up ~12.5 % of the total community. The final proportions of each strain at different grazer levels are presented in Fig. 4. Clonal proportions changed significantly during the experiment (initial vs. final proportions) but was unrelated to grazer concentration (PERMANOVA, time $p$ <0.001, grazer $p$ =0.46, time × grazer $p$ =0.46). Individual proportions of strains within treatments were significantly different (PERMANOVA, strain $p$ <0.001). Strain C1407 had a significantly higher proportion in the medium grazer level compared to the control and the high grazer level (pair wise comparison). Also, strains C1417 and C1419 were not detected at all at the end of the experiment in the medium grazer treatment. However, these two strains were poorly represented in the low and high grazer treatments as well, with an average proportion ranging from 1 % to 4 %. Strains C1407 and C1402 dominated the community irrespective of grazer density. Strain C1407 dominated the population with proportions ranging between 38 % and 59 % in all treatments and the control. Strain C1402 had the second highest proportions (14–35 %) throughout the experimental gradient.

At day 12, there was a non-linear correlation between clonal richness (defined as the total number of strains in a population) and grazer level ($R^2$=0.88). Clonal richness decreased from the start of the experiment (PERMANOVA, time $p$ <0.0001, grazing $p$ <0.0001, time × grazing $p$ <0.0001), and it was significantly lowest in the medium grazer level compared to the control and the two other grazer levels (pair wise comparison, $p$ <0.001; Fig. 5a).

For each treatment we calculated evenness (Pielou) based on clonal proportions. There was a non-linear correlation between grazer level and evenness of clonal proportions ($R^2$=0.69). Evenness decreased in all treatments (PERMANOVA, time $p$ <0.0001, grazing $p$ <0.001, time × grazing $p$ <0.001) during the experiment and was significantly lower (pair wise comparison, $p$ <0.001) in the medium grazer level compared to the control and the other grazer levels (Fig. 5b).

## Changes in *S. marinoi* Chain Length

The number of cells per chain was significantly reduced in all treatments over time, but not in the control. The chain length at all grazer levels was significantly different from the control at day 8 (PERMANOVA, time $p$ <0.05, grazer $p$ <0.05, time × grazer $p$ <0.05) and day 12 (PERMANOVA, time $p$ <0.001, grazer $p$ <0.001, time × grazer $p$ <0.001; Fig. 6).

**Table 1** Mean values of phytoplankton density and chl *a* on days 0, 5, 8, and 12

| | day | cells $l^{-1}$ | SE | chl *a*, μg $l^{-1}$ | SE | fmol PUA $cell^{-1}$ | SE | diss. PUA, nM | SE | temp. (°C) | SE | pH | SE |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| C | 0 | $3.8\times10^6$ | $1.0\times10^6$ | 7.0 | 0.0 | 0.0 | – | 0.6 | – | 10.6 | <0.1 | 7.6 | 0.0 |
| | 5 | $14.5\times10^6$ | $1.1\times10^6$ | 15.9 | 1.4 | 0.07 | <0.01 | 0.7 | – | 10.4 | <0.1 | 7.8 | 0.1 |
| | 8 | $23.6\times10^6$ | $5.1\times10^6$ | 24.1 | 7.0 | 0.2 | <0.1 | 0.7 | – | 10.3 | <0.1 | 8.3 | 0.1 |
| | 12 | $56.1\times10^6$ | $12.8\times10^6$ | 39.8 | 12.1 | 1.6 | 0.1 | 1.0 | – | 10.5 | <0.1 | 8.8 | 0.2 |
| LG | 0 | $4.7\times10^6$ | $1.4\times10^6$ | 7.0 | 0.2 | 0.0 | – | 0.6 | | 10.4 | <0.1 | 7.8 | 0.0 |
| | 5 | $14.4\times10^6$ | $0.5\times10^6$ | 14.1 | 1.6 | 0.06 | <0.01 | 0.6 | <0.1 | 10.1 | <0.1 | 7.9 | 0.1 |
| | 8 | $20.4\times10^6$ | $2.5\times10^6$ | 24.4 | 5.8 | 0.3 | <0.1 | 0.8 | <0.1 | 10.4 | <0.1 | 8.4 | 0.2 |
| | 12 | $53.1\times10^6$ | $7.6\times10^6$ | 41.5 | 8.7 | 4.0 | 1.9 | 1.1 | 0.3 | 10.2 | 0.1 | 8.8 | 0.1 |
| MG | 0 | $4.5\times10^6$ | $0.7\times10^6$ | 7.1 | 0.3 | 0.0 | – | 0.6 | – | 10.1 | <0.1 | 7.6 | 0.0 |
| | 5 | $11.2\times10^6$ | $2.0\times10^6$ | 14.2 | 1.0 | 0.08 | <0.01 | 0.6 | – | 10.0 | <0.1 | 7.8 | 0.2 |
| | 8 | $21.2\times10^6$ | $4.0\times10^6$ | 22.1 | 1.6 | 0.7 | <0.1 | 0.7 | <0.1 | 9.9 | <0.1 | 8.3 | 0.1 |
| | 12 | $57.1\times10^6$ | $19.5\times10^6$ | 39.6 | 3.7 | 3.9 | 2.7 | 1.0 | – | 10.2 | <0.1 | 8.9 | 0.2 |
| HG | 0 | $3.6\times10^6$ | $1.5\times10^6$ | 6.0 | 1.4 | 0.0 | – | 0.6 | <0.1 | 10.2 | <0.1 | 7.6 | 0.0 |
| | 5 | $9.1\times10^6$ | $1.7\times10^6$ | 11.0 | 2.1 | 0.1 | <0.01 | 0.6 | <0.1 | 10.6 | <0.1 | 7.7 | 0.1 |
| | 8 | $15.0\times10^6$ | $2.8\times10^6$ | 18.3 | 4.0 | 0.9 | <0.1 | 0.7 | <0.1 | 10.3 | <0.1 | 8.4 | 0.1 |
| | 12 | $45.9\times10^6$ | $8.8\times10^6$ | 31.6 | 7.4 | 3.2 | 1.2 | 0.8 | <0.1 | 10.0 | 0.2 | 8.7 | 0.1 |

