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Grønning, Josephine Bøgeskov

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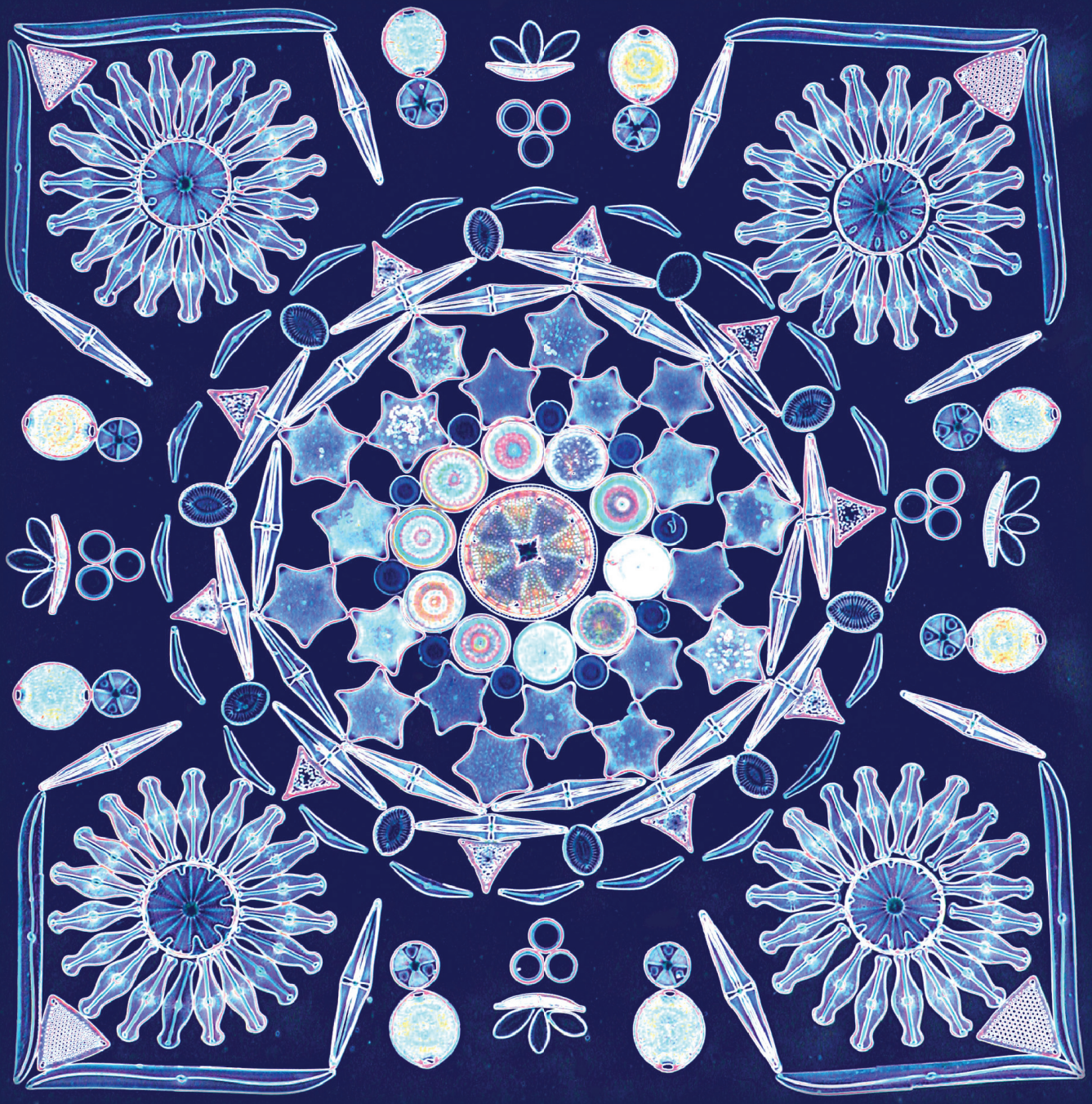
PhD Thesis



Ph.D. Thesis
Doctor in Philosophy

DTU Aqua
National Institute of Aquatic Resources

Defence in diatoms: mechanisms and trade-offs

Josephine Grønning

Supervisor:
Thomas Kiørboe

Co-supervisors:
Erik Selander
Per Juel Hansen

Kongens Lyngby 2022

DTU

DTU Aqua
National Institute of Aquatic Resources
Technical University of Denmark

Kemitorvet
Building 202
2800 Kgs. Lyngby
www.dtu.dk/english

Summary

Defence plays an important role in shaping the planktonic food web. The combination of predation and defence allows for the co-existence of defence- and competition-specialists and is hence an important mechanism in maintaining species diversity. For predation to promote species diversity defence must come at a cost, otherwise all species would be equally defended. Phytoplankton have developed a large array of defence mechanisms ranging from morphological to biochemical or behavioural. Many of the defence mechanisms are inducible, i.e., harnessed or intensified when grazer cues are present. This has been demonstrated for different defence strategies such as toxin production in dinoflagellates or diatoms, bioluminescent capabilities in dinoflagellates and chain-length reduction and/or shell-thickness increase in diatoms. Inducible defences are believed to evolve when defences are costly. However, the demonstration and quantification of phytoplankton defences are rare and have been notoriously difficult to establish.

This PhD thesis explores defence mechanisms and trade-offs in diatoms through three different projects: (i) Inducibility of shell-thickening by grazer cues and the associated trade-offs, (ii) effect of shell-thickness on prey selection by grazers, and finally (iii) grazer-induced aggregation as a defence mechanism in diatoms.

Diatoms are characterized by having a siliceous outer shell that provides protection against grazing. In fact, the shell of the diatom appears to be the strongest known biological material relative to its density. In my first project, we demonstrate for seven species of diatoms that the presence of chemical grazer cues induces thickening of their silica shells at the cost of reduced growth rates. The response is proportional to the concentration of cues, although it is highly variable, both between and within species. From previous quantification of the defensive value of thicker shells, we demonstrate that the trade-off is near neutral, i.e., that the benefits (lower mortality rate) is near equal the costs (lower growth rate), and that this may help explain why diatoms are a particularly diverse group.

It is now well documented how a thicker shell decrease mortality rate in diatoms. However, the exact mechanism of how a thicker shell is beneficial has yet to be demonstrated. It is therefore unknown if the reduced mortality rate is due to the cells being de-selected by the grazer or handled for such a long time, that the general foraging time by the copepod is reduced. By using direct video-observations, we demonstrate how thick-shelled diatoms captured by a copepod are not only handled for much longer but are also far more likely to be rejected by the copepod, compared to thin-shelled diatoms. Overall, the presence of thick-shelled diatoms, whether due

to light limitation, grazer presence, or both, reduces the grazing mortality of diatoms because copepods spend more time handling cells, leaving less time for actually feeding.

Diatoms play an important role in the ocean carbon cycle. Rapid mass sedimentation by the end of a bloom moves great amounts of carbon from the surface to the deep. However, sinking is also of crucial survival value to diatoms and a part of their life cycle. When conditions change from replete to deplete, diatoms form aggregates that sink rapidly to the bottom. Here, the predation pressure is much less of that in the water column. This enables the transition from vegetative cells in the surface to resting stages that can survive the winter in the benthic zone. The common assumption is that aggregation is initiated by nutrient depletion; cells then become sticky and due to high cell concentrations, they collide, stick together, and sink out. In my third project, we discovered that some diatoms form aggregates in the presence of grazer cues. We explored the inducibility of stickiness in six species of diatoms. Two species increased their stickiness when exposed to copepod cues and the response was proportional to the cue concentration until saturation. In one species, the increase in stickiness was additionally proportional to the duration of exposure. We further tested the effect of nutrient limitation (Si and N) on the stickiness of five diatom species and found to our surprise that nutrient limitation did not increase the stickiness. In fact, one of the species had higher stickiness in high-nutrient conditions, compared to low-nutrient conditions.

Dansk resumé

Forsvar spiller en vigtig rolle i udformningen af det planktoniske fødenet. Kombinationen af prædation og forsvar muliggør en sameksistens mellem forsvars- og konkurrencespecialister, og er derfor en vigtig mekanisme til at opretholde artsdiversiteten. For at dette skal være tilfældet, må der nødvendigvis være omkostninger ved et forsvar. Hvis ikke, ville alle arter udvikles til at være forsvaret i lige høj grad. Planteplankton har udviklet en lang række forsvarsmekanismer, der varierer lige fra morfologiske til biokemiske eller adfærdsmæssige. Mange af forsvarsmekanismerne er inducerbare, hvilket betyder, at de udtrykkes eller intensiveres, når der er prædatorer til stede. Dette er blevet demonstreret for forskellige forsvarsstrategier såsom toksinproduktion i dinoflagellater eller kiselalger, bioluminescerende egenskaber i dinoflagellater, reduktion af kædelængde, samt øget skaltykkelse i kiselalger. Inducerbare forsvar menes at opstå, når omkostningerne ved et forsvar er store. Dog er demonstrering og kvantificering af forsvar i planteplankton vanskelig og de er derfor sjældent rapporteret i den videnskabelige litteratur.

Denne ph.d.-afhandling har til formål at udforske forsvarsmekanismer og de tilhørende omkostninger i kiselalger gennem tre forskellige projekter: (i) Inducerbarheden af øget skaltykkelse og de tilhørende omkostninger, (ii) effekt af skaltykkelse på vandloppers udvælgelse af kiselalger, og endelig (iii) vandloppe-induceret aggregering som en forsvarsmekanisme i kiselalger.

Kiselalger er kendetegnet ved at have en kiselholdig ydre skal, der giver beskyttelse mod græsning. Faktisk er kiselalgens skal det stærkeste kendte biologiske materiale i forhold til dets densitet. I mit første projekt demonstrerer vi for syv forskellige arter af kiselalger, at tilstedeværelsen af kemiske signaler fra vandlopper inducerer en øget skaltykkelse. Omkostningen ved dette er reducerede vækstrater og størrelse. Responsen er proportional med koncentrationen af signaler, selvom den er meget variabel – både mellem arter og indenfor den samme art. Fra tidligere kvantificering af den defensive værdi af tykkere skaller viser vi, at fordelene (lavere dødelighed) er næsten lig med omkostningerne (lavere vækstrate), og at dette kan være med til at forklare, hvorfor kiselalger er en særlig forskelligartet gruppe.

Det er veldokumenteret hvordan en tykkere skal mindsker dødeligheden hos kiselalger. Den nøjagtige mekanisme for, hvordan en tykkere skal er gavnlig, er dog endnu ikke demonstreret. Det er derfor uvist, om den reducerede dødelighed skyldes at cellerne fravælges af vandlopperne eller om de håndterer algerne i så lang tid, at den generelle byttesøgningstid hos vandloppen reduceres. Ved at bruge direkte video-observationer demonstrerer vi, hvordan tykskallede kiselalger, som fanges af en vandloppe, håndteres

i meget længere tid end tyndskallede kiselalger. Derudover har de tykskallede kiselalger også en langt større sandsynlighed for at blive afvist af vandloppen sammenlignet med tyndskallede kiselalger. Samlet set reducerer tykskallede kiselalger deres græsningsdødelighed, hvad end den øgede skaltykkelse skyldes lysbegrænsning, tilstedeværelse af vandlopper, eller begge dele. Dette er fordi vandlopper bruger længere tid på at håndtere celler, hvilket efterlader mindre tid til faktisk at spise alger.

Kiselalger spiller en vigtig rolle i havets kulstofkredsløb. Hurtig massesedimentering af kiselalger i slutningen af en opblomstring flytter store mængder kulstof fra overfladen til dybet. Men at synke dybere ned i vandlagene har også en afgørende overlevelsesværdi for kiselalger og en del af deres livscyklus. Når forholdene ændrer sig fra gunstige til ugunstige, danner kiselalger aggregater, der synker hurtigt til bunden. Her er prædationstrykket meget mindre end i den øvre del af vandsøjlen. Dette muliggør en overgang fra vegetative celler i overfladen til hvilestadier, der kan overleve vinteren i dybet. Den almindelige antagelse er, at aggregering igangsættes når næringsstoffer udtømmes. Celler bliver derefter klistrede og på grund af de høje cellekoncentrationer, støder de sammen, klæber til hinanden og synker. I mit tredje projekt opdagede vi, at nogle kiselalger danner aggregater i når signalstoffer fra vandlopper er til stede. Vi undersøgte inducerbarheden af klæbrighed i seks forskellige kiselalgearter. To arter øgede deres klæbrighed, når de blev udsat for vandloppersignalstoffer, og responsen var proportional med koncentrationen af signalstoffer indtil en vis grænse. Hos én art var øgningen i klæbrighed desuden proportional med eksponeringens varighed. Vi testede yderligere effekten af næringsstofbegrænsning (Si og N) på klæbrigheden af fem forskellige kiselalgearter og fandt til vores overraskelse, at næringsstofbegrænsning ikke øgede klæbrigheden. Faktisk havde en af arterne højere klæbrighed under forhold med høj næringsstofkoncentration sammenlignet med forhold med lav næringsstofkoncentration.

Preface

This Ph.D. thesis was prepared at the National Institute of Aquatic Resources at the Technical University of Denmark, in fulfilment of the requirements for acquiring a Doctor degree in Philosophy. The research was carried out from September 2018 until March 2022 (including leave of absence) at the Centre for Ocean Life, Kongens Lyngby, Denmark. During these years I was supervised by Thomas Kiørboe, Erik Selander and Per Juel Hansen. Six weeks in total was spent as external research stays at the Department of Marine Sciences at the University of Gothenburg, Sweden. This Ph.D. was funded by the Centre for Ocean Life, a Villum Kahn Rasmussen Centre for Excellence funded by the Villum Foundation. Additional support for conference participation was received from the Otto Mønsted Foundation.

Kongens Lyngby, 11th March 2022

Josephine Grønning

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I also wish to thank my two co-supervisors, Erik and Per. Erik, thank you for some great times in Gothenburg, for your help and assistance in extracting copepodamides and for introducing me to the world of chemical ecology. Per, although we did not meet as much as I would have liked to, you still manage to plant a couple of seeds in my head every time we met. Seeds that got to grow over the course of my PhD and some that gave me a slightly different perspective, so thank you!

A very, very special thanks to Fredrik, my fellow lab-rat. During the last couple of years, we have shared many laughs and frustrations, (too?) long coffee breaks, hours in the lab and trips to Gothenburg. I enjoyed working with someone who is at least as much of a perfectionist as I am, and someone I could always count on for help. I am grateful to have had you by my side as a colleague and a friend.

Although the pandemic turned out to play a big role during my PhD, being a part of the Ocean Life Centre at DTU Aqua has truly been a pleasure. Whenever possible, the young researchers created a fantastic social life through e.g. everyday cookie breaks, international dinners, retreats, etc., and this was indeed supported by the all the PI's. Such an environment is special, and I wish to thank especially Aurore, Jérôme, Camila, Daniël, Marina, Mridul, Subhendu, Emily, Neil, Tommy, Rocío, Sei, Federica, Louise and Rémy for being part of it. I wish to give a further couple of thanks to people at DTU Aqua: Lilian, thank you for your eternal patience and help with all the administrative and Karin, for your help especially towards the end of my PhD. Colin, thank you for helping out with all my chemical analysis, and Hans, for building and repairing laboratory equipment. Finally, a special thanks to Jack for always being helpful in the lab and for assisting me in the cold rooms during countless experiments!

