Limnol. Oceanogr., 57(1), 2012, 318–324

doi:10.4319/lo.2012.57.1.0318

Grazer-induced chain length plasticity reduces grazing risk in a marine diatom

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Abstract

We show that *Skeletonema marinoi* suppresses chain formation in response to copepod cues. The presence of three different copepod species (*Acartia tonsa*, *Centropages hamatus*, or *Temora longicornis*) significantly reduced chain length. Furthermore, chain length was significantly reduced when *S. marinoi* was exposed to chemical cues from caged *A. tonsa* without physical contact with the responding cells. The reductions in chain length significantly reduced copepod grazing; grazing rates on chains (four cells or more) were several times higher compared to that of single cells. This suggests that chain length plasticity is a means for *S. marinoi* to reduce copepod grazing. In contrast, chain length was not suppressed in cultures exposed to the microzooplankton grazer *Gyrodinium dominans*. Size-selective predation may have played a key role in the evolution of chain formation and chain length plasticity in diatoms.

The size of phytoplankton organisms is of fundamental importance for their interactions with grazers. Most grazers on phytoplankton are confined to limited ranges of prey sizes (Hansen et al. 1994). The lower limit of this size range is set by the grazer's ability to detect, capture, and handle the prey, whereas the upper limit may be set by superior evasion capabilities of the prey or by morphological constraints on handling of larger prey such as, e.g., diatom chains (Hansen et al. 1994). Overall grazing rates therefore vary significantly with prey cell size, and this introduces important effects on the flow of energy and material to higher trophic levels (Smetacek 1999). In general, diatoms are among the most important primary producers in the world's oceans. They are responsible for a third of oceanic carbon fixation and $> 10\%$ of global carbon fixation (Raven 2003). Species of the genus *Skeletonema*, in particular, often dominate during spring blooms in temperate waters. These diatoms therefore contribute extensively to the biogeochemical cycle of carbon and other elements in temperate waters.

Most chain-forming diatoms display variability in size due to extensive plasticity in chain length. They may occur as single cells or chains of up to > 20 cells (Smayda and Boleyn 1966), and such chain length plasticity may provide an effective refuge from size-dependent grazing. It has previously been shown that grazing rates of copepods in the genus *Calanus* were higher on diatom chains than on single-celled diatoms (Richman and Rogers 1969; Meyer et al. 2002). These size-related changes in grazing impact may thus have large effects on pelagic ecosystems and energy flows, especially when found among ecologically dominant species (Smayda and Boleyn 1966).

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In the present study we explore the hypothesis that chain-forming diatoms are able to adjust their chain length to minimize losses to dominant grazers. It has previously been reported that grazing copepods reduce the chain length in chain-forming diatoms (Martin 1970; O'Connors et al. 1976; Deason 1980), but the observed reduction was assumed to result from direct mechanical chain breakage by the copepods. On the other hand, if the response by diatoms is an active response to reduce grazing loss, diatom cells must be capable of remotely sensing potential grazers. We therefore exposed *S. marinoi* to mesozooplankton and microzooplankton grazers both by direct incubation in the presence of grazers, and indirectly, by incubation with caged copepod grazers separated from the diatom cells. The resulting changes in chain length were quantified and the effect on grazing rates of mesozooplankton grazers was tested.

Methods

Organisms—S. marinoi strain G4 was obtained from the University of Bergen (isolated in Raunefjord, Norway, 2006). Cultures were grown at 16°C in f/2 media aerated with sterile filtered air in cell culture bottles. Light intensity was ~ 50 μmol m^{-2} s^{-1} with a 16 : 8 h light : dark (LD) period. Aeration was vigorous, and normally only single cells and short chains are formed in bubbled cultures. The stock culture of *S. marinoi* was diluted with K/10 media to a starting concentration of 15,000 cells mL^{-1}, equivalent to ~ 200 μg C L^{-1} (Strathmann 1967) in all experiments. We determined cell concentrations from triplicate samples analyzed on a particle counter (Beckman Coulter Multisizer III, https://beckmancoulter.com) and by scanning flow cytometry (CytoBouy, http://cytobuoy.com/), to allow for automatic chain length determination. *Rhodomonas baltica* was obtained from the Danish Technical University, National Centre For Aquatic Resources (DTU-Aqua).