PUA concentration given as per cell content and in dissolved form. Average values for basic water parameters collected with a Seaguard and a Hanna sond were also given

*C* control, *LG* low grazing, *MG* medium grazing, *HG* high grazing

## Discussion

We conducted a mesocosm experiment to study potential effects of grazer presence and abundance on intraspecific variation of a diatom population consisting of eight genetically distinct strains of *S. marinoi*. Phenotypic variation of genetically distinct *S. marinoi* strains was examined by two monoclonal experiments, including grazer and non-grazer treatments. The genotyping and phenotypic characterization of our experimental strains confirm that diatom populations consist of genetically distinct strains, which differ significantly with regard to phenotypic traits, such as physiological functions or morphological characters. This variation was generally affected by grazer presence and the response was strain specific. Our mesocosm data show that grazers are able to affect clonal richness and evenness of clonal proportions within the range of natural densities of our studied predator–prey system. Genetic diversity was significantly reduced at the medium grazer level implying that grazers affect intraspecific diversity of *S. marinoi* at specific copepod/diatom ratios.

### Grazer Relevant Phenotypic Traits in Experimental Strains and Grazed Mesocoms

Phenotypic variation among strains of phytoplankton increases the ability of a species to respond and adapt to changes in their environment [49, 50]. Phenotypic variation of populations is subsequently also known to enhance ecosystem stability and recovery from disturbance [51]. Variability of physiological characters as shown here for the experimental Baltic Sea *S. marinoi* population may enable their dominating status during the spring bloom. For instance, the population exhibited variation of maximum growth rate. The growth rate of an individual is governed by numerous biochemical processes occurring in the cell [52] making it a relevant trait to compare between strains. The most dominating strains in the mesocosms, C1402 and C1407, showed significantly different maximum growth rates (C1402 $\mu=1.75$ $day^{-1}$ vs. C1407 $\mu=1.16$ $day^{-1}$). Our data support findings in earlier studies showing that certain strains (strain C1402 in this case) might become dominating because of their fast growth rates [4]. However, strains (C1407 in our study) do not necessarily rely only on fast growth but may use other strategies. *S. marinoi* may for instance reduce its chain length and thereby reduce the grazing risk [53]. However, C1407 had the longest chains at grazer presence. Strain C1407 was one of the strains producing most PUAs, but our other data suggests that PUA is unrelated to grazers, i.e., other strategies must be involved. The ecological significance of PUAs is not clear. Some studies have shown that PUAs have negative effects on copepod reproduction [e.g., 25] while others have reported negligible effects in field conditions [54–58]. It is therefore plausible that factors which have not been quantified here, e.g., clonal interactions, affect the structuring of the diatom populations. Strains with different fatty acid content and quality may also be grazed upon at different rates [59]. Such a dynamic and phenotypically plastic population may respond quickly to day-to-day fluctuations in the environment and consequentially prevail over longer periods [5].