Although not being an official part of my PhD, I wish to thank Sigrún, Andy and Torkel for luring me into the world of plankton during my earlier studies, as well as introducing me to the joy (and frustrations) of field and laboratory work.

I also want to thank some people outside DTU Aqua. Thanks to Anna for some deep talks on work-life and life in general, and to Kristie and Aubrey for company and assistance during my trips to Sweden. Thank you to all my friends for listening to me talk about plankton and for the most part smile and nod appreciatively. Finally, I wish to thank my close family, whom I can always rely on for love and support. Last, but definitely not least, I want to thank my best friend and partner-in-life Martin. I could easily dedicate a full page to thanking you, but instead I will keep it simple: thank you! The last couple of years has been great, but I know the next will be even better for us!

List of publications

This PhD thesis is based on the following publications:

I. **Grønning, J.**, Kiørboe, T., 2020. Diatom defence: Grazer induction and cost of shell-thickening. Funct. Ecol. 34, 1790–1801. doi:10.1111/1365-2435.13635

II. **Grønning, J.**, Ryderheim, F., and Kiørboe, T., 2022. Thicker shells reduce copepod grazing on diatoms. Limnol. Oceanogr. Lett. doi:10.1002/lol2.10243

III. **Grønning, J.**, Kiørboe, T. Grazer-induced aggregation in diatoms. (in preparation)

Additionally, the following manuscripts represent minor contributions to this thesis:

- Rigby, K., Kinnby, A., **Grønning, J.**, Ryderheim, F., Cervin, G., Berdan, E. and Selander E. Species specific responses to grazing and acidification in phytoplankton – winners and losers in a changing world. (submitted)

Contents

CHAPTER 1

Introduction

1.1 General introduction

All organisms face three 'Darwinian challenges' in life: to acquire resources, to survive, and to reproduce. An individual's fitness depends on the success of doing so and will indeed materialize differently for different life forms (Litchman et al., 2013; Kiørboe et al., 2018). For example, a shark acquires resources very differently from a bacterium. The fundamental activities are thus depending on the details of the biology of a given organism. Trying to describe an ecosystem or a community by defining each single species becomes an extremely complex task, and this traditional species-centric approach often fails in addressing more global ecological questions. Therefore, using a trait-based approach, where individuals are characterized by a few essential traits, one can describe complex ecosystems in a more simple manner (Kiørboe et al., 2018). A trait is a *"well-defined, measurable property of organisms, usually measured at the individual level and used comparatively across species"* (McGill et al., 2006). There are a few key traits applicable to all life-forms, that can describe most of the Darwinian fitness: body size, resource acquisition mode and defence. These key traits are inextricably linked by trade-offs. That is, in an environment where resources are finite, organisms much 'chose' where to invest the acquired resources. The currency of trade-offs are eventually growth and mortality rates (Kiørboe et al., 2018). This thesis evolves around describing defence mechanisms and the associated trade-offs in planktonic diatoms.

1.1.1 Defence mechanisms in terrestrial ecosystems

The evolution of terrestrial plants is first of all driven by competition for space, i.e. competition for light in the air and water and nutrients in the ground (Smetacek, 2012). This pattern is consistent throughout and moving along the habitat-gradient from deserts to rainforests, two types of trees exist: the classic tree with a branching trunk, and a crown of leaves and the palm tree with its single stem and large leaves in a ring on top. These two shapes appear to present the optimal solutions to competition for resources. Grasses, on the other hand, are easily overgrown by trees and bushes

and are therefore dependent on processes preventing this, e.g., grazing. Opposite to trees and shrubs, that grow at the tip of the plant, grasses grow at the base and are thus not prevented from growing when grazed by e.g. mammals (Smetacek, 2012).

Secondly, speciation in land plants is largely driven by animals. Since plants are not motile, they rely to some extent on animals to spread their pollen or seeds. Furthermore, to meet the Darwinian challenges of life, plants have developed defence mechanisms to avoid or reduce grazing by herbivores, which also generates and maintains species diversity (Kempel et al., 2011; Smetacek, 2012). Following are a few examples of defence mechanisms in plants.

First in line of defence are mechanical defences such as impenetrable barriers like bark or waxy cuticles, or even adaptions like spines and thorns. Some examples are the tough bark of palm trees, slippery leaves of holly plants, or thorns and spines of roses and cactuses. Another is the silicification of grass leaves, often implemented as phytoliths or other forms of amorphous silica, either deposited in the leaf epidermis, or in spines trichomes or hairs on the leaf surface (Hartley and DeGabriel, 2016). By toughening, and functioning as abrasive, silicification of leaves acts as a physical deterrent on herbivores, as well as decrease digestibility and even cause wear and tear on the teeth of herbivores (Massey and Hartley, 2009; Smetacek, 2012; Mitchell et al., 2013; Hartley and DeGabriel, 2016; Johnson et al., 2022).

If the mechanical barriers should be breached by herbivores, next in line of defence are secondary metabolites that are often toxic compounds, harmful to the herbivore. For example, many plants produce alkaloids like nicotine, caffeine, or morphine that have pharmacological effects on herbivores – including humans. Other molecules become toxic when eaten and can cause diarrhea, salivation, or irritation of the mouth. Some even cause lathyrism, a paralytic disease triggered by overconsumption of, e.g., seeds from grass peas (Manna et al., 1999). If attacked by herbivores, damaged plant tissue can also release hormones that promote the release of volatile compounds, attracting parasitoids, which in turn uses the grazers as hosts and eventually kill them. Another 'biotic' defence is based on species coexistence, like for some acacia tree species that have developed mutualistic relationships with colonies of ants. The thorns of the trees are swollen and hollow at the basis and the extrafloral nectaries produces nectars, providing both housing and food for the ants. In turn, it is suggested that the ants provide a defence for the leaves of the trees (Young et al., 1996).

Marine phytoplankton experiences much of the same challenges as terrestrial plants: bottom-up control of evolution by competition for resources and top-down control driven by predation and mortality. However, defence mechanisms in marine phytoplankton are in many ways fundamentally different from defence mechanisms in terrestrial plants. Many marine phytoplankton are motile, and contrary to terrestrial plants, they have a chance to escape grazers through different escape mechanisms. Moreover, while terrestrial plants are multicellular, they can afford to lose parts, e.g., buds or leaves, to grazers, which in turn can induce a defence in the plants. Contrary, marine phytoplankton are unicellular: either a cell is eaten, or it is not. A collective

defence in marine phytoplankton like toxicity (discussed later) is thus difficult to comprehend in an evolutionary context, as the cells of a particular species in e.g. a phytoplankton bloom are not genetically identical, like the leaves of a tree. Now, leaving the plants on land and moving underwater to marine algae, I wish to cite Smetacek (2001), who very elegantly describe this exact transition:

> *"Imagine yourself in a light forest looking upwards, seeing in your mind's eye only the chlorophyll-bearing cells of the canopy floating in mid-air, free from the attachment of leaves, twigs, branches and trunks. Now forget the forest and the trees, and see only blurred clouds of tiny green cells obscuring the blue sky beyond. You are looking at a phytoplankton bloom of a density typical of lakes and coastal oceans."*

CHAPTER 2

Defence mechanisms in phytoplankton

Marine phytoplankton, including both protistan phototrophs and photosynthetic bacteria, are arguably the most important group of primary producers on the planet, as they account for roughly half of the primary production (Field et al., 1998). They form the base of the marine food web and play a vital role in the global biogeochemical cycling of carbon, nitrogen, phosphorous, and silicate. The protists differ in resource acquisition, being either autotrophic, heterotrophic or mixotrophic, and thus, protists are an extremely diverse group of organisms with immense ecological importance (Fig. 2.1).

Simple competition models and experiments show that the number of coexisting species utilizing the same resources cannot exceed the number of resources (Hardin, 1960; Tilman, 1981). Imagine an aquatic system, where two species are living of and competing for the same limiting resource. At steady state, the one species with the lowest requirement for that nutrient to sustain growth will outcompete the other. Now, add multiple species utilizing the same nutrient to that system and you get the same result: One species will outcompete the rest. Unless, though, two or more species have the exact same nutrient requirement (Tilman, 1981). This is known as the 'Competitive Exclusion Principle'. Indeed, in the marine environment, only a small number of limiting resources exists for phytoplankton and yet the diversity of species utilizing them is close to incomprehensible. This puzzling fact gave rise to Hutchinson's 'Paradox of the plankton' (Hutchinson, 1961).

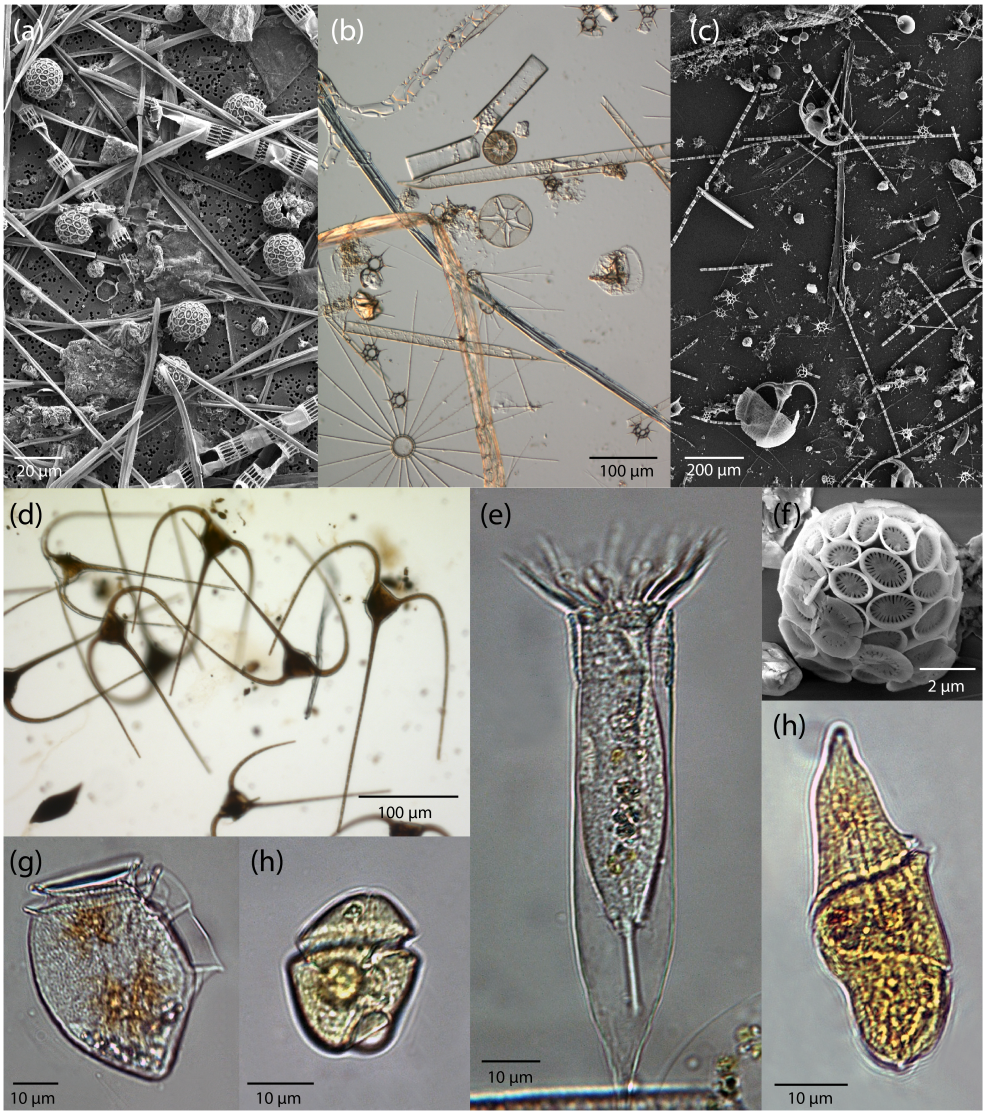

Figure 2.1: Mosaic of different phytoplankton species. (a) Scanning electron-microscopy (SEM) image of a natural phytoplankton assemblage from a plankton tow in the Norwegian Raunefjord close to Bergen. Dominating species are the diatoms *Skeletonema* sp. and *Pseudo-nitzschia* sp. and the coccolithophore *Syracosphaera* sp. (b) Cleaned diatom shells from an Antarctic plankton tow. (c) SEM image of phytoplankton assemblages also from the Norwegian Raunefjord, including e.g. long chains of *Skeletonema* sp. and *Ceratium tripos* dinoflagellates. (d) Dinoflagellates of the species *Ceratium macroceros* sampled at Dogger Bank in the North Sea. (e) A tintinnid ciliate from the North Sea. (f) SEM image of the coccolithophore *Syracosphaera nodosa* sampled in the Danish Øresund. (g) The dinoflagellate *Dinophysis norvegica* sampled in the North Sea. (h) A *Gyrodinium aureolum* dinoflagellate sampled in the North Sea. (i) A dinoflagellate of the species *Gyrodinium spirale* also sampled in the North Sea. Photo credits: (a), (c), Mikal Heldal, University of Bergen; rest, Helge Abildhauge Thomsen, Technical University of Denmark.

A common solution to this paradox is the introduction of predators to the above (imaginary) aquatic system and thereby an incentive for prey to develop a defence (e.g Paine, 1966; Smetacek, 2001; Leibold et al., 2017). Now, return to our aquatic system with two species sharing the same resources, make one of them a competition specialist with low nutrient requirement, and the other a defence specialist, that divides its energy investment between resource acquisition and defence. Obviously, the competition specialist will quickly outcompete the defence specialist. However, if we further introduce predators to the system, a collective loss of the competition specialist will prevent them from displacing the defence specialist, allowing coexistence of the two species, a hypothesis known as 'Killing the winner' (Fig. 2.2) (Winter et al., 2010). That phytoplankton diversity is maintained by the presence of grazers have been demonstrated theoretically (Ehrlich and Gaedke, 2018; Våge et al., 2018; Cadier et al., 2019) and is indeed supported by several experiments showing, that if predators are manipulated or removed from natural plankton communities the diversity decrease (Paine, 1966; Sarnelle, 2005; Leibold et al., 2017).

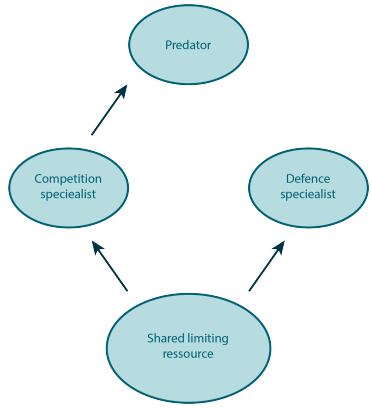

Figure 2.2: Diagram illustrating the structure of the 'Killing the Winner'-principle. The competition and defence specialist both compete for the same limiting resource. Predation on the competition specialist release resources to the defence specialist, allowing the co-existence of the two. Modified from Våge et al. (2014).

2.1 Types of defence mechanisms in phytoplankton

Defence mechanisms in phytoplankton are very divers and are generally divided into three types: morphological, physiological, and behavioural (Pančić and Kiørboe, 2018). Following are some examples of different defence mechanisms, however the list is far from exhausted nor detailed. Further, diatom specific defence mechanisms will be described more thoroughly in the later Chapter 3.3.