Female *Acartia clausi* and *Centropages hamatus* used in the first experiment were collected from the Skagerrak using a 200-μm plankton net deployed near the Sven Lovén

Centre for Marine Sciences–Tjärnö, Sweden, a day prior to the start of the experiment. *Acartia tonsa*, *Centropages hamatus*, and *Temora longicornis* used in all subsequent experiments were collected from cultures at DTU-Aqua. *Gyrodinium dominans* is identical to the clone used previously by Jakobsen and Tang (Jakobsen and Tang 2002) and was obtained from a culture at DTU-Aqua based on isolates from the northern inlet of Øresund.

Effects of grazers—In the first experiment, diluted *S. marinoi* ($\sim$ 200 μg C L^{-1}) were added to triplicate 620-mL glass bottles and the concentration was determined by triplicate measurements using the particle counter. These bottles received either 1 mL of filtered seawater (*fsw*) (control), 1 mL of *G. dominans* at 2500 cells mL^{-1} (final concentration 5 *G. dominans* mL^{-1}), or 0.01 adult *A. clausi* mL^{-1} and 0.004 adult *C. hamatus* mL^{-1} in a small volume of *fsw*. The bottles were incubated on a plankton wheel (0.5 rpm) for 48 h at 16°C with $\sim$ 16 : 8 h LD periods. At the end of the incubation, a well-mixed sample was carefully withdrawn from each bottle and analyzed on the particle counter.

In a second experiment, diluted *S. marinoi* ($\sim$ 200 μg C L^{-1}) were added to triplicate 620-mL glass bottles, which received either 1 mL *fsw* (control), 30 *G. dominans* mL^{-1}, 0.01 *A. tonsa* mL^{-1}, 0.006 *T. longicornis* mL^{-1}, or 0.006 *C. hamatus* mL^{-1}. This was to determine if the change in chain length was a general reaction to copepod grazers. The bottles were incubated on a plankton wheel (0.5 rpm) for 96 h (copepods) or 72 h (*G. dominans*). Subsequently, *S. marinoi* cell concentrations and chain lengths were analyzed on the particle counter and by scanning flow cytometry.

Effects of grazer cues—Copepod feeding could potentially cause shifts in size distribution through mechanical damage such as chain fragmentation or selective grazing on size classes. An experiment was therefore designed to test if the changes in chain formation could be attributed to direct grazing from copepods, or if waterborne cues from grazers or damaged conspecifics alone could induce the effect. Cages were constructed from 15-mL polypropylene centrifuge tubes with the apical ends replaced by 2-μm polycarbonate filters. The transport across the 2-μm cage membranes was tested visually with food coloring and showed complete mixing after 2 h on the plankton wheel. Thus, *S. marinoi* cells outside the cages receive waterborne cues from caged grazers, but the caged grazers were separated from physical contact with the diatom cells by the membrane filter. The cages contained either five *A. tonsa* in *fsw*, five *A. tonsa* feeding on *S. marinoi*, five *A. tonsa* feeding on *Rhodomonas*, or *fsw* alone to control for cage effects. The copepods had been acclimatized to the type of food used in treatments for 24 h prior to the start of the experiments. The cages were placed inside five replicate 300-mL glass bottles containing diluted *S. marinoi* and incubated on a plankton wheel (0.5 rpm) for 48 h. Subsequently, *S. marinoi* cell concentrations and chain lengths were analyzed on the particle counter and by scanning flow cytometry.

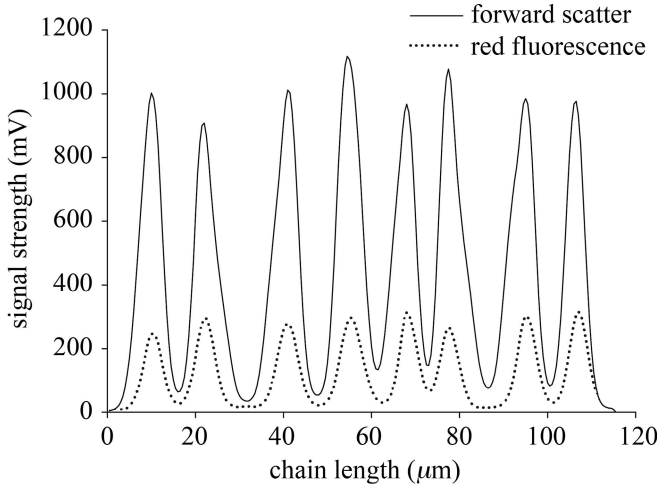

Fig. 1. Example of particle scans of *Skeletonema marinoi* recorded by the scanning flow cytometer. The forward scatter detector records the individual cell frustules and red fluorescence detector collects information about presence of chlorophyll-containing chloroplasts within diatom frustules, subsequently used to determine chain length.