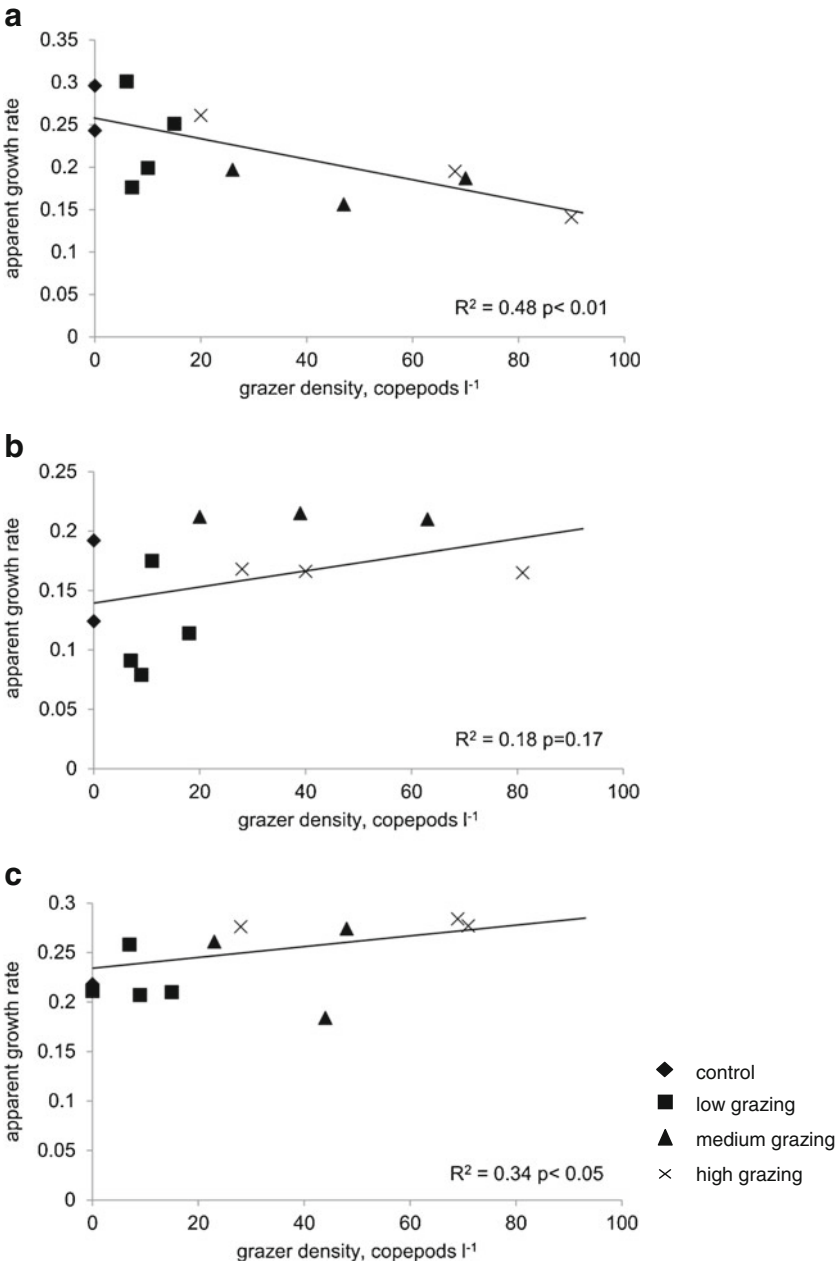


**Fig. 3** Apparent growth rates vs. grazer density during days 0–5 (**a**), 5–8 (**b**), and 8–12 (**c**). In the beginning of the experiment (days 0–5), increasing grazer density decreased the apparent growth rate of *S. marinoi* populations. This relationship was not found during days 5–12