2.1.1 Morphological defences

Morphological defences, as indicated by the name, includes defences related to the morphology of the cell and is therefore very species-specific. Proposed defences include size, shape, and structure of the cell. Indeed, size can be interpreted as a defence mechanism: the larger the size of a cell, the fewer predators with that specific prey size spectrum exists (Smetacek et al., 2004; Kiørboe, 2011). Consequently, it can be argued that the morphology of some species of phytoplankton seek to increase their size. This by e.g. having inflated cell volume due to the presence of a vacuole as for diatoms or the dinoflagellate *Noctiluca lucens* (Menden-Deuer and Lessard, 2000; Kiørboe, 2013), or by forming colonies of either two-dimensional chains (many diatoms) (Fig. 2.1a,c, Fig. 3.2) or three-dimensional structures, like the prymnesiophyte *Phaeocystis spp.* (Hessen and van Donk, 1993; Jakobsen and Tang, 2002; Pančić and Kiørboe, 2018). The shape of the cell, including spine-like structures may also play a defensive role (Fig. 3.2), although there are no studies demonstrating lowered mortality due to spines (Pančić et al., 2019). In contrast, the structure of the phytoplankton cell has been demonstrated to provide defence against predators. This includes, e.g., the calcification of coccolithophores (Fig. 2.1a,f) (Harvey et al., 2015; Monteiro et al., 2016) and silicification of diatoms (Fig. 2.1b, Fig. 3.1) (Liu et al., 2016; Pančić et al., 2019; Xu et al., 2021), which will be discussed in greater details later in this thesis.

2.1.2 Physiological defences

Several physiological traits of phytoplankton are proposed to be of defensive value. These include e.g. bioluminescence and/or production of chemical compounds (secondary metabolites) (Pančić and Kiørboe, 2018). Bioluminescent dinoflagellates can reduce mortality due to copepod grazing by flashing (White, 1979; Hanley and Widder, 2017). The bioluminescent mechanism is believed to have multiple purposes: act as an aposematic signal to warn predators of potential toxicity, a startle display to startle the predators, or a 'burglar alarm' that draws attention to the predator from larger, visual predators (Hanley and Widder, 2017; Prevett et al., 2019). The latter is the most accepted, however there must be a cell density threshold for this to be true (Prevett et al., 2019).

Toxin production of phytoplankton have been studied thoroughly and are found in a wide range of species including dinoflagellates, diatoms, and cyanobacteria. Many of these secondary metabolites are toxic to humans, like paralytic shellfish toxins (PST) produce by various species of *Alexandrium* dinoflagellates, and blooms of these harmful algae are a known challenge for aquaculture and shellfish consumers (Trapp et al., 2021). Generally, there are two ways that toxins can affect predators: the first is a deterring effect, causing the predator to not eat a particular prey (Teegarden and Cembella, 1996; Colin and Dam, 2002; Selander et al., 2006). The second is a toxic effect, where the predator is affected after having ingested the toxic prey, either by

reducing its ability to feed on further prey or even by killing the predator (Colin and Dam, 2003; Sopanen et al., 2011; Pančić and Kiørboe, 2018).

Whether the toxins function as a predator deterrent that prevents the cell form being eaten, or the cell is consumed with subsequent toxic effects on the grazer, opens the discussion of whether a defence mechanism is a 'private good' or a 'public good'. If the toxin prevents the cell that produces the toxin from being consumed, it is a 'private good'. That is, the predator selects against the toxic cells and instead feeds on alternative prey, a potential competitor. The benefit to the toxic cell is then two-fold: mortality rate is lowered and competition for resource is reduced. If the toxin, on the other hand, reduces the further feeding capability of the predator only after being consumed, the toxin-producing cell must sacrifice its own life only to the benefit of the surrounding cells, i.e., 'public good'. A similar situation applies to toxins that leak into the environment where they affect predators (or competitors). Arguably, the latter could only evolve if the prey population consist of mainly clonal cells, like leaves on a tree (Pančić and Kiørboe, 2018). However, blooms of toxic phytoplankton are not comprised of single clones. In fact, bloom-forming species hold substantial genetic diversity (De Vargas et al., 2015; Rengefors et al., 2017), and thus the evolution of toxin production as a public good becomes difficult to understand, and must be associated with private good (Thornton, 2002; Driscoll et al., 2016).

2.1.3 Behavioural defences

Suggested behavioural defences in phytoplankton include resting stages and motility. Production of dormant stages are a common trait among phytoplankton, such as spores in diatoms or cysts in dinoflagellates, and a defensive means towards parasites and predators (Montresor et al., 2003; Toth et al., 2004; Kuwata and Tsuda, 2005). This can be seen as a predator avoidance strategy, where cell germination is supressed to some extend when predators are present, or as a morphological defence strategy, as the resting cells are relatively inedible (Rengefors et al., 1998; Ellegaard and Ribeiro, 2018). Motility as defence against predators may decrease mortality rate due to an ability to escape predators in efficient escape jumps, for example, when caught in feeding currents of zooplankton (Jakobsen, 2001, 2002). However, being a motile prey also increases the chances of being encountered by a predator (Pančić and Kiørboe, 2018).

2.2 Defence-associated trade-offs

As described earlier, one of the key factors in shaping the plankton community is the employment of defence against predators and/or pathogens. This can only be true, however, if there is a cost to a defence, otherwise all species would likely be equally defended and the diversity of species would again compare to the number of resources available (Thingstad et al., 2014; Våge et al., 2014). Thus, there is no free lunch and trade-offs to a defence ensures that there is no such thing as a Darwinian daemon (Litchman and Klausmeier, 2008; Litchman et al., 2013).

Trade-offs of defence mechanisms in phytoplankton have seldom been demonstrated, and more rarely quantified, and many experiments even suggest defences to be costless (Pančić and Kiørboe, 2018 and references therein). One explanation to this conflict between theory and practice could be that defence experiments are most often carried out without any limitations in terms of nutrients, light, temperature, etc. If trade-offs only materializes under natural (limiting) conditions, as widely accepted for terrestrial plants (Coley, Bryant and Chapin, 1985; Hahn et al., 2021), such experiments will be unable to demonstrate trade-offs (e.g. Kapsetaki and West 2019; Chakraborty et al., 2019; Wang et al., 2015; Ryderheim et al., 2021).

The fitness of an organism is as earlier mentioned determined by the difference between growth and mortality, and these should be the currency of the trade-off between the benefit and the cost of a defence. Ideally, experiments exploring defence and associated trade-offs should result in a mechanistic understanding of the underlying processes of the two. A mechanistic understanding of the trade-offs would better allow one to generalise from the few species one can examine experimentally, which, in turn would facilitate the development of mechanistically underpinned trait-based models of ecosystems (Kiørboe et al., 2018)

2.3 Inducibility of defence and copepodamides

Many phytoplankton defences are inducible, meaning that they are upregulated or harnessed when predators are present. This suggests that there must be a significant cost to a defence. According to defence theory, inducible defences should be favoured in variable environments, i.e. defences should be inducible when the risk is variable and when the defence is costly (Mole, 1994; Tollrian and Harvell, 1999; Stamp, 2003).

Unlike terrestrial plants or marine macroalgae, whose defence is often triggered by herbivores or pathogens damaging the plant tissue, the life of a phytoplankton cell depends on its ability to immediately defend itself against grazers or pathogens (van Donk et al., 2011). Phytoplankton must therefore rely on chemical cues, more than on physical interaction, to induce its defence. One can distinguish between three main types of induction cues; (i) chemical cues released upon mechanical damage, (ii)

grazer-specific cues released by the grazer and (iii) feeding-specific cues (van Donk et al., 2011).

Chemical cues released by mechanical damage of phytoplankton cells can be sensed by other cells, that in turn can deploy or upregulate their defence. The released cues can e.g. be from sloppy feeding by copepods or from cells lysed by viruses. Apparently, the prey response differs, depending on whether the chemical cues stem from closely related species or un-related, historically, and geographically co-occurring species. This has been well studied for animals (e.g. Chivers et al., 1997; Wisendenet al., 1999; Hazlett and McLay, 2005; Scherer and Smee, 2016), however almost no studies exist on single celled phytoplankton. Recently, a study by Brown and Kubanek (2020) examined the importance of phylogenetic relatedness on the dinoflagellate *Alexandrium minutum*'s assessment of predation risk. They found, that when exposed to chemical cues released by unrelated but historical, geographical co-occurring species, *A. minutum* significantly increased toxin production and decreased growth rate. Contrary, when exposed to cues from more closely related species, *A. minutum* decreased toxin production and increased growth rate. This response differs from the general trend that cues from conspecifics induce stronger defence than cues from heterospecifics (Scherer and Smee, 2016). The authors argue that cues from conspecifics may indicate senescence of a phytoplankton bloom and therefore it would be more advantageous for *A. minutum* to utilize nutrients from lysed cells to increase growth rate to above mortality rate. Two other studies on *Alexandrium catenella*, found opposite responses: One found toxin production to decrease when exposed to cues from conspecifics in line with the study by Brown and Kubanek (2020) (Griffin, Park and Dam, 2019), whereas the other found the toxin production to increase in response to lysed cells of two conspecifics (Senft-Batoh et al., 2015). Responses to dead conspecific thus appears to be species-specific and the ability to discriminate between cues from con- or heterospecifics, and subsequently the ability able to regulate investment in defence or growth, accordingly, would indeed add to the success of a given species of phytoplankton.

The second type of induction cues are grazer-specific cues released from grazers, such as sex pheromones or aggregation pheromones that prey cells can spy on. These types of pheromones are very species-specific and would be a valuable source of information to the phytoplankton. However, disentangling the exact mechanisms that governs defence induction in specific species have proved to be difficult (van Donk et al., 2011).

Feeding-specific cues are chemical compounds associated with the digestion of prey. Indeed, releasing such chemicals for prey to spy on is of no benefit to the predator, yet predators cannot be in full control of chemical compound released when ingesting and digesting prey (van Donk et al., 2011). One such compound of chemical cues are the copepodamides. These are fairly recently discovered polar lipids, released by feeding copepods, common predators of phytoplankton (Selander et al., 2015, 2016; Grebner et al., 2019). Induction of defence mechanisms by the presence of copepods have been demonstrated for many marine phytoplankton groups: increase in shell-thickness

of diatoms (Pondaven et al., 2007), increased toxin production in dinoflagellates and diatoms (Selander et al., 2006, 2012; Lundholm et al., 2018; Ryderheim et al., 2021), elevated bioluminescent capacities of dinoflagellates (Lindström et al., 2017), or changes in morphology of colony-forming phytoplankton (Ryderheim et al., submitted; Long et al., 2007; Selander et al., 2011; Bergkvist et al., 2012; Rigby and Selander, 2021).

CHAPTER 3

Diatoms

Diatoms are unicellular or colonial, purely autotrophic microalgae. They form an extremely diverse and evolutionary successful group with an estimated number of species between 100,000 and 200,000 (Armbrust, 2009; Mann and Vanormelingen, 2013). Diatoms play a key role in the global carbon fixation as they account for 20-25 % of the Earth's global primary production and around 40 % of the marine primary production, which is equivalent to the amount produced by the terrestrial rainforests (Field et al., 1998; Sarthou et al., 2005; Armbrust, 2009). Thus, diatom production is one of the key sources of carbon for marine, costal and estuarine food webs, supporting the most productive fisheries in the world, (Armbrust, 2009).

Additionally, diatoms play a key role in the major biogeochemical cycles of the Earth. For example, diatoms typically dominate the biomass in phytoplankton blooms, however only a small fraction of species are known to constitute total abundance (Smetacek, 2012; Behrenfeld et al., 2021). Following these blooms, which are typically terminated by nutrient-depletion, is an event of aggregation and mass sinking, facilitating the export of carbon to the depth. Thus, diatoms are a key contributor to the biological pump (Tréguer et al., 2018). As a function of their silica shell, which will be discussed below, diatoms are moreover key drivers of the silicon cycle (Tréguer and De La Rocha, 2013). It is estimated that a silica atom is incorporated into a diatom shell 39 times from the time of entering the ocean until it is buried in the sea floor (Armbrust, 2009).

3.1 Living in a glass shell

Diatoms are found in almost all illuminated aquatic environments and are divided into two main groups, centric and pennate diatoms (Fig. 3.1). Differences between these two groups include both cytological, biological, and ecological aspects, with one of the most obvious differences being the shape. Centric diatoms are radially symmetrical, while pennate diatoms typically have a well-defined main axis (Serôdio and Lavaud, 2020). They can be with or without spines that can be many times the length of

the cell. Most diatom species grow as solitary cells, however some species also forms colonies of e.g. fan-, ribbon-, or 'pearl-on-a-string' shaped chains (Fig. 3.2).

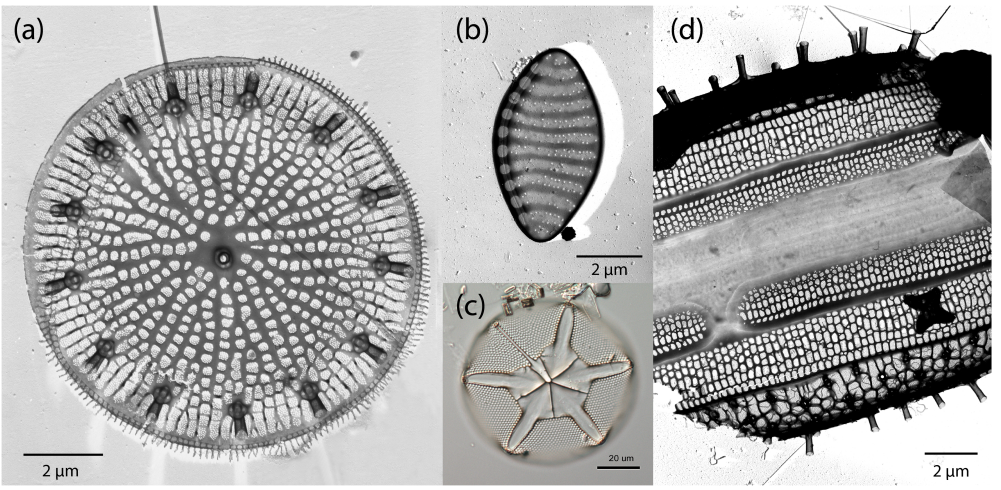

Figure 3.1: Shells of Antarctic diatoms. (a), (d) Transmission electron-microscopy (TEM) images from above and the side of the centric diatom *Thalassiosira* sp. shell (b) TEM image of the pennate diatom *Fragilariopsis* sp. shell (c) The shell of the centric diatom *Asteromphalus* sp. Photo credits: Helge Abildhauge Thomsen, Technical University of Denmark.

What is probably the most characteristic feature of diatoms is their beautifully ornamented glass shell ('frustule'), which encapsulates the cell (Fig. 3.1). This siliceous shell is composed of two halves ('valves') that partially overlap each other, like two Petri-dishes (Finkel and Kotrc, 2010; De Tommasi et al., 2017). The characteristic shell is thought to be a substantial factor explaining the success of diatoms and the benefits appear to be many-fold: it enhances nutrient uptake (Mitchell et al., 2013), facilitates light harvesting (Romann et al., 2015), protects the cell from harmful UV-radiation (Aguirre et al., 2018), and it protects against pathogens, parasitoids and grazers (Smetacek, 2012; Liu et al., 2016; Pančić et al., 2019). The latter defensive role against grazers will be further examined later. Finally, the siliceous shell may provide support for a central water-filled large vacuole, which takes up a significant fraction of the cell volume. Like for the silica shell, the benefits of having a vacuole are multiple and to some extend all tied to the inflation of the cell. First of all, the vacuole partly counter-balances the increased cell density due to the silica shell and acts as a buoyancy control (Hansen and Visser, 2019; Behrenfeld et al., 2021). Secondly, it stores nutrients for the cell which are then in turn sequestered from competitors (Smetacek, 1999). Finally, by increasing its size, it also reduces the number of potential grazers and increases the specific nutrient affinity and light capture potential (Raven, 1987; Smetacek, 1999; Smetacek et al., 2004).