Effects of chain lengths on grazing rates—*A. tonsa* females were acclimated to experimental conditions (*S. marinoi* concentration and temperature) for 24 h prior to the tests. Five female *A. tonsa* were added to two groups of five replicate 300-mL glass bottles filled with either short-chain or long-chain *S. marinoi* ($\sim$ 200 μg C L^{-1}). Short-chain *S. marinoi* was obtained by growing stock cultures in the presence of copepods; following this treatment, there were no chains longer than four cells. Long-chain *S. marinoi* was obtained by growing stock cultures without copepods. The bottles were incubated on a plankton wheel (0.5 rpm) for 12 h in the dark. Subsequently, *S. marinoi* cell concentrations and chain lengths were analyzed on the particle counter and by scanning flow cytometry.

Clearance rates on short- and long-chain *S. marinoi* were calculated from the disappearance of *S. marinoi* biovolume using a modification of Frost's equation (Frost 1972):

$$F = \frac{V}{tn} \ln\left(\frac{v_1}{v_0}\right)$$

where V is the bottle volume, t is the incubation time, n is the number of copepods, and v_0 and v_1 are *S. marinoi* biovolume concentrations (μm^3 mL^{-1}) after incubations with or without grazers.

Scanning flow cytometry—Scanning flow cytometry is particularly useful for studies of chain-forming diatoms (Takabayashi et al. 2006). The frustules and chloroplasts of individual cells within each chain generate distinct peaks in forward scatter and red fluorescence, and this enables direct detection of the number of cells per chain with each particle scan (Fig. 1). Using the cell count algorithm in the instrument's software (CytoClus, http://cytobuoy.com), we constructed a gating set that determined the presence of a cell when peaks in either forward scatter, red fluorescence, or both were detected. This gating set enabled the detection

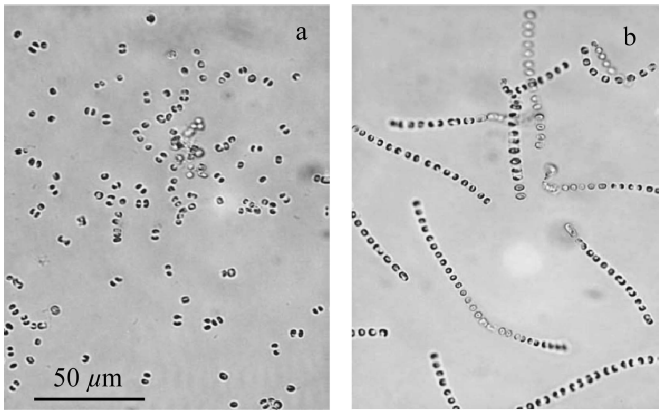

Fig. 2. Effects of grazers on chain length in *S. marinoi*. (a) *S. marinoi* incubated with the copepods *Acartia clausi* and *Centropages hamatus*; (b) controls without grazers.

of chain lengths of 1, 2, 3, 4, 5, 6, 7, and 8 or more cells long. Triplicate counts of 0.5 mL from each incubation were analyzed.

Statistics—In the first experiment, the effects of grazers (data from the particle counter) were analyzed by multivariate analysis of size spectra with principal components analysis (Wold et al. 1987) using the Soft Independent Modeling of Class Analogy (SIMCA) software (Umetrics). This calculation was done with each population objects and the total cell volume in each cell size as variables. Each variable was scaled to unit variance. Statistical significance of presented components was judged by cross validation within SIMCA.

The effect of grazers in the second experiment and the effect of grazer cues (data from the flow cytometer) were tested by two-factor ANOVA with copepod species (effects of grazers) or copepod treatment (effects of cues) as one factor and chain length as the other factor. Tukey's post hoc test at p-values < 0.05 was used to test for significant differences between means.

The effects of chain lengths on grazing rates were tested by one-factor ANOVA among chain lengths. Tukey's post hoc test at p-values < 0.05 was used to test for specific differences in grazing among chain lengths.