Several factors have been shown to control chain length in diatoms, e.g., temperature, amount of light, growth rate, and co-occurrence of grazers [60–62]. The plastic response, i.e., reduction in chain length in *S. marinoi*, decreases grazing efficiency by copepods because of reduced encounter rates. Chains of *S. marinoi* with four cells have a 16-fold risk of being grazed upon by copepods compared to individuals with one cell. [53]. Strains of *S. marinoi* are morphologically variable [63] as was seen in the significant variation of chain length among clones in the non-grazer conditions. Grazer presence reduced the chain length in all strains, but the response of different strains was variable. Some were reduced significantly more than others. In the mesocosms, chain length was reduced in all grazer treatments but increased in the control. Bergkvist et al. [53] showed that water-borne cues from grazers reduced chain length in *S. marinoi*. However, the clear reduction of chain length as a response to grazer presence is unique in our large experimental enclosures. Previous studies reporting effects of grazers on chain length or colony size of protist prey have used significantly smaller experimental volumes [9, 53, 64–66]. Our results indicate that reduction of chain length in *S. marinoi* as a consequence of grazer presence may also be relevant as an escape mechanism in field conditions. Our experimental set-up does not rule out the possibility that chain length reduction was in part affected by mechanical breakage by grazers. However, the maximum

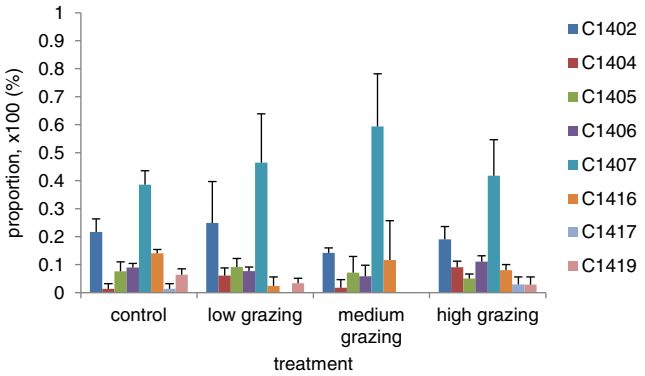


**Fig. 4** The proportion of individual clones in each mesocosm treatment based on the microsatellite genotyping of isolated clones at the end of the experiment (day 12). On average, 36 clones out of 50 (success rate, 72 %) initially isolated, yielded genetic data. *Error bars* represent SE

chain length of *S. marinoi* in the mesocosm conditions, even without copepods, was on average between two and three cells. Most of the copepods feeding in the mesocosm can ingest chains of few cells. In addition Bergkvist et al. [53] showed that chain length suppression is triggered by chemical signals rather than physical damage from grazers. The mechanical breakage of chains caused by zooplankton should thus be of minor concern. It has been suggested [25, 67–70] that production of PUA compounds by diatoms may be involved in grazing deterrence or have an impairing effect on copepod recruitment. Our experimental strains differed with respect to PUA content when grown in monocultures. The presence of grazers neither increased nor decreased PUA content per cell suggesting that the production of PUA compounds was not related to grazers. In the mesocosms, PUA content per diatom cell increased towards the end of the experiment but no significant differences were found between the control and the grazed treatments. Therefore, our results indicate that PUAs are more likely to be coupled to growth phase of the diatom population [71] and are not, e.g., induced by the presence of grazers. This is supported by our data showing that levels of dissolved PUA increased towards end of the exponential growth phase. Our results also indicate that grazing by copepods on a suite of *S. marinoi* strains with varying PUA production is not likely to be selective since no correlation between clonal PUA production and final proportion of respective clones in the mesocosm was found. This is supported by earlier findings of, e.g., Amin [72] who observed mainly non-selective grazing by copepods on a diverse diet of PUA producing *S. marinoi* strains.