As discussed earlier, no benefits come without costs. In terms of the vacuole, costs are mostly related to the extra material for building and maintenance, however these are generally exceeded by the benefits of a vacuole, especially under resource-limited conditions. In non-limited resource conditions, however, having a vacuole may reduce the maximum specific growth rate due to part of the cells energy being allocated to vacuole building and maintenance, and not to growth (Raven, 1997). Another requirement for being a vacuolated cell is a cell wall. In the case of diatoms, a silicified shell, which itself is associated with a number of costs. Firstly, diatoms have evolved an obligate requirement for dissolved silica, and thus, in environments with low dissolved silica concentration, growth of diatoms will be limited and other phytoplankton groups may proliferate (Finkel and Kotrc, 2010). However, when dissolved silica concentrations are sufficient, the energetic cost of silica precipitation is suggested to be low; for a diatom of which 25 % of the dry weight is composed of silica, silicification only consumes app. 2 % of the cell's total energy budget (Finkel and Kotrc, 2010). Another trade-off of the silica shell is higher density and subsequently higher sinking rate compared to similar sized cells, that does not have a silicified (or calcified) shell (Raven and Waite, 2004). However, the density also depends on the physiological state of the cells: nutrient and energy limitations increase the sinking rate of the cells by several folds (Sarthou et al., 2005, and references therein). Other costs of the diatomic silica wall are related to physical constraints. Being enclosed in a tightly sealed silica shell, phagotrophy is prevented and diatoms rely only on autotrophy for energy production (Smetacek, 2012)

3.2 Diatom cell division and life cycle

Living in a silica shell further affects the life cycle of diatoms. When the cells divide, the two valves form the basis of two daughter cells, each with a new valve made inside the parent. Consequently, one daughter cell is smaller than the parent cell and over successive divisions, cells are decreasing in size (Armbrust, 2009; Smetacek, 2012; Behrenfeld et al., 2021). To regain the original size, sexual reproduction is necessary and if this is not triggered within a specific size range of the diatom, it may lose the ability to divide (Mann et al., 2003). Sexual reproduction differs slightly between centric and pennate diatoms. In centric diatoms, small cells can become either sperm or eggs. Sperm breaks free of the cell wall and swim to an egg, still encased within the wall, enter the cell wall, and fertilize the egg nucleus. The resulting zygote swells to a specialized cell, an auxospore, and loses its old cell wall. Finally, the auxospore produce a new and much larger cell wall, eventually restoring the cell size. Sexual reproduction in pennate diatoms is also triggered by a specific size range, however instead of forming sperm and eggs, pennate cells produce morphological identical gametes, which fuse to create the zygote and auxospore that again form a new and much larger cell wall. Sexual reproduction imposes a risk for especially centric diatoms:

if sperm fails in finding an egg, the gametes will die. The risk is less for pennate diatoms, as they only form gametes when they find an appropriate mate (Armbrust, 2009).

Diatoms have a high maximum growth rate compared to other phytoplankton groups, and when environmental conditions become favourable, they bloom quickly and can increase their density by many orders of magnitude in just a few days (Armbrust, 2009; Behrenfeld et al., 2021). Blooms often occur at high latitudes in spring, following deep winter mixing or at low latitudes in areas with upwelling of nutrient rich deep water. A bloom is usually terminated when nutrients become limited and grazers and pathogens abundant. As the sinking rate increases and cells become more sticky, diatoms form aggregates and sink out of the water column (Brzezinski et al., 1990; Jackson, 1990; Kiørboe et al., 1990; Burd and Jackson, 2009).

This mass sinking event is considered to be of survival value to many diatom species (Smetacek, 1985) as it enables a transition from vegetative cells in the surface to resting stages in the deeper pelagic or benthic regions, where predation is much less (Smetacek, 1985; Riebesell, 1991; Passow and Alldredge, 1995; McQuoid and Hobson, 1996; Hansen and Josefson, 2001, 2004). Diatom resting stages are formed as a response to environmental cues, signalling that the viable growth season is over (McQuoid and Hobson, 1996; Ellegaard and Ribeiro, 2018).

Resting stages are characterized by having a highly resistant and thick cell wall and by the ability to undergo physiological dormancy. For diatoms, they can generally be divided into two types: resting spores and resting cells. Resting spores are morphologically different from vegetative cells, whereas resting cells are similar. Both types, however, differ from vegetative cells in both physiological and cytoplasmic parameters (McQuoid and Hobson, 1996; Ellegaard and Ribeiro, 2018). Studies on the longevity of resting stages of diatoms in sediments demonstrated germination of cells estimated to have resided in the sediments between >55 and $>$ 90 years. A small portion of viable resting stages is enough to seed a diatom bloom when environmental conditions again becomes favourable (McQuoid, Godhe and Nordberg, 2002; Härnström et al., 2011; Lundholm et al., 2011; Ellegaard and Ribeiro, 2018).

3.3 Defensive strategies of diatoms

The primary grazers of diatoms are various protists, in particular dinoflagellates, crustacean zooplankton like copepods and krill, but also small filter-feeding fish like sardines or anchovies (Smetacek, 2012). Copepods are the most abundant zooplankton group in the oceans (Smetacek et al., 2004) and will be the grazers of main focus in this section. Diatoms have evolved a range of proposed defences mechanisms to withstand predation, with the most obvious or characteristic being the silica shell described above. Whereas dinoflagellates have the ability to engulf and digest whole

cells, grazers like copepods and krill rely on silica-lined mandibles or gizzards to crush each cell. If the diatom shell is not crushed before ingestion by copepods, cells are known to be able to survive gut passage (Fowler and Fisher, 1983). Indeed, the frustule has the highest mechanical strength relative to its density of any known material – could be imagined as the shell of a nut – and experiments show a decrease in grazing mortality by copepods with increasing silica content of the diatom shell (Smetacek, 2012; Aitken et al., 2016; Liu et al., 2016; Pančić et al., 2019). Thus, remarkably force is needed to crush the shell, something that would arguably cause wear and tear on the 'teeth' and could be a mechanism affecting copepod selectivity (Hamm et al., 2003; Michels et al., 2012). The shell of the diatom and the teeth of the copepods has indeed given rise to an evolutionary arms race between a predator and a prey (Smetacek, 2001, 2012).

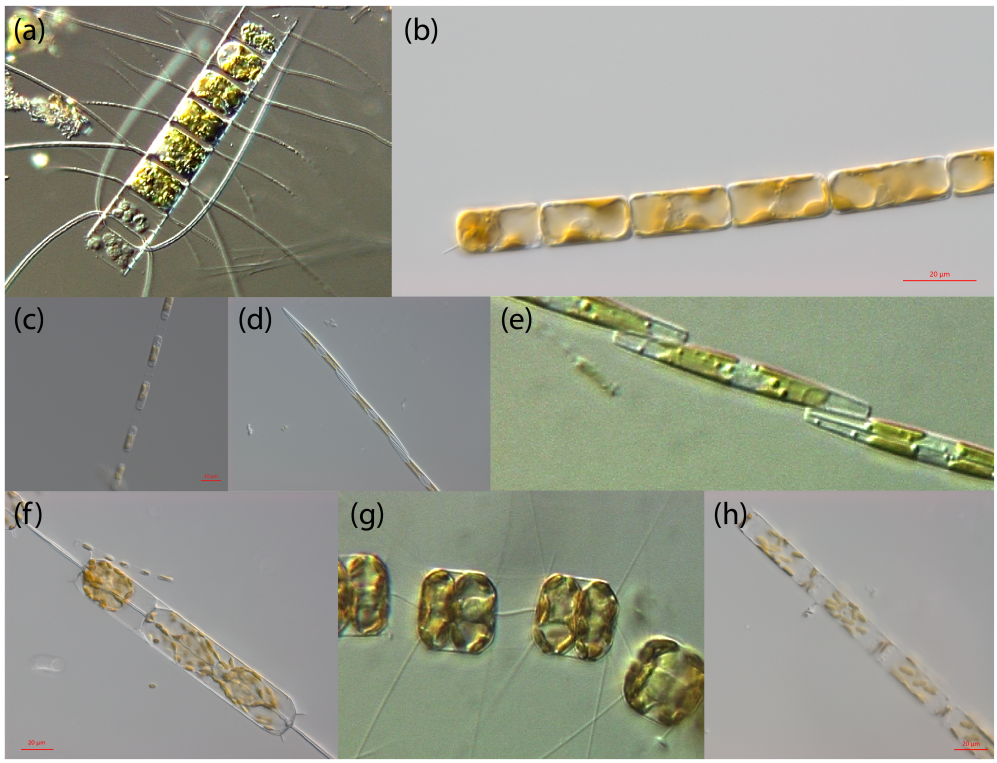

Figure 3.2: Mosaic of chain-forming diatoms. (a) *Chaetoceros* sp. (b) *Gunardia delicatula*. (c) *Skeletonema* sp. (d), (e) *Pseudo-nitzschia* sp. (f) *Ditylum* sp. (g) *Thalassiosira* sp. (h) *Cerataulina* sp. Photo credits: Niels Daugbjerg, University of Copenhagen.

Some diatom species form colonies consisting of morphologically identical, clonal cells, which has been suggested to be an antipredator behaviour (Fig. 3.2). Through colony formation, the diatoms can increase their size by several orders of magnitude and potentially move outside the prey size-preference of smaller predators like ciliates. However, this makes them susceptible to larger grazers like copepods. Multiple studies have shown that chain length of chain-forming diatoms species is significantly reduced when exposed to copepod predators. In fact, recent experiments demonstrated a decrease in chain length of the diatoms *Thalassiosira rotula* and *Chaetoceros curvisetus* by 79 % and 49 % respectively, when exposed to copepodamides (Rigby and Selander, 2021). This decrease was accompanied by a decrease in grazing mortality. Significant reductions in chain length of *Skeletonema marinoi* in response to copepods has also been demonstrated (Bergkvist et al., 2012), however similar response was not found in *Chaetoceros affinis*, suggesting that the response is somewhat species-specific (Rigby and Selander, 2021). A study by Ryderheim et al. (submitted) explored the relationship between chain-length of four species of diatoms (C*haetoceros affinis*, *Chaetoceros* sp., *Skeletonema marinoi* and *Thalassiosira nordenskioeldii*) and the size of three grazers (copepodites, copepod nauplii and dinoflagellates) (Ryderheim et al., submitted). They observed that long chains of diatoms were defended against smaller grazers (nauplii and dinoflagellates) but not against the larger copepodite grazers. Contrary, shorter chains were consumed by the smaller grazers. This suggests that the defensive value of chain formation depends on the grazer community composition and the plastic response of chain formation/suppression an efficient defence. In addition to colony formation, spines are another morphological feature found in diatoms, that may act as a predator deterrent, however this no direct evidence exists (Pančić and Kiørboe, 2018).

Toxin production is found in some diatom species, e.g. of the genus *Pseudo-nitzschia* or the closely related *Nitzschia* (Bates et al., 2018). They produce domoic acid (DA), a neurotoxin that accumulates in the body tissue of e.g. copepods. Studies have showed that in the presence of copepods, different species of *Pseudo-nitzschia* increased their toxin production by orders of magnitude. However this did not seem to affect the grazing rate or fecundity of the copepods, and thus have no 'private good' effect on the diatoms (Harðardóttir et al., 2015; Tammilehto et al., 2015; Lundholm et al., 2018; Cook et al., 2022). The only demonstrated effect of DA on copepods is a temporal effect on the feeding and mortality of copepods, a 'public good' also beneficial to the competitors of the DA-producing diatoms (Lundholm et al., 2018). An explanation for the somewhat lacking effect on copepods could be adaptation to DA, suggesting that the copepods could be one step ahead of the diatoms in the evolutionary arms race. In a unpublished study, Olesen and Ryderheim et al. succeeded in demonstrating both a private and public benefit of DA production in *Pseudo-nitzschia seriata* (Olesen and Ryderheim et al., submitted). They exposed eight different strains of *P. seriata* cultures to copepod cues and observed increased toxin production and decreased growth. Through direct observations, they demonstrated a private benefit of DA production by an up to 75 % increased rejection rate of cells caught by copepods. They

further demonstrated a public benefit through reduced feeding activity by copepods fed with toxic cells. However, copepods showed no reduction in feeding activity when they were fed a mix of toxic and non-toxic prey, suggesting that the public benefit is reduced in more 'natural' conditions. It further suggests that evolution is driven to a higher extend by the private benefit of toxin production.

When ingested by copepods, diatoms produce polyunsaturated aldehydes (PUA) within seconds of the cell being disrupted. The presence of these PUAs in the maternal diet of some copepod species, has been found to induce arrested development, increased mortality rates and/or birth defects of the copepod nauplii. Additionally, some field studies observed decreased hatching success of copepods during diatoms blooms (Ianora et al., 2003; Miralto et al., 2003; Leising et al., 2005). The toxic effect on copepods and further inhibition of their embryos from developing normally, has led to the hypothesis of PUA as a defence mechanism. However, despite the obvious adverse effects, this is difficult to understand in an evolutionary context (Jonasdottir et al., 1998). If the diatoms producing the PUA must be sacrificed to achieve the negative effect on copepods, the defence becomes a public good. Again, this is only sensible, if a diatom bloom consisted of genetically identical cells, which is not the case.

Arguably, besides being a mechanism to cope with unfavourable environmental conditions, resting stages in diatoms can also be considered a defence mechanism. Though defence against predation has been the main focus of this section, it is worth to briefly touch upon a study by Pelusi et al. (2021), who demonstrates induction of spore formation by virus infection. In fact, they observe that infection in the diatom *Chaetoceros socialis* induce up to 80 % of the cultures to form spores (Pelusi et al., 2021). Moreover, they find the propagation of infectious virus to be dramatically minimized upon germination by spores. Although there are limited studies of biological interaction as triggers of spore formation in diatoms, one could speculate that predators may induce a similar response as viruses. As described earlier, diatom resting stages have highly resistant and thick cell walls, and a study by Kuwata and Tsuda (2005) observed a strong grazing selectivity by two species of copepods, preferring vegetative cells over resting spores of *Chaetoceros pseudocurvesitus*. They further demonstrated the ability of resting spores to germinate after gut passage and thereby surviving gut passage (Kuwata and Tsuda, 2005). Similar results have been found for two other species of diatoms grazed by copepods: *Chaetoceros socialis* and *Detonula confervacea*, as well as for multiple diatom species ingested by other predators like Pacific oysters and freshwater crabs (Barillé and Cognie, 2000; Kuwata and Tsuda, 2005; Devercelli and Williner, 2006), all further indicating a survival value of resting spores.