Results

Effects of grazers—*S. marinoi* not exposed to grazers formed long (> 8 cells) chains. In contrast, *S. marinoi* exposed to a mixture of the copepod grazers *A. clausi* and *C. hamatus* were dominated by single cells and chains of two cells (Fig. 2). Microzooplankton grazers (*G. dominans*) did not have the same effect; in fact, mean biovolume in *G. dominans*–exposed cultures were slightly larger than grazer-free controls mainly in the lower-size bins (200–1000 μm^3), probably due to size-specific grazing on shorter chains by *G. dominans* (Fig. 3). Principal components analysis showed that this difference was significant in both the first and second components (Fig. 4), and post hoc test showed significant differences among all three groups.

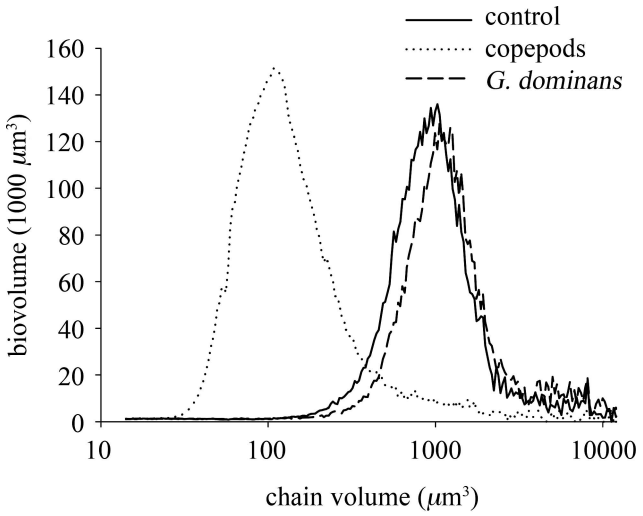

Fig. 3. Size distribution (equivalent spherical diameter) of *S. marinoi* incubated with the copepods *A. clausi* and *C. hamatus*, the heterotrophic dinoflagellate *Gyrodinium dominans*, and controls without grazer.

To test if decreasing chain length is a general reaction to copepod grazers, we exposed *S. marinoi* individually to three different copepod species, *A. tonsa*, *C. hamatus*, and *T. longicornis*. The copepod treatments all resulted in significantly higher number of single cells and a significant reduction in the number of chains of three cells or more compared to the control (Fig. 5).

Effects of grazer cues—To test if the observed change in chain length was triggered by copepod-derived chemical cues or the result of differential grazing on longer chains, or copepods fragmenting chains into subfragments, *S. marinoi* was exposed to caged *A. tonsa* without prey or caged *A. tonsa* feeding on *S. marinoi* or the cryptophyte *R. baltica*. The presence of caged *A. tonsa* feeding on either of the

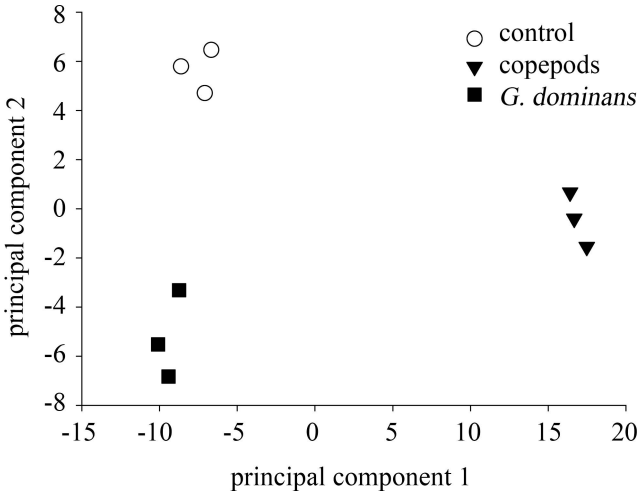

Fig. 4. Score plot from principal components analysis of size spectra from *S. marinoi* incubated with the copepods *A. clausi* and *C. hamatus*, the heterotrophic dinoflagellate *G. dominans*, and controls without grazer.