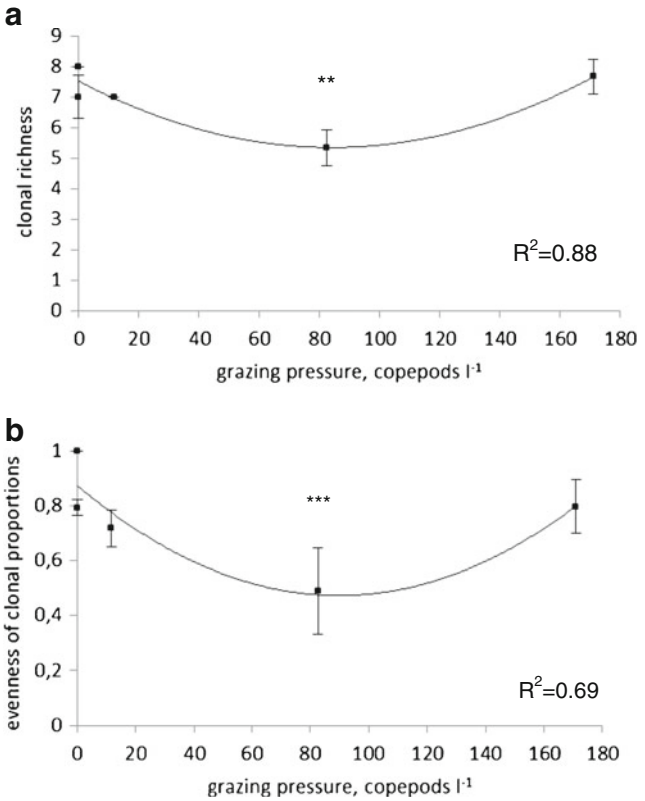


**Fig. 5** Estimates of genetic diversity based on microsatellite genotyped isolates at the end (day 12) of the mesocosm experiment. Correlation (best-fit curve, quadratic) analysis was performed in order to investigate potential relation between grazer level and genetic diversity. **a** Clonal richness. **b** Evenness of clonal proportions. Level of significance, $^{**}p<0.001$, $^{***}p<0.0001$. Initial and control values positioned at zero on *x* axis

## Effects of Grazer Presence on Genotypic Diversity

Clonal composition (the proportion of each strain in a population) changed over time in all treatments but was unrelated to grazer concentrations. Clonal proportions per se seem insensitive with regard to grazer effects. Other estimates revealed grazer effects on the genetic diversity. It was most clearly observed in the medium grazer level, which had significantly reduced richness and evenness of clonal proportions. It appears that effects of grazing on genotypic diversity most likely occurred at the beginning of the experiment when diatom densities were low. The *S. marinoi* population was much denser during the later part of the experiment, and no effect of grazers in terms of reduced apparent growth rate could be observed.

Strain C1407 dominated throughout the experimental gradient even though its maximum growth rate at optimal conditions was among the lowest. Interestingly, this strain had the longest chains in all monoclonal experiments, including non-grazer and grazer treatments. With respect to chain length one would expect this strain to be most vulnerable to grazing in the mesocosms [53]. The long chains of C1407 indicate that factors or strategies other than chain length reduction and baseline growth rates determine clonal proportions when grazers are present. Strain C1407 may have unfavorable fatty acid composition that makes copepods graze on the other strains. It is also possible that this strain produces allelopathic substances that prevent grazing similar to other phytoplankton [73]. Generally, strains that showed highest maximum growth

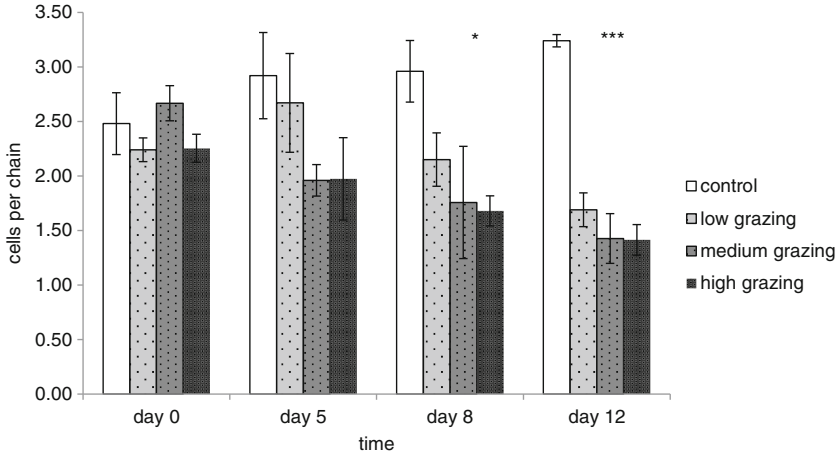


**Fig. 6** Chain length (number of cells per chain) of *S. marinoi* in the mesocosms was measured four times (day 0 October 13th, day 5 October 17th, day 8 October 20th, day 12 October 24th) during the mesocosm experiment

rates (without grazers) were not dominating the population in the mesocosms indicating that they were grazed upon at different rates, or alternative grazer—independent mechanisms such as intraspecific rivalry [74, 75], or intraspecific autotoxic interactions [15] shaped the final clonal composition. The growth of *S. marinoi* was not limited by nutrients, i.e., intraspecific nutrient competition did not occur.