CHAPTER 4

Objectives

The work of this thesis centres around defence mechanisms in marine diatoms, specifically those of silicification of the shell and aggregation. The thesis is based on three projects with the overarching aim of expanding our knowledge on the inducibility of the two proposed defences, as well as the underlying mechanism behind copepod selectivity of defended diatoms. I further attempt to estimate to potential costs associated with the benefits of a thicker shell. To meet these aims, the following three topics are explored:

I. *The inducibility of silicification of diatoms shells by the presence of copepods and the associated costs.* We hypothesise, that given the defensive benefits and potential costs of a thicker shell, shell-thickening in diatoms can be induced by the presence of copepods (Fig. 4.1). We expose different species of diatoms to chemical cues extracted from copepods and monitor biogenic silica content, growth rate, and size of the cells. Finally, we discuss the benefits, costs and trade-offs associated with silification.

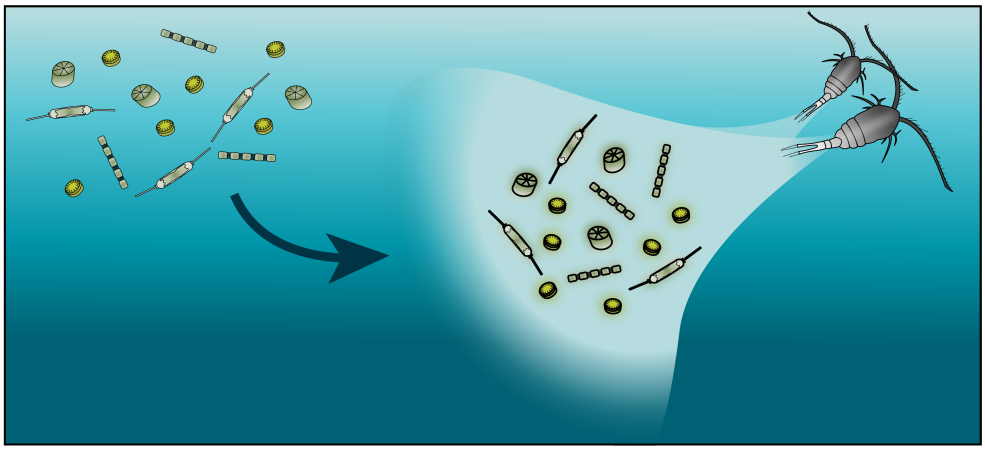

Figure 4.1: Schematic of hypothesis of Project I: Chemical cues released by copepods will induce shell-thickening in diatoms at the expense of reduced growth.

II. *The underlaying mechanism of copepod selectivity of diatoms with difference shell thickness.* We hypothesise that copepods fed with diatoms with thick and thin shells, will reject thick-shelled cells to a greater extent than the thin-shells (Fig. 4.2). We further hypothesise, that handling time of thick-shelled cells is longer than that of thin shelled-cells. We examine this by using high-speed video microscopy and discuss the implication of our findings on seasonal blooms and subsequent carbon sequestration by diatoms, as well as on the evolutionary arms race between diatoms and copepods.

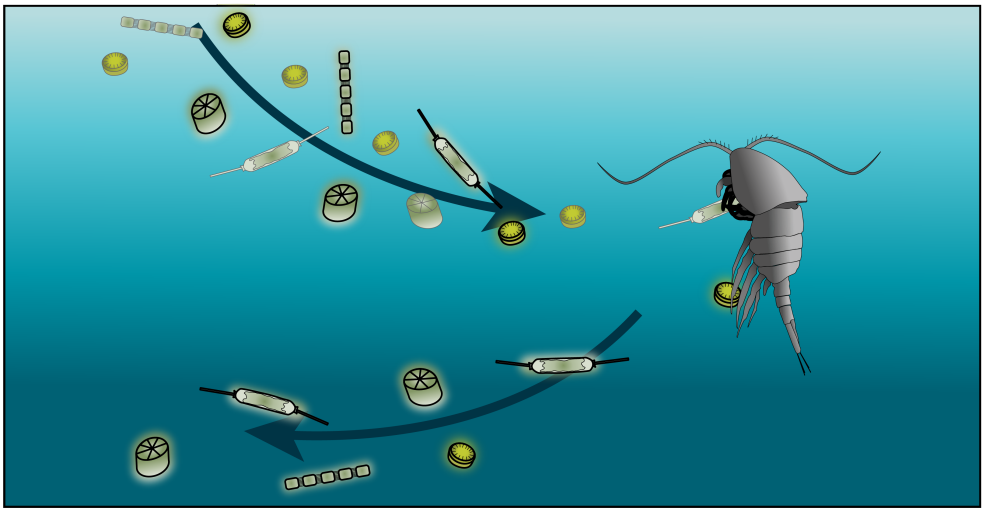

Figure 4.2: Schematic of hypothesis of Project II: Copepods fed thick- and thin-shelled diatoms will reject thick-shelled cells more frequently than thin-shelled cells. Additionally, handling time of thick-shelled cells will be longer than that of thin-shelled cells.

III. *Aggregation as a defence mechanism.* During experiments of our first project, we found that a culture of diatoms exposed to chemical copepod cues aggregated to a much larger extant than a non-exposed culture of the same species – and that this was indeed repeatable. We therefore hypothesised that aggregation could serve as a defence mechanism against copepod grazing (Fig. 4.3). We examined the relative increase of stickiness of six different species of diatoms when exposed to copepod cues and the additional effect of nutrient limitation and discuss the potential costs and benefits of aggregation as a defence mechanism.

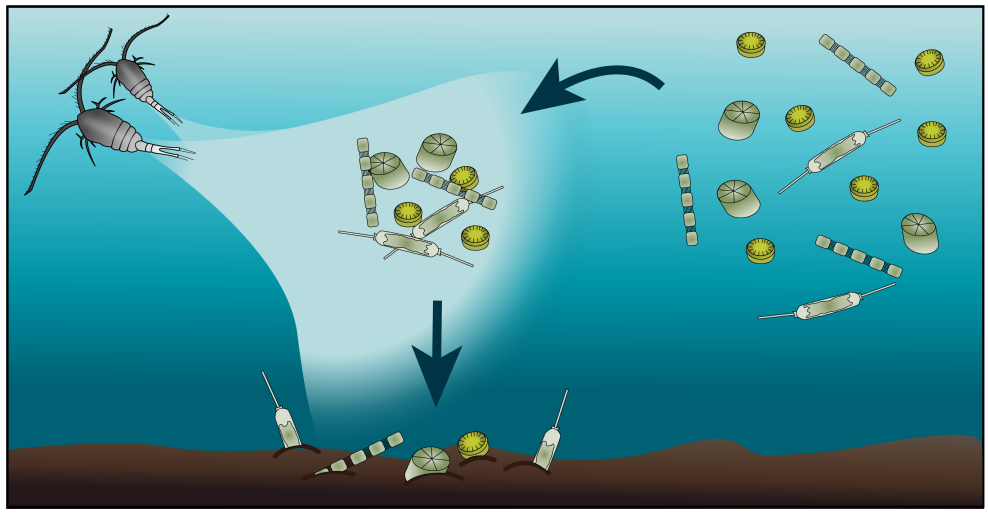

Figure 4.3: Schematic of hypothesis of Project III: Copepod cues induce stickiness in diatoms and thus facilitates aggregation.

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CHAPTER 5

Discussion and perspectives

This chapter summarizes the main findings of my three projects. It discusses the defensive value of silicification of diatoms shells and increased stickiness followed by aggregation. Finally, it comments on the ecological relevance of the projects.

5.1 Paper I

The defensive role of diatom's silica shell has previously been debated, however the general consensus is now that the shell serve as protection against grazers and pathogens. This is partly based on studies examining the strength of the shell, which has been found to have the highest mechanical strength relative to its density for any known biological material and require substantial force to break (Smetacek, 2001; Hamm et al., 2003; Raven and Waite, 2004; Aitken et al., 2016). Moreover, studies have found shells to provide partial protection against grazers, as the mortality rate of diatoms decrease with increasing silica content of the shell (Liu et al., 2016; Pančić et al., 2019). One study have found silicification to be inducible by grazers but at no costs (Pondaven et al., 2007), and is thus conflicting with general trade-off theory.

In my first project, we examined the inducibility and cost of silicification. We hypothesised that diatoms would increase their shell thickness (biogenic silica content) and in turn lower their growth rate, all in response to the presence of grazers. To test this, we exposed seven diatom species to chemical cues extracted from copepods and sampled the biogenic silica content, growth rate and size (volume) of the cells. We used three different experimental set-ups to test three different aspects: (i) the effect of exposure over time, (ii) the effect of copepod cue concentration, and (iii) the effect of silica limitation.

We found that all species increased their silica content when exposed to cues, that the response was proportional to the concentration of cues but also that the response

was variable, both between and within species. Growth rate and cell volume also decreased, however this response was again variable.

We discussed the benefits, costs, and trade-offs of shell-thickening. Based on a relationship between biogenic silica concentration and grazing mortality established by Pančić et al. (2019), we estimated that the average increase of biogenic silica content by 16 % found in our experiments would result in an average decrease in grazing mortality of 11 %, and that this would be 'paid' for by a 10 % reduction in growth rate. Thus, the changes in mortality and growth rate are of similar magnitude, suggesting and near-neutral trade-off and subsequently flat fitness-landscape. The latter may be an explanation to the high variabilities found in this study and further adds to the explanation for the high diversity of coexisting species.

5.2 Paper II

The main grazers of diatoms, copepods and krill, have silicified 'teeth' that enables them to crack the hard shell and subsequently digest the diatoms, unless the shell is too thick and strong (Liu et al., 2016; Pančić et al., 2019). Black box incubation experiments have demonstrated decreased grazing by copepods as a function of increased silicification of the diatom shell, but these have not revealed the exact mechanism of the shell-defence: is the grazing reduction due to the shells being unbreakable, are they de-selected by the copepods or are the cells handles for so long, that the actual time for grazing is reduced?

In my second project, we explored the mechanism behind a reduced grazing mortality experienced by thicker-shelled diatoms. We used direct observations to demonstrate the selectivity by the copepod *Temora longicornis* fed three differently sized species of diatoms: *Cyclotella cryptica*, *Thalassiosira weissflogii* and *Ditylum brightwellii*. Prior to the experiments, diatom cultures had been manipulated to have thick or thin shells, by culturing them under low or high light, respectively. We carried out experiments, where we observed the fraction of cells rejected by copepods after capture. We further tested the effect of prey density on the copepod selectivity and finally measured the prey handling time of thick- versus thin-shelled cells.

We found the fraction of captured cells rejected by the copepod to be significantly higher for thick-shelled compared to thin-shelled diatoms for all three diatoms species. Furthermore, the larger species of *D. brightwellii* had a higher rejection rate compared to the two smaller species of *T. weissflogii* and *C. cryptica*, a pattern particularly evident for the thick-shelled cultures. Prey density was found to have no effect on prey selectivity.

The prey handling time depended both on the outcome of the capture, on the prey size, and on shell-thickness. For the two larger species, *D. brightwellii* and *T. weissflogii*, handling time of rejected cells were less than that of ingested cells. For

the small *C. cryptica*, handling time did not differ between rejected or ingested cells. Shell-thickness affected handling time significantly for the two large species, where thick-shelled cells were handled for longer than thin-shelled. This trend was not observed for the small *C. cryptica*.

Our results show, that both copepod selectivity and prey handling time depend on diatom shell-thickness, however the size of the diatoms is also an important factor. We discussed the importance of silicification of diatom shells as a defence mechanism and argue that this is both a 'private good', as defended shells increase the odds of being rejected, but also a 'public good', as the long handling time of defended cells reduce the grazing time of the copepods and hence the grazing rate on diatoms. The defence mechanism of silicified diatom cell walls and the ability to select and handle prey by copepods is much likely part of an ongoing arms race, affecting the evolution and succession of the predator on its prey.

5.3 Paper III

As a part of their life cycle, diatoms typically form aggregates by the end of a bloom. Aggregates sink and thus facilitate transport of the cells from the upper ocean to the bottom. When conditions again become favourable, the cells can re-colonize the pelagic. Formation of aggregates happens by physical coagulation: cells collide due to turbulence or other processes, and if the cells are 'sticky', they may adhere to one another.

During experiments related to my first project, I observed heavy aggregation in diatom cultures exposed to copepodamides, compared to non-exposed cultures. We wanted to explore this phenomenon further and formulated the hypothesis that diatoms will aggregate, when exposed to copepod cues.

To examine this, we measured the stickiness of six species of diatoms exposed to copepodamides. This was done in a 'Cuette device': a fixed inner cylinder and a rotating outer cylinder with a gap in-between for diatom suspension. Knowing the rotational speed of the outer cylinder, we could calculate the shear. Combining this with the observed decrease in cell concentration of the suspension (when two particles stick together, there will be one less in the suspension), we were able to calculate the stickiness of the culture. We tested the effect of exposure time on stickiness of one diatom species, the effect of cue concentration on stickiness six species and the effect on nutrient limitation (Si and N) on stickiness of five species.

We found that two out of six species responded to copepod cues by increasing the stickiness and argued that this could potentially be a defence mechanism found in some species of diatoms. Forming aggregates increases the sinking speed, allowing for the cells to reach the bottom, where mortality is lower that of the surface. Additionally, we found the chain forming *Skeletonema marinoi* to reduce its chain length when

exposed to cues. Although acting in opposite ways, the changes in size may reduce grazing mortality and we argue that the effects of these defences depend on the cell density. Indeed, increasing stickiness would be beneficial when grazing mortality exceeds growth, as aggregation may increase the number of cells overwintering at depth. Based on observations of post bloom, massive aggregation events, it is often assumed that nutrient limitation increases stickiness and hence aggregation. However, we found to our surprise the opposite pattern for several of the species: nutrient deplete cells were less sticky than nutrient replete ones. We suggested the explanation to be found in coagulation theory; collision rate increases with the number of particles squared. Thus, collision rates increase rapidly during a bloom and in order not to aggregate and sink out too soon, cells may decrease stickiness to fully utilize the favourable growth conditions.

5.4 Overall discussion and perspectives

A trait of an organism can be constitutive or inducible, i.e. always expressed or only expressed when necessary. This is true for defence mechanisms in both terrestrial plants and marine phytoplankton. Many experiments have demonstrated defences in phytoplankton to be inducible, e.g., toxin production, bioluminescence capabilities and suppression of chain formation, as discussed in Chapter 2.3. The fact that many of these mechanisms are inducible indeed support the assumption that they are of defensive value. Moreover, it supports the theory by suggesting a substantial costs: Why else would a defensive trait only be activated when needed?

It has been notoriously difficult to experientially demonstrate costs of defences, and even more difficult to quantify them. One explanation could be, that experiments most often only examine one defence mechanism at a time, i.e. only expose prey organisms to one predator (or predator cue) at a time. When prey deploys their defence, it would be very reasonable to only activate the one targeting the specific predator, and perhaps this is after all not that costly. For example, one of our studies demonstrated that the cost in terms of reduced growth is warranted by the benefit of increased shell-thickness (Paper I). However, if one would expose prey organisms to the entire suite of danger it experiences in natural and not laboratory environment, deploying the entire array of defence mechanisms could indeed be a costly affair. Certainly, also a very complex affair in terms of laboratory experiments, where assigning specific costs to specific defences and untangle these from the effects of nutrient- and light-availability, temperature, etc., is near impossible.