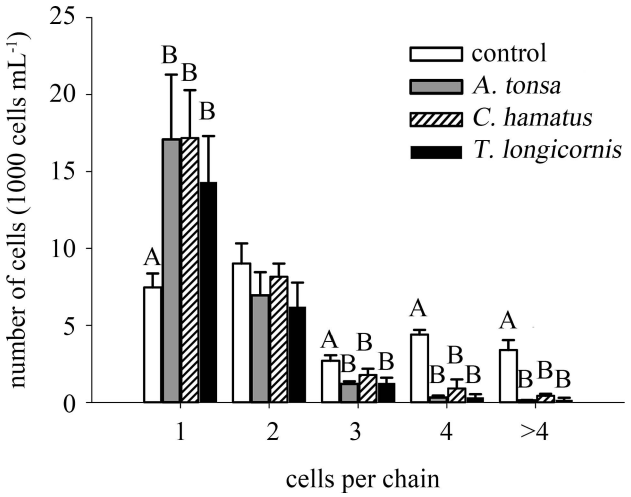

Fig. 5. Individual effects of the copepods *Acartia tonsa*, *C. hamatus*, or *Temora longicornis* on chain length distribution of *S. marinoi*. Letters above bars indicate significant difference in chain length. Values are means + SD.

algae resulted in a significantly higher number of single cells and a reduction in the number of chains of three cells or more in the surrounding *S. marinoi* than in grazer-free controls (Fig. 6). In contrast, there were no significant effects of caged starving *A. tonsa* on the chain lengths in the surrounding *S. marinoi* (Fig. 6).

Effects on grazing rates—We tested the effects of chain length on copepod grazing rates by measuring the clearance rates of *A. tonsa* on *S. marinoi* chains of different lengths in both the control culture and in a culture that had been induced to shorter chain lengths with copepod cues. In both cultures, clearance rates on individual chain lengths were obtained using scanning flow cytometry. Clearance rates

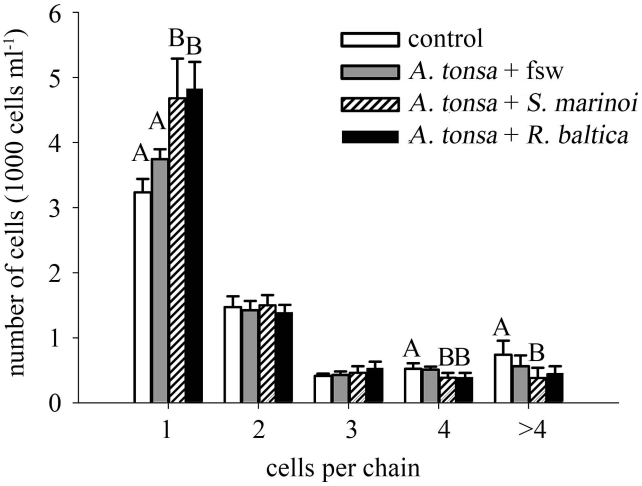

Fig. 6. Chain length distribution of *S. marinoi* cells after incubation with cages holding either *fsw* (control), *A. tonsa* in *fsw*, *A. tonsa* in a suspension of *S. marinoi*, or *A. tonsa* in a suspension of the cryptophyte *Rhodomonas baltica*. Letters above bars indicate significant difference in chain length. Values are means + SD.

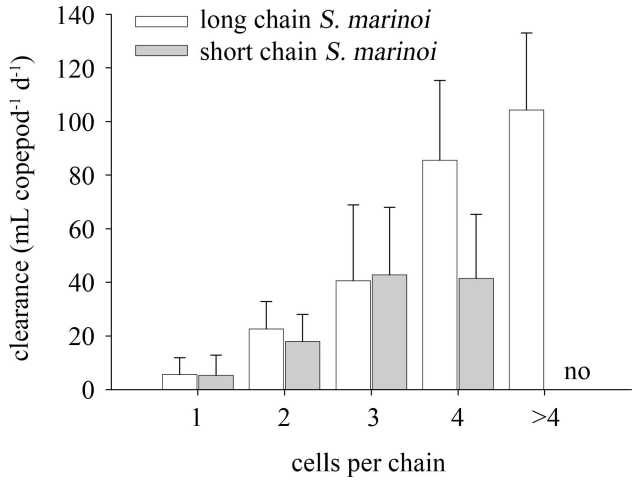

Fig. 7. Copepod clearance rate (mL copepod^{-1} d^{-1}) on control culture *Skeletonema marinoi* (control *S. marinoi*) and *S. marinoi* culture that had been induced to the shorter chain length by copepod grazers (grazer-induced *S. marinoi*). Clearance rate increases significantly with chain length in both treatments, but there were not enough chains to calculate clearance for chains > 4 cells in the grazer-induced culture. Values are means + SD. no = not observed.

increased significantly with chain length in a similar way in both treatments (Fig. 7). The total cell loss was 30% higher in long-chain *S. marinoi* compared to short-chain *S. marinoi*. To avoid confounding from grazer-induced chain length plasticity the incubation time was short (overnight) compared to the experiments conducted to observe grazer-induced chain length plasticity.