Data obtained from the mesocosms indicated that clonal richness was maintained close to initial levels. Clonal richness declined minimally in the control and in the low and high grazer levels. A significant decrease of clonal richness was only observed in the medium grazer treatment emphasizing that selective grazing was most intense at this phytoplankton/copepod ratio (10:1 based on initial μg C). This treatment was the only one having significantly lower apparent growth rates compared to the control in the early phase of the experiment when effects on diversity were most likely imprinted. In the medium grazer level, grazing pressure and selective grazing was apparently high enough to eradicate strains C1417 and C1419 thereby reducing the genetic diversity significantly. Nevertheless, these strains were poorly represented in the other treatments as well (1–4 %), hence selective grazing might not be the only explanation for their disappearance in the medium grazer level treatment.

In contrast to the medium grazer level, grazing in the high grazer level treatment was not effective in terms of shaping genetic diversity of the prey population. One reason for the lack of an effect might be that the amount of chemical cues of grazers may have surpassed a threshold in *S. marinoi* triggering anti-predatory mechanisms. Knowledge about such thresholds launching defense mechanisms in phytoplankton is limited. It has been discussed as a strategy of rotifers to avoid predation [76]. The concept of thresholds could follow a cost-benefit model where the costly anti-predatory defense mechanisms only are induced at a certain level of threat in order not to waste energy. The response to grazer presence may also differ among strains [77], e.g., the chain length of C1417 was reduced most drastically at grazer presence in the monoclonal experiments. Strain C1419 was also one of the most heavily affected in question of chain length reduction at grazer presence. A boosting of anti-predatory engagement may explain their reappearance in the high grazed treatment. Generally, it is well accepted that selective grazing [78, 79] plays a part in the processes together with other factors [79] configuring the genotypic structure of prey populations. In diatom populations the other factors may involve, e.g., differential use of resources among clones [80] or clonal allelopathic interactions [74].

An interesting detail was also that generally genetic diversity was maintained and that the population structure was similar in all other treatments except one. Gsell et al. [17] showed that equilibration of diversity may be a result of variable temperature optimum among clonal lineages. This is plausible when temperature is continually changing (e.g., seasonal fluctuation) which obviously was not the case in our experiment with stable temperature conditions. Grazers also have the ability to maintain diversity in prey populations by, e.g., differential grazing on variable size classes of the prey population [9]. Proportions of strains differed within treatments but were generally similar between treatments, with a few exceptions (C1407 higher proportion in medium grazing compared to control and high grazing treatment, also C1417 and C1419 were not present in medium treatment), implying the existence of some stabilizing factor.

The genotypic composition of a population and its level of genetic diversity are known to affect several processes of ecological relevance, e.g., primary production, population recovery from disturbance, interspecific competition, community structure, and fluxes of energy and nutrients [81]. This has also been showed with phytoplankton species. For example the productivity of *Chlamydomonas* sp. (Chlorophyta) is higher when the population consists of a mixture of genotypes rather than a monoculture [82]. The effect of genetic diversity on ecosystem functions may be comparable to that of species diversity and is most pronounced in systems with low species richness [83], like in the Baltic Sea. Thus, data implying that grazers are involved in configuring the level of genetic diversity in Baltic

diatom populations is intriguing also in the perspective of ecosystem functions of phytoplankton populations.

## Conclusions

Our results confirm that genetically distinct individuals of *S. marinoi* are phenotypically diverse. When grazers are incubated together with these variable populations the diversity is configured even further. Clonal richness and evenness of clonal proportions was reduced at medium grazer level. Strains that were weakly represented in all other treatments were not observed at all in the medium grazer level. Contrary, genetic diversity was well maintained in the other treatments suggesting existence of some stabilizing factor. Chain length of *S. marinoi* was significantly reduced at grazer presence but the extent of the response was strain specific. We did not observe any coupling between grazer presence and production of PUA in *S. marinoi* supporting the theory that PUA compounds are linked to growth-phase dependent events.

Our results also have a general relevance for phytoplankton research where single clone incubations are frequently used for, e.g., estimating species-specific growth rates or toxin production. These kinds of experiments neglect to demonstrate the wide interspecific variation. Our study show that eight strains isolated at the same day from the same small aliquot of sediment exhibited significant intraspecific baseline variation in ecologically important traits such as growth rate, production of PUA compounds, and chain length. This variation was furthermore configured when grazers were present implying that single strain incubations used for forecasting the autecology of a species in nature is simplified to an extent where the relevance of ecological interpretations becomes questionable.

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