5.4.1 Defensive role of the silica shell

Silicification of the diatom shells is an inducible defence against copepod grazers, however variable between and within species (Paper I). Increased thickness of the shell does indeed increase the chance of being rejected if caught by a copepod (Paper II), which further supports the defensive role of the silica shell – although perhaps not the initial role. Deposition of dissolved silica from the environment happens in a specific part of the diatom cell cycle (G2 phase) and depends on the growth rate: Fast growth shortens the silica uptake phase and thus less is deposited for the shell. Contrary, slow growth elongates the uptake phase resulting in more silicon for the shell (Martin-Jézéquel et al., 2000). Consequently, induced silicification of diatom cells should come at a cost of reduced growth rate, which was also observed in our study, although lower than expected (Paper I).

Another cost associated with induced silicification appears to be reduced cell size. In our study, the average cell volume decreased in cultures that increased the shell thickness in response to copepod cues (Paper I). Cells of smaller sized phytoplankton experience higher grazing pressure than those of larger, simply because the biomass of smaller grazers is larger. Therefore, reduced cell volume is arguable a cost to increased shell thickness.

In our study, we indeed found higher rejection rate by copepods with thicker shells, but we also found that small cells were ingested irrespective of the silica content (Paper II). A recent study presents the same results, although not their main objective: Xu et al., (2021) tested the effect of biogenic silica content on copepod feeding by presenting *Paracalanus pravus* copepods with high- and low-silica cells of *Thalassiosira weissflogii* and *Amphora coffeaeformis*. They found the copepods to prefer low-silica cells of *T. weissflogii* but high-silica cells of *A. coffeaeformis* and explained the latter with a lower frustule elasticity of high-silica cells of *A. coffeaeformis*. However, the results could also be explained by the same as observed in our study: the efficiency of silicification as a defence vanishes for small cells. However, thick-shelled cells ingested whole by the copepods have higher chance of surviving gut passage than thin-shelled, and thus increased shell-thickness of smaller diatom species may still be beneficial protection.

5.4.2 Aggregation as a defence

Indeed, aggregation and sinking is already a part of the diatom life cycle and is recognised as one of the reasons for the success of diatoms. Our study suggests that there is an additional aspect to the survival value of mass sinking events: a defensive aspect (Paper III). Aggregation in diatoms showed to be inducible by predators (cues), however only in some species (Paper III). For the two responding species (*Cyclotella cryptica* and *Skeletonema marinoi*), stickiness increased with increasing concentration of predator cues, suggesting the ability to adapt to the immediate predation pressure.

As for the silicification, one can argue increased stickiness and subsequent aggregation as a defence mechanism based on the inducibility: When grazing pressure reaches a certain level, diatoms may benefit from escaping this by aggregating and sinking out. Further, as viral infection has been demonstrated to induce formation of fast sinking resting spores in diatoms, it is not difficult to imagine a similar response to predators.

As our study only explored the inducibility of stickiness in various species of diatoms, no costs are directly observed or reported. Obviously, there are some costs associated with aggregation: loss of growth opportunity. However, if a greater number of cells survive until the next growth opportunity window, this cost is potentially balanced out. Indeed, this would only be true if the aggregating cells form resting stages able to survive in the deeper pelagic or in the sediments. Thus, further investigation of the physiological state of the aggregating cells are needed – ideally experiments following the cells through a full 'cycle': from viable cells in the surface to sticky, aggregating cells, potentially followed by a dormant period and a subsequent germination and growing of the cells.

Another aspect of stickiness and aggregation worth exploring, is exactly the 'how'. How are diatoms increasing their stickiness? It is fairly well established, that aggregation in diatoms involves production of sticky transparent exopolymeric particles (TEP) which form the aggregate matrix and promote aggregation (Alldredge and Passow, 1993). Is the abundance of TEP increasing, when increased stickiness is induced by copepod (cues)? Additionally, what are the costs associated with this potential production?

5.4.3 Combination of defences and public/private good

As briefly touched upon in the beginning of this chapter, experiments most often explore one defence at a time. Indeed, future experiments could benefit from observing multiple potential defences in diatoms, when exposed to predators. Take for example *Skeletonema marinoi*, the chain-forming diatom frequently used as 'lab-rat'. We know from previous studies that chain formation is suppressed in the presence of copepod grazers. Our study demonstrated increased stickiness and decreased chain length of *S. marinoi* when exposed to predator cues. What would happen if one would expose *S. marinoi* to copepods and monitor the chain length, the stickiness *and* the shell thickness? Potentially, the 'cheapest' defence would be deployed first, followed by the others, if the grazing pressure were high enough. However, besides the level of grazing pressure, the defensive responses of *S. marinoi* would arguably also depend on grazer composition, potentially also competition from other phytoplankton and environmental conditions like nutrient and/or light availability, etc. As discussed earlier for cost experiments, carrying out experiments like the above would indeed be very complicated, and disentangle the different mechanisms and factors affecting them extremely difficult.

A defence can be viewed as a public good or a private good, however they seldom exclude each other. An increase in shell-thickness of a cell is indeed a private good to the defended cell, clearly demonstrated by the lower mortality rate. Nevertheless, shell thickening of defended cells must also benefit the public, as the defended cells experience slower growth and is thus a lesser competitor for nutrients to the non-defended cells. Further, non-defended cells benefit from the longer handling time of defended cells by copepods, allowing less time to forage on other cells. Similarly, increasing stickiness of a cell is obviously a private good as the sticky cell aggregates with others and sink. However, it arguably only takes one sticky cell for two colliding cells to stick together and thus plenty of room is left for cheaters. Induced stickiness of some species must also be a public good, and one can ask if a true 'private good' even exits in a closely coupled ecosystem.

5.4.4 Ocean cycles and evolutionary arms race

Diatoms play a large role in the carbon and silicon cycles of the oceans, and both silicification and aggregation of diatoms are impacting factors. In an *in situ* iron fertilization experiment, Assmy et al (2013) suggested a division of diatoms species into two different life cycle strategies: a 'boom-and-burst strategy' of thin-shelled, fast-growing species such as *Chaetoceros dichaete* and a 'persistence strategy' of thick-shelled, slower-growing species like *Fragilariopsis kerguelensis*. The first group were defined as 'carbon sinkers' as they quickly bloom and produce fast sinking aggregates, that partly due to low Si:C ratios (thin shells) contributes to the deep carbon flux. Contrary, the second persistent group of diatom species are defined as 'silica sinkers', that are responsible for silicon export, partly due to high Si:C ratios (Assmy et al., 2013). If the presence of grazers can induce diatoms to produce thicker-shelled, sticky cells that aggregate, variation in the grazing pressure could cause a shift in the distribution of carbon and silica sinkers, as thin-shelled species (carbon sinkers) could increase shell thickness and potentially move towards being silica sinkers, ultimately blur the lines between the two types.

The evolution of diatoms, i.e. the two strategies as described above, is likely due to both bottom-up and top-down controlling effects. The thin-shelled, fast growing (often pennate) species developed in consistence with decreasing availability of silicon in the oceans and is thus likely to be bottom-up controlled (Finkel and Kotrc, 2010; Conley et al., 2017; Petrucciani et al., 2022). Contrary, the evolution of thick-shelled, slower growing species is probably resulting from top-down control by grazers like copepods. There has been, and still is, an ongoing evolutionary arms race between diatoms and their grazers, and silicification and aggregation are two means for the diatoms to reduce grazing mortality.

5.4.5 Final remarks

In this thesis, I attempted to expand the knowledge on defence mechanisms in diatoms, more specifically silicification of the shell. Moreover, due to a discovery of increased aggregation in copepod-cue induced diatom cultures, this thesis also explored aggregation as a defence mechanism. One important thing to keep in mind when discussing and extrapolating findings from laboratory experiments to e.g. global marine ecosystems, is the ecological relevance of the experiments. That is, are they carried out under conditions that can be directly translated to natural environments? I.e., are densities of diatoms or copepods, or concentrations of nutrients comparable to natural environments? In my experiments, the answer must be 'yes' and 'no'. Yes, diatom densities and copepodamides are comparable in most experiments, however nutrient concentrations were in most experiments saturated – therefore no. Though, when the experimental aim was to either quantify costs (Paper I), test effects of nutrient limitation (Paper I and III), or the effect of prey density (Paper II), nutrients and cells were, or at least attempted to be, added with respect to natural concentrations. In other experiments, ecological relevance was of lesser importance, as the main objective was to 'provoke' and explore the inducibility (Paper I and III). Indeed, caution should always be taken when speculating on global implications, based on simple, laboratory experiments with only a few species.

While it is my hope that this thesis has added to the general knowledge about defence mechanisms in diatoms, it has raised a number of questions yet to be answered. In general, understanding underlying mechanisms and trade-offs of defence diatoms through studies of a few species, enables the transition from a species approach to a trait-based approach to ocean life. Thus, quantifying the associated benefits and costs of defence is of crucial importance and indeed calls for further research. Additionally, combined defences in diatoms, and protists in general, due to multiple stressors like grazers/predators and environmental limitations should be explored further. Moreover, while there has already been focus on the extensive implications of post-diatom bloom aggregation, this thesis suggests aggregation as a defence mechanism, an interpretation which should be further explored.

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CHAPTER 6

Paper I

Diatom defence: Grazer induction and cost of shell-thickening

Josephine Grønning & Thomas Kiørboe

Centre for Ocean Life, Technical University of Denmark, DTU Aqua, Kemitorvet B201, Kongens Lyngby, DK-2800, Denmark

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Abstract

1 Diatoms account for 40% of the ocean primary production and play a key role in the oceans' ability to sequester carbon. The evolutionary success of diatoms and their role in ocean biogeochemistry are related to the siliceous shell that provide partial protection against grazing.

2 The structure and function of phytoplankton communities are governed by environmental constraints and organismal trade-offs. Defence mechanisms may help explain the high diversity of phytoplankton (incl. diatoms) in the ocean, but only if the defence comes at a cost. Defence costs have been notoriously difficult to demonstrate and quantify in marine phytoplankton.

3 Here, we demonstrate for seven species of planktonic diatoms that their shell thickens and their growth rate declines when cells are exposed to chemical cues from copepods, important predators of diatoms. The responses are proportional to the concentration of grazer cues, but are also highly variable, both between and within species.

4 At our standard experimental condition, the typical decline in growth rate is 10%, and the typical increase in cellular biogenic silica is 16%. The latter value corresponds to a decline in grazing mortality due to small copepods of 11%. Thus, silicification in response to grazers is exactly warranted.

5 The similar magnitude of the costs and benefits of silicification suggests a flat fitness landscape along the competition-defence axis. This may help explain the high diversity of coexisting diatoms in the ocean.

6 The significant but variable contribution of diatoms to the downward flux of organic carbon in the ocean depends to a large extent on the silica content of the cells. This is due less to the ballasting effect of silica, but mainly to the different life histories of more or less defended cells that are governed by evolutionary adaptations and – as demonstrated here - plastic responses to grazers.

6.1 Introduction

Predator-prey interactions are key in shaping ecosystem structure and function (Belgrad and Griffen, 2016; Creel, 2018; Lima, 1998). In the oceans, zooplankton predation is believed to be an important mechanism maintaining phytoplankton species diversity by allowing the co-existence of defence- and competition specialists, as demonstrated both theoretically (Cadier et al., 2019; Ehrlich and Gaedke, 2018; Våge et al., 2018) and experimentally (Leibold et al., 2017; McCauley and Briand, 1979). Indeed, phytoplankton have evolved a large range of defence mechanisms, ranging from morphological to biochemical or behavioural (Pančić and Kiørboe, 2018; van Donk et al., 2011). However, the costs and benefits of these different defence mechanisms have rarely been quantified, or even demonstrated, and hence the defence trade-offs hypothesised to promote diversity are most often unknown (Pančić and Kiørboe, 2018)

Diatoms are a diverse and important group of phytoplankton. They account for about 20% of the Earths photosynthesis (Armbrust, 2009) and are a major contributor to the biological carbon pump (Smetacek, 1999). Diatoms are characterised by an external silica shell, the frustule, that has many hypothesised functions, ranging from enhancement of light and nutrient harvesting to mechanical protection of the cell against grazing (Hamm et al., 2003; Mitchell et al., 2013; Romann et al., 2015). The defensive role of the shell has been suggested based on its unparalleled strength (Hamm et al., 2003; Milligan and Morel, 2002; Raven and Waite, 2004; Smetacek, 2001). The shell has the highest mechanical strength relative to its density of any known biological material (Aitken et al., 2016) and the consequent force required to crush the cell wall is remarkable (Hamm et al., 2003; Wilken et al., 2011). Recent experiments have indeed demonstrated that the shell provides partial protection against copepod grazing (Liu et al., 2016; Pančić et al., 2019). Thus, a 6-fold increase in silica content in a variety of diatoms decreased the predation mortality due to a copepod grazer by 4-fold and entirely prevented small copepod nauplii from consuming the diatoms (Pančić et al., 2019).

For predation to promote diversity, defence must come at a cost. Else, defended species would outcompete less defended species, and all species would be equally defended. Defence costs have been notoriously difficult to quantify in phytoplankton and the magnitude of the costs may depend on whether or not the cells are resource limited (Pančić and Kiørboe, 2018). However, many defence mechanisms in phytoplankton are inducible, i.e., are harnessed or intensified in response to grazer cues (Selander et al., 2019), suggesting significant costs to the defence (Karban, 2011; Tollrian and Harvell, 1999). There is some experimental evidence that silicification in diatoms is inducible by grazer cues, suggesting that silicification is costly (Pondaven et al., 2007). The biochemical costs of silica deposition in the shell are minute (Raven, 1983), but the deposition of silica is rate-limited. Thus, slower growth allows heavier silicification (Martin-Jézéquel et al., 2000) and one may hypothesise that heavier silicification may

be at the cost of slower growth (Pančić et al., 2019). This, however, remains to be tested.

Here, we examine the potential inducibility and cost of silicification. We hypothesise that diatoms increase their contents of biogenic silica (shell thickness) and decrease their growth rate in response to diffusible copepod cues. We expose cells grown under both silica replete and deplete conditions to copepod grazer cues and record potential changes in silica contents and growth rates of the cells. To examine the generality of this hypothesis, we test seven different species of diatoms. We demonstrate increases in silica content and decreases in growth rates of induced vs. non-induced cells in all species tested, but also that the response is highly variable both within and between species.

6.2 Materials and methods

Diatom cultures and culturing conditions

We examined seven species of diatoms: *Amphiprora paludosa* (CCMP125), *Cyclotella cryptica* (CCMP331), *Cyclotella meneghiniana* (CCMP336) (which later DNA analysis demonstrated to be a different strain of *C. cryptica*), *Ditylum brightwellii* (unknown strain), *Navicula incerta* (CCMP542), *Nitzschia laevis* (CCMP559), and *Thalassiosira weissflogii* (unknown strain). None of the species are known to produce toxins. *Thalassiosira weissflogii* and *D. brightwellii* were obtained from DTU Aqua, Denmark, while the other species were obtained from NCMA at Bigelow Laboratory, Maine. All diatoms were grown in B1 medium with silica ($\sim$ 500 µM Si) (Hansen, 2007) at 16 °C and a L:D cycle of 16:8 (120 µmol m^{-2} s^{-1}). Cells in exponential growth were used to initiate experiments. Experiments were run in continuous light at the same light intensities and temperature conditions as the cultures.

Copepod cues

We used two different sources of copepod cues, either filtered water from a culture of copepods (*Acartia tonsa*), or copepod signal molecules extracted from freeze dried copepods, *Calanus finmarchicus* ('Copepodamides', Selander et al., 2015). Copepodamides induce a variety defence mechanisms in phytoplankton (Selander et al., 2019).