Discussion

In this study we showed that the common chain-forming diatom *Skeletonema marinoi* adjusts its chain length in the presence of copepod grazers to reduce grazing loss. These changes were triggered without physical contact between grazer and diatom, and derive from cues associated with feeding grazers. The fact that the chain length of *S. marinoi* changes as a reaction to grazing by copepods, but not to the presence of non-grazing copepods or *G. dominans*, suggests that the cue is specific and only released when copepods are actively foraging. Exposing *S. marinoi* to water containing copepods grazing on *R. baltica* or on *S. marinoi* resulted in reduced chain length. This is most likely a result of a general chemical cue(s) released from grazing copepods or grazed algal cells and not specifically related to *S. marinoi*.

Significant increases were observed in the abundances of single cells in the presence of feeding *A. tonsa*. Single *S. marinoi* cells (2–4 μm) are close to the minimum retainable size for *A. tonsa* (Berggreen et al. 1988; Hansen et al. 1994), and significantly lower copepod grazing rates were observed on single- or two-celled chains than on longer chains. This suggests that attaining a single- or double-cell modus is an advantageous adaptation for *S. marinoi* populations developing in environments with abundant copepod grazers. The marine dinoflagellate *Alexandrium tamarense* responds to grazer cues with simultaneous

reduction in chain length, swimming speed, and increased toxicity (Selander et al. 2011; Selander et al. 2012). The lower grazing rates on grazer-induced *Skeletonema* cells could therefore have resulted from other defense mechanisms correlated to the changes in chain length. Grazing experiments with both induced and non-induced *Skeletonema* chains of different length, however, indicated similar clearance rates between induced and non-induced *Skeletonema* chains of the same length (Fig. 7), which suggests that the chain length and not toxicity is the main mechanism that reduces grazing on induced *Skeletonema* cells.

Previously, several authors have noted chain length effects on chain-forming diatoms in the presence of grazing (Martin 1970; O'Connors et al. 1976; Deason 1980). However, they all concluded that the observed chain length reductions resulted from mechanical cleavage by grazing copepods. However, taking our results into account, it is likely that the reduction in chain length, at least in part, was triggered by chemical cues released from grazers also in these studies. Deason (1980) specifically noted that the suppression in chain length does not increase with increased grazing activity, which would otherwise have been expected if chain length decrease resulted solely from physical interactions with grazers. Martin (1970) measured chain length and total zooplankton grazing simultaneously over several months and noted that high grazing pressure is typically accompanied by shorter chain length in *Skeletonema costatum* (now subdivided into six different species including *S. marinoi*, author's comment). This suggests that grazer-induced chain length reduction operates also in the open sea, although it is not clear if the correlation is statistically significant. Direct experimental evidence in more natural conditions is still needed.

Grazing pressure on phytoplankton is high in the pelagic environment (Cyr and Pace 1993; Calbet 2001; Calbet and Landry 2004) and provides the evolutionary drive to develop defensive mechanisms that reduce grazing. In diatoms, intensive grazing by copepods may constitute an important evolutionary pressure triggering the development of chain length plasticity. There are several examples of how changes in cell or colony size are a means to avoid grazing. Cues released by grazing *Daphnia* induced colony formation in green algae belonging to the family Scenedesmaceae (Hessen and Van Donk 1993; Lampert et al. 1994). The bacterium *Flectobacillus* sp. shifted from small cells to filaments when grazed by the bacterivorous flagellate *Ochromonas* sp., and small suspended cells were more susceptible to grazers than the filamentous cells (Corno and Jurgens 2006). Moreover, while Jakobsen and Tang (2002) found that colony formation in *Phaeocystis globosa* increased when grazed by the heterotrophic dinoflagellate *Gyrodinium dominans*, Long and colleagues (Long et al. 2007) found that *P. globosa* suppressed colony formation by 60–90% in response to cues from grazing copepods. The dinoflagellate *Alexandrium tamarense* splits up chains and reduces swimming speed in response to grazer cues, which reduces grazer encounter rates several-fold (Selander et al. 2011). Dominant copepod grazers are typically omnivorous (Turner 2004), and ideally any defensive mechanism should not aim to reduce grazer abundances per se because co-occurring, competing phytoplankton would benefit equally. Instead, defensive adaptations should entail the deterrence of grazers specific to that species and ultimately direct those grazers to feed on other species, to reduce competition from undefended phytoplankton. This may be achieved by producing grazer-deterring compounds in dinoflagellates (Selander et al. 2006) or by escape behavior in ciliates and flagellates (Jakobsen 2002). Structural changes can also be a defense mechanism; Pondaven and colleagues (Pondaven et al. 2007) found that the marine diatom *Thalassiosira weissflogii* increased the cell wall silicification in response to cues from grazing copepods. We hypothesize that a shift in chain length in order to fall outside the optimal prey size range of the dominant grazers is another such strategy successfully employed by *S. marinoi*. However, single *S. marinoi* cells and short chains are likely to be exposed to significant grazing from microzooplankton and larval forms of mesozooplankton.