The filtrate was water from a continuous culture of the copepod *Acartia tonsa* with about 100 individuals L^{-1} and maintained on a mixture of phytoplankton (*Heterocapsa triquetra*, *Rhodomonas salina* and *Thalassiosira weissflogii*). Culture water was first passed through a 20-µm filter and then through a GF/C filter. The filtrate with

nutrients added to B1 concentrations was used in experiments immediately after filtration and served as a positive control.

Copepodamides were purified from freeze dried *Calanus finmarchicus* as described in Selander et al. (2015). Briefly, *C. finmarchicus* were extracted with methanol and crude extracts were subjected to liquid-liquid partitioning between methanol/water and heptane. The methanol-soluble fraction was then fractionated on C18 SPE columns. Impurities were eluted in 70% methanol (aq) and copepodamides in 100% methanol. Finally, the methanol extract was concentrated by rotary evaporation, dissolved in a small volume and fractionated by reversed phase gradient elution HPLC (Selander et al 2015). The copepodamide content of each fraction was determined through direct infusion mass spectrometry. Positive fractions were pooled and the final concentration determined by mass spectrometry.

The copepodamides were dissolved in methanol and added to the empty culture flasks, and the methanol was then evaporated under a stream of nitrogen (Selander et al., 2015). Diatom cultures were then immediately added. Nominal concentrations of copepodamides varied between 1-10 nM, but effective concentrations were at least 2 orders of magnitude less due to a short half-life time and unknown losses (Selander et al., 2019).

Experimental designs

We ran three different types of induction experiments to test the effect of exposure time, copepodamide concentration, and nutrient depletion on the potential inducibility. (1) Repeated batch experiments, where diatoms were exposed to copepod cues over a period of 7-11 days; (2) dose-response batch experiments, where diatoms were exposed to different concentrations of copepodamides for 48 hours; and (3) continuous culture experiments with exposure to copepodamides (Table 1). Nutrients were replete in the two batch experiments, while silica was limiting in the chemostat experiment. Cell concentrations and sizes (volume) were measured by a Coulter Counter (Beckman Coulter, Multisizer 3, USA). Cells of *C. cryptica* and *C. meneghiniana* tended to aggregate during incubations, and samples (20 mL) were therefore sonicated for 120 s before counting.

Repeated batch experiments

In these experiments, cell cultures were diluted daily to the same, low cell concentration with fresh growth medium such that the cells never were resource limited. Slowly growing species (*D. brightwellii*) were diluted only every 2nd day. Control and treatment cultures were run in duplicate (*D. brightwellii* and *T. weissflogii*) or triplicate (all others). In experiments with copepodamides, cultures were transferred to new, freshly coated culture bottles every day with a nominal concentration of copepodamides of 10

nM. Control cultures were similarly transferred to bottles coated with pure methanol. In experiments with filtrate, the slow-growing species (*D. brightwellii*) were diluted with fresh filtrate by minimum 20 %, and fast growing species (*T. weissflogii*) by minimum 50 %. Samples for cell counts for growth rate estimation and measurements of biogenic silica in cells and dissolved silica concentrations were taken daily, except from *D. brightwellii* in filtrate, where samples were taken daily initially and every second day.

Table 1: Experimental information. The table lists the experimental design of experiment together with the tested species, the duration and the source of copepod cues.

Experiment nr.	Species	Duration	Copepod cues
Repeated batch experiments			
1	*D. brightwellii* or *T. weissflogii*	11 days	Filtrate
2	*C. cryptica* or *C. meneghiniana*	7 days	Copepodamides
3	*A. paludosa* or *D. brightwellii*	8 days	Copepodamides
Dose-response experiments			
4	*C. cryptica* or *T. weissflogii*	48 hours	Copepodamides
5	*C. cryptica* or *C. meneghiniana*	48 hours	Copepodamides
6	*N. incerta* or *N. laevis*	48 hours	Copepodamides
7	*A. paludosa* or *D. brightwellii*	48 hours	Copepodamides
Continuous culture experiments			
8	*T. weissflogii*	5 days	Copepodamides
9	*A. paludosa*	5 days	Copepodamides

Dose-response experiments

For the dose-response experiments, triplicate diatom cultures were grown at four different nominal concentrations (0, 2, 5 or 10 nM) of copepodamides for 48 h. Bottles were coated at the start and after 24 h as described above. Cell concentrations were measured at 24 and 48 h for growth rate estimates and samples for biogenic silica concentrations were taken at start and termination of the experiment. Cell densities were low throughout the incubation and cultures never became resource limited.

Continuous culture experiment

To test silicification and growth rates of diatoms in response to copepodamides under silica limitation, continuous culture experiments were carried out with two diatom species (*T. weissflogii* and *A. paludosa*). Cultures were first grown to $\sim$ steady state in

chemostats with a continuous inflow of B1 medium with a low silica concentration of 20 µM to ensure that silica became limiting. The dilution rate was 0.66 d^{-1}. During the subsequent 5 days, triplicate chemostats of each species were exposed to copepodamides along with triplicate controls. Cultures were daily transferred to new bottles, freshly coated with methanol containing copepodamides (10 nM) or pure methanol, as above. Each culture bottle was aerated to ensure complete mixing and to prevent carbon limitation. Samples for cell concentration, biogenic silica content, and dissolved silica were collected every 24 h. To avoid any silica contamination, no glassware was used during the experiments.

In a chemostat at steady state the cell growth rate is fixed by the dilution rate (D), and a growth rate response therefore instead materialises as a change in the concentrations of cells and dissolved silica in the culture bottle. Thus, a decrease in growth in response to copepod cues would result in lower cell concentration and higher silica concentration in the culture. Specifically, by assuming Michaelis-Menten kinematics to describe the functional response of growth rate to silica concentration, one can estimate the maximum growth rate (μ_{Max}) from the steady state cell ($\widehat{B}$) or ambient silica ($\widehat{S}$) concentration in the culture (both in units of µM silica) (Ryderheim et al., 2020):

$$\mu_{\mathrm{Max}} = \frac{D\alpha(S_{\mathrm{i}} - \widehat{B})}{\alpha(S_{\mathrm{i}} - \widehat{B}) - D} \quad or \quad \mu_{\mathrm{Max}} = \frac{\widehat{S}\alpha D}{(\widehat{S}\alpha - D)} \tag{1}$$

where D is the dilution rate, α the affinity for silica (0.41 L d^{-1} µmol Si^{-1}, Paasche, 1973), and S_i the concentration of silica in the inflow growth medium. This allows one to compare the maximum growth rate of induced relative to non-induced cells. While the estimate of the maximum growth rate is sensitive to the (assumed) magnitude of the affinity, the relative change is not.

Biogenic silica analysis

For analysis of cellular biogenic silica content, the method of Paasche (1980) and Pančić et al. (2019) was followed. 10 mL samples were filtered on 3-µm polycarbonate filters and washed twice with acidic MilliQ water to reduce the dissolution of silica. The pH of this acidic MilliQ water was adjusted by adding hydrochloric acid until a pH of 5.0. Hereafter, the filters were dried at 65 °C for 90 min and stored at -20 °C until dissolved in 15 mL 0.5 % (w/v) sodium carbonate solution and reheated at 85 °C for 90 min. When cooled, the pH was adjusted to 5-6 by adding concentrated sulfuric acid and the reactive silica concentration was analysed in a SmartChem 200 wet chemistry analyser (Unity Scientific, MA) following the molybdate method of Strickland and Parsons (1972). For analysis of dissolved silica in cultures and medium, 10 mL samples were passed through a 0.2 µm syringe filter and stored at -20 °C until

analysed in a SmartChem 200 as above. Biogenic silica content is here expressed per cell volume, because the strength of the shell scales approximately with silica per volume rather than with silica per surface area (Pančić et al., 2019).

Statistical analysis

The dose response relationship between copepodamide concentration and silica content, growth rate, or cell volume was analysed with linear regression. The effects of copepodamides in batch and chemostat experiments were analysed with a linear mixed model. The models took treatment and time as fixed effects and replicate as a random effect. Day 0 is not included in the statistical analysis of repeated batch or chemostat experiments.

To test within each approach whether the proportion of experimental trials that responded positively to induction was significant, a binomial one-tailed test was used. A t-test was used to further test whether the overall mean responses were different from zero. A significance level of 5 % was used throughout. All statistical analysis was performed in R (Version 1.2.5001). All mean values in this study are presented with ± standard deviation.

6.3 Results

Dose-response experiments

Of the seven species tested in the dose-response experiments, six showed a significant increase in silica per cell volume with increasing concentration of copepodamides (Fig. 1, Table 2, Table S1). The increase varied from 3-36 % compared to controls (Fig. 1, Table S1). The effect of copepodamides on growth was more variable; 3 species significantly decreased their growth with increasing amount of copepod cues whereas the other four did not. Finally, cell sizes (volume) decreased by 3-12 % in response to copepodamides in 4 out of 7 species. Two species showed the opposite effect after 48h and increased volume by 3-11 %. The last one, *T. weissflogii*, did not change volume in response to copepodamides.

When comparing the results across all species tested in the dose-response experiments, the proportion of experiments showing increased silica content of cells exposed to copepod cues is significant (binominal test, $p = 0.016$) with an overall mean change of 18.7 ± 11.1 %, which is also significantly different from zero ($p = 0.004$, Fig. 2a). The proportion decreasing growth rate is not significant ($p = 0.18$ and $p = 0.06$, respectively) and neither is the overall mean change of -17.3 ± 38.7 % ($p = 0.1171$) (Fig. 2b). Finally, the proportion of experiments with decreased cell volume is significant ($p = 0.013$), as is the overall mean change of -3.8 ± 6.5 % ($p = 0.050$).

Repeated batch experiments

Repeated batch experiments tested the inducibility of a species over multiple cell divisions (7–11 days). In all species tested in these experiments, the silica content per cell volume was higher in induced than non-induced cells, and the difference was statistically significant in 4 of 6 species (Fig. 3, Table 3). The average relative increase in silica content in induced cells over the course of the experiments was comparable to the results in the dose response experiments and varied between 5-35 % (Table 3). The growth rate and cell volume of induced cells were significantly reduced in 3 and 4 out of 6 species, respectively (Table 3).

When comparing across all species and treatments in the repeated batch experiments, the proportion of cells with higher silica content in induced relative to non-induced cells is significant ($p = 1.405\text{e-}07$) (Fig. 4a). The mean change in silica is 16.3±17.3 %, which is statistically different from zero ($p = 1.55\text{e-}07$). Likewise, the proportion of cells decreasing their growth rate or cell volume ($p = 0.04877$ and $p = 0.0004$, respectively), and their overall means of -10.1±21.5 % ($p = 0.003$) and -1.9±3.6 % ($p = 0.0008$), respectively, are all statistically significant (Fig. 4b-c).

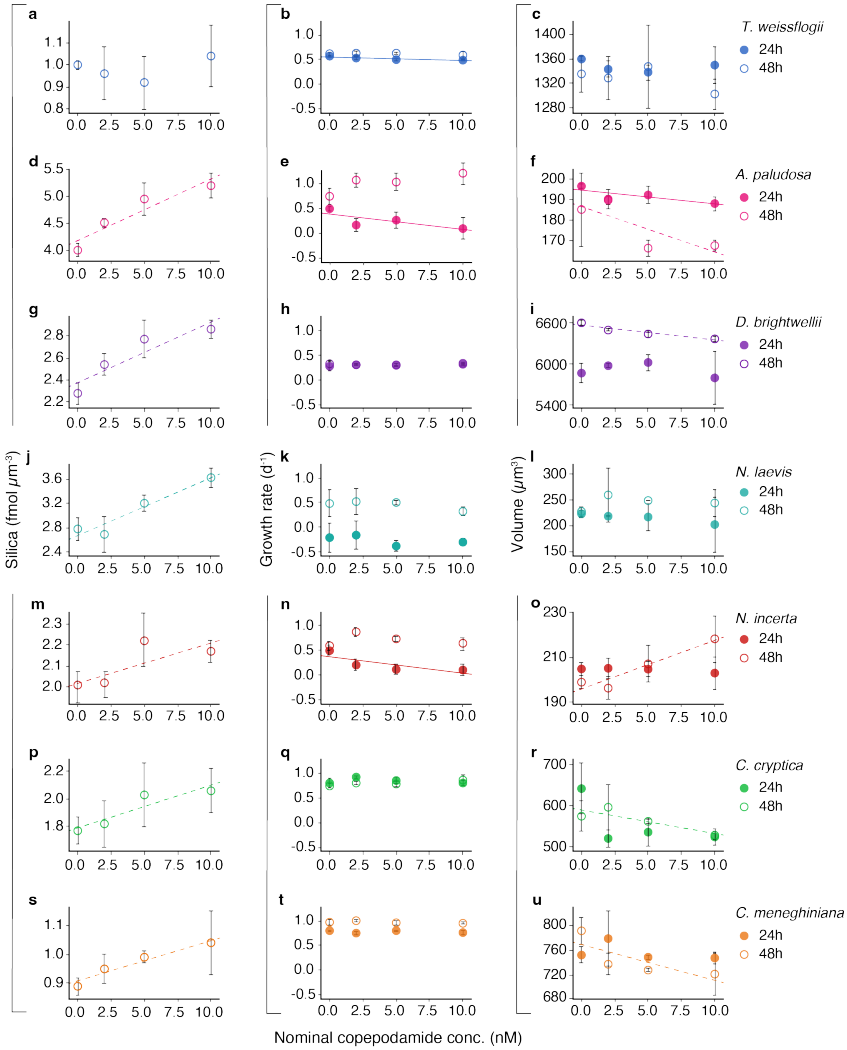

Figure 1: Relationship between copepodamide concentration and silica (left column), growth rate (mid column) and cell volume (right column). Points represents the mean of triplicates, and error bars the standard deviation. Each row represents the species present in the legend of that row. Dots and open circles colours represent samples taken after 24 h and 48 h, respectively. Solid and dashed lines denote linear regressions for 24 h and 48 h and are plotted in cases where they were statistically significant ($p > 0.05$). Note the different scales on y-axis.

Table 2: Dose-response experiment. Percentage difference in silica content, growth rate and cell volume between lowest (0 nM) and highest (10 nM) concentration of copepodamides based on their regressions. Differences are relative to control treatments, i.e. positive or negative differences means an increase or decrease in induced cells, respectively. $*p < 0.05$; $**p < 0.01$ and $***p < 0.001$.

Species	△Silica (µm^{-3})	△Growth (d^{-1})		△Volume (µm^3)	
	48h	0-24h	24-48h	24h	48h
T. weissflogii	2.7	-12.4 *	-11.1	-0.5	-2.1
A. paludosa	27.3 ***	-85.5 *	45.2	-3.4 *	-11.9 *
D. brightwellii	23.1 ***	9.8	3.1	-1.6	-3.3 ***
N. laevis	35.6 ***	-66.7	-32.7	-9.1	2.8
N. incerta	9.5 *	-90.2 **	-5.7	-1.0	11.1
C. cryptica	17.3 *	4.0	13.4	-14.4	-9.7*
C. meneghiniana	15.2 *	-2.1	-3.9	-2.0	-7.4*

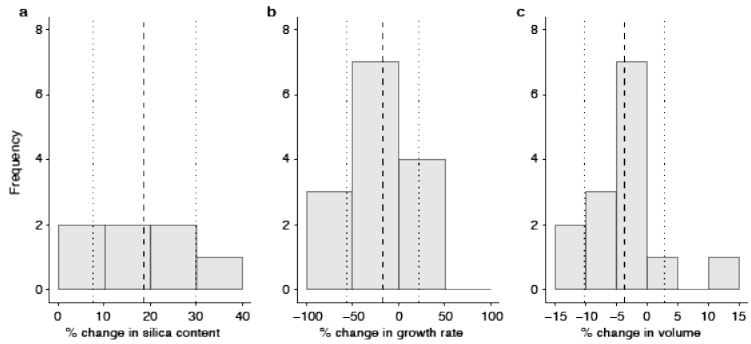

Figure 2: Frequency distribution of percent change in silica content (a), growth rate (b) and cell volume (c) of all species in the dose-response experiments. The dashed line represents the overall mean and the dotted lines its standard deviation. Mean percent change in silica content, growth rate and cell volume are 18.7 ± 11.6, -17.3 ± 38.7 and -3.8 ± 6.5, respectively.

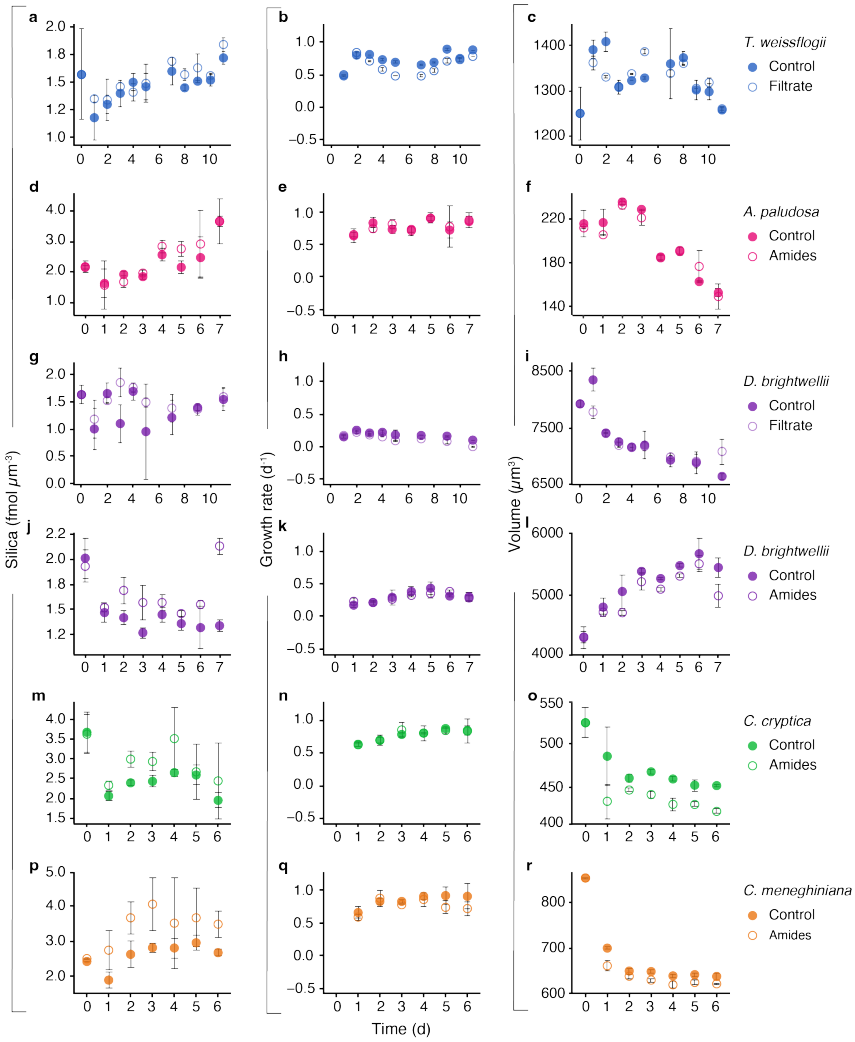

Figure 3: Variation in silica content (left column), growth rate (mid column) and cell volume (right column) over time of induced (open circles) and non-induced (dots) cells in repeated batch experiments. Points represents the mean of triplicates, and error bars the standard deviation. Each row represents the species present in the legend of that row. Note the different scales on y-axis.

Table 3: The relative difference between induced and non-induced cells (%), averaged over the time course of each repeated batch experiment, in silica content, growth rate, and cell volume. Positive differences reflect increase in induced cells, and vice versa. Differences are presented as means ± SD (number of samples). $*p < 0.05$; $**p < 0.01$ and $***p < 0.001$.

Species	△Silica (μm^{-3})	△Growth (d^{-1})	△Volume (μm^3)
T. weissflogii (F)	4.9 ± 5.3 * (20)	-13.3 ± 12.2 * (20)	0.1 ± 2.0 (20)
A. paludosa	6.8 ± 13.8 (14)	1.4 ± 7.0 (14)	-0.5 ± 4.4 (14)
D. brightwellii (F)	19.3 ± 28.1 (16)	-34.3 ± 33.8 ** (16)	-0.1 ± 3.6 * (16)
D. brightwellii	15.8 ± 10.0 ** (14)	0.9 ± 20.4 (14)	-3.9 ± 2.7 ** (14)
C. cryptica	24.7 ± 9.6 ** (12)	0.5 ± 4.3 (12)	-6.1 ± 2.5 *** (12)
C. meneghiniana	34.8 ± 9.4 *** (12)	-9.4 ± 10.0 * (12)	-3.1 ± 1.3 *** (12)

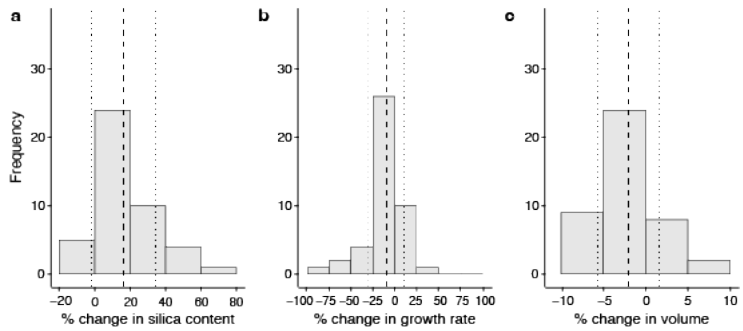

Figure 4: Frequency distribution of percent change in silica content (a), growth rate (b) and cell volume (c) of all species in the repeated batch experiments. The dashed line represents the overall mean and the dotted lines its standard deviation. Mean percent change in silica content, growth rate and cell volume are 16.3 ± 18.2, -10.1 ± 21.5 and -2.1 ± 3.6, respectively.

Continuous culture experiments

Two species, *T. weissflogii* and *A. paludosa*, were tested in a continuous culture set-up with silica as the limiting resource (Fig. 5). Only *T. weissflogii* showed a significant response to the presence of copepodamides (Table 4). As hypothesised, induced cells had a significantly higher biogenic silica content than non-induced cells and showed a significant negative growth rate response (Table 4). In a chemostat set-up, where the growth rate is prescribed, a negative growth response will materialise as a decrease in cell concentration and an increase in the concentration of the limiting nutrient (Si). Both of these properties differed significantly in induced vs. non-induced cells in a way consistent with a negative growth rate response of *T. weissflogii* cells (Table 4). A reduction in the maximum growth rate of induced compared to non-induced cells of about 20 % accounts for the observed change in both steady-state cell and silica concentrations (cf. equation 1). Cell volumes decreased slightly in induced cells of both species, but none of them significantly (Table 4).

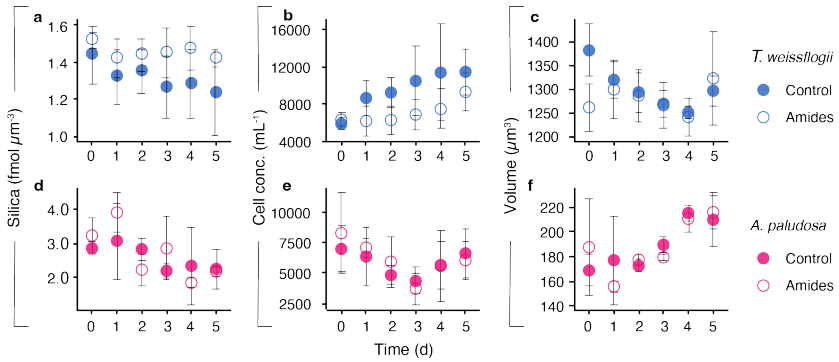

Figure 5: Silica (left column), growth rate (mid column) and cell volume (right column) over time of induced (open circles) and non-induced (dots) cells. Each row represents the species present in the legend of that row.

Table 4: Average percentage difference between control and induced treatments in continuous culture experiments. Differences is based on control treatment, i.e. positive or negative differences means an increase or decrease in induced cells, respectively. Differences are presented as means ± SD (number of samples). $*p < 0.05$; $**p < 0.01$ and $***p < 0.001$.

Species	△Biogenic Silica (μm^{-3})	△Cell conc. (d^{-1})	△Volume (μm^3)	△Biogenic Silica (μm^{-3})
T. weissflogii	9.6 ± 4.4 ** (12)	-23.4 ± 16.3 ** (12)	-9.0 ± 27.1 (12)	37.5 ± 23.9 * (12)
A. paludosa	4.1 ± 22.6 (12)	5.2 ± 15.0 (12)	4.0 ± 17.3 (12)	-29.5 ± 10.1 (12)

6.4 Discussion

It has long been suggested but only recently experimentally demonstrated that the silica shell in diatoms provides partial protection against copepod grazing and that the protective value increases with the silica content of the shell (Liu et al., 2016; Pančić et al., 2019). Here, we have demonstrated for seven species of diatoms and using three different approaches, that diatoms adjust this defence in response to copepod cues by thickening their shell. Although the response is quite variable, between both species and approaches, the average response is positive for all species, and significant for most (Tables 2-4 and Table S1). Even silica-limited cells of one of the two species examined in the chemostat experiment increased their silica content in response to grazer cues. Many other defence mechanisms are intensified in plankton in response to the presence of predator cues. These include zooplankton that modify their morphology and vertical migration patterns (De Meester et al., 1999; Lass and Spaak, 2003) and phytoplankton that intensify toxin production (Selander et al., 2006), modify colony and chain formation (Bergkvist et al., 2012; Tang, 2003; Tang et al., 2008; van Donk et al., 2011), or change their motile behaviour (Selander et al., 2011).

The response found here, up to 35 % increase in silica content in response to grazer cues, is somewhat less than the response reported for the only species examined previously, *T. weissflogii* (Pondaven et al., 2007). *Thalassiosira weissflogii* cells doubled their silica content when growing cells were exposed to growth media that had been conditioned by feeding *Calanus finmarchicus* at a concentration of 40 adults L^{-1}. This concentration corresponds to a grazer biomass at least 5 times higher than that in the copepod cultures that delivered the filtrate in our experiments. This is consistent with our further demonstration that shell thickening increases with the concentration of the copepod cue.

The observations that shell thickening can be induced and that the induction is proportional to the concentration of copepod cue both suggest that shell thickening comes with a cost: defence theory predicts that defences should be inducible when the risk is variable and the defence is costly (Tollrian and Harvell, 1999). Defence costs are often cryptic and difficult to quantify in phytoplankton (Pančić and Kiørboe, 2018). However, we have here demonstrated that all the species examined indeed reduce their growth rate and/or their cell volume in response to copepod cues, although the response is quite variable.

Costs and benefits of shell thickening

Below we discuss the benefits and the costs of shell thickening.

Benefits

There is now clear experimental evidence that heavily silicified cells are grazed less by small, mm-sized copepods than lightly silicified cells, and the magnitude of the grazing reduction with silicification is consistent between the two available studies (Liu et al., 2016; Pančić et al., 2019). Pančić et al. (2019) found that copepod grazing mortality (δ) varied inversely as a power function of biogenic silica content, roughly as $\delta \sim \mathrm{Si}^{-0.79}$, with different species of diatoms following the same relation. Thus, the fractional reduction in grazing mortality ($\triangle\ \delta$) varies with the fractional increase in silica content ($\triangle$S) approximately according to

$$\Delta\delta = 1 - (1 + \triangle\delta)^{-0.79} \tag{1}$$

The fractional increase in silica content of induced relative to non-induced cells at a nominal concentration of copepod amides of 10 nM varied between 3-35 % with a mean of 16 % (from the repeated batch experiment, Fig. 4) corresponding to a fractional decrease in grazing mortality between 2-21 % (average 11 %) (from equation 2). This reduction in grazing mortality of induced vs. non-induced cells varies in proportion to the concentration of signal molecules and, presumably, therefore in proportion to the concentration of copepod grazers. That is, the higher the predation risk, the better protected the cells become.

It is not trivial, however, to translate our nominal concentrations of copepod signals to actual bulk concentrations of copepods and copepodamides in the ocean. The latter, however, are substantially lower than our nominal values. Thus, Selander et al. (2019) observed the actual bulk concentration of copepodamides off the Swedish west coast to vary between 40 fM – 2 pM over the course of a year, largely in proportion to the density of copepods. In our study, copepodamides were introduced by coating the culture flasks, which yields an effective concentration in solution about 10 times lower, and this concentration subsequently declines exponentially and rapidly. Selander et al. (2019) found the effective concentration to be just 1 % of the nominal after 12 hours and 0.1 % after 24 h, but depending on the temperature and on the concentration and type of bacteria in suspension, this may be more or less. Thus, our experimental concentrations are much less the nominal concentration but possibly in the high end of the natural range. The experiments using copepod culture filtrate may be more in line with natural concentrations of signal molecules. The CF/C filter removes most of the cue and the cue concentration decreases rapidly over the course of the 24 h induction periods.

Grazing by other zooplankton than copepods may also be impaired by elevated silica content. Similar to copepods, krill are equipped with siliceous mmouthparts that together with powerful mandibles (copepods) or gizzards (krill) appear adapted to break diatom shells (Hamm et al., 2003). However, thicker shells are less crack-able, and even krill may be unable to digest the particularly thick-shelled diatoms, such as *Fragilariopsis kerguelensis*, that dominate large regions of the southern Ocean (Assmy et al., 2013). Also, the siliceous mouthparts of grazers might wear down, like is known for terrestrial grass-feeders. Protistan grazers, particularly dinoflagellates, appear to feed on diatoms independent of their silica content (Pančić et al., 2019) although some species of dinoflagellates and ciliates may prefer diatoms with low silica content (Zhang et al., 2017).

Costs

Induced cells grow slower than non-induced cells, on average by ~ 10 % (repeated batch experiment), and this may represent the physiological costs of increased silicification. The growth penalty may have two components, i.e., the metabolic cost of assimilating and depositing silica that allocates resources away from growth, and/or a necessary prolongation of the cell cycle to allow time for accumulating additional silica. Allocation costs are small: the synthesis cost of biogenic silica is low, about 2 % of the energy content of the cell (Pančić and Kiørboe, 2018; Raven, 1983), and a 16 % increase in the contents of biogenic silica would thus amount to a 16 % of 2 % = 0.3 % decrease in growth rate.

It is well established that diatoms limited by resources other than silica achieve a higher silica content, simply because a reduced growth rate allows more time to deposit biogenic silica (Martin-Jézéquel et al., 2000). The same allocation pattern is common in terrestrial plants that invest surplus carbon in carbon-based defence molecules when