Microzooplankton are typically the most important grazers in the pelagic environment (Calbet and Landry 2004) and thus reducing chain length may not be a mechanism to avoid grazing in general, as at high grazing pressure from flagellates longer chains would be advantageous instead. The increase in the mean *S. marinoi* size observed in the *G. dominans* treatment (Fig. 3) most likely results from *G. dominans* preferably grazing on single cells and shorter chains, and suggests that longer chains are not ingestible for *G. dominans*, although we cannot rule out cues from *G. dominans* being responsible for this increase in size. Copepods, however, generally have higher clearance rates for microzooplankton than nonmotile phytoplankton, and microzooplankton grazing pressure is typically relaxed during high copepod abundances, which suggests that breaking up into smaller units can still be functional in periods of high copepod grazing. Chain length plasticity is intimately connected with cell divisions. Chains are formed by daughter cells staying attached after division. Similarly, we suspect that chain splitting occurs when new cells form that are not attached after division. Thus, grazer-induced chain length plasticity is probably fast enough to track population fluctuations of copepod grazers but too slow to track changes resulting from dynamic patchy distributions and vertical migrations of zooplankton.

While chain length plasticity is clearly an advantageous adaptation to life in the pelagic environment, the evolutionary cause of chain formation in diatoms is unclear. Chain length has been correlated with growth rate and is, to some extent, dependent on nutrient availability and temperature (Pahlow et al. 1997; Takabayashi et al. 2006). Because longer diatom chains sometimes sink at a slower rate (Smayda and Boleyn 1966), it has been suggested that the ability to form chains is an adaptation to improve flotation, which enables diatoms to remain for longer periods of time in the euphotic zone and, as a result, attain a higher growth rate (Takabayashi et al. 2006). But buoyancy also seems to be correlated with the status of the cells. The diatom *T. weissflogii* was shown to be almost neutrally buoyant under favorable nutrient conditions, but became less buoyant after nitrate depletion (Richardson

and Cullen 1995). This suggests that diatoms are able to control their buoyancy with mechanisms not only related to chain formation. Nutrient limitation may also favor formation of single cells or shorter chains in diatoms (Takabayashi et al. 2006). The experiments shown here were all performed in nutrient-replete concentrations, and the presence of copepods probably increased nutrient concentration further. Thus, the effect of grazers is probably not mediated by nutrient concentrations, although it is possible that the effect of grazers would have been different in nutrient-limited conditions.

The current study implies that chain length plasticity may be an evolutionarily adopted trait in chain-forming diatoms and suggests that size-selective predation may have played a key role in the evolution of chain formation and chain length plasticity. Further, this change in size as a response to cues from grazers could affect the food web structure and the flow of energy and materials in the pelagic ecosystem (Hay and Kubanek 2002). Identification of the copepod grazer cues and the system with which the diatom detects them are important goals toward developing a better understanding of predator–prey interactions in pelagic ecosystems.

Acknowledgments

We thank two anonymous reviewers who provided comments that improved this manuscript. This work was supported by Marine Chemical Ecology (MARICE, an interdisciplinary research platform at the Faculty of Science, University of Gothenburg) and EU Marie Curie project 237517-LIDPOP (Linking Inducible Chemical Defences and Phytoplankton Population Dynamics). We thank the Technical University of Denmark-Aqua and the Sven Lovén Centre for Marine Sciences–Tjärnö for hospitality.

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Associate editor: Michael R. Landry

Received: 16 March 2011
Accepted: 07 November 2011
Amended: 01 November 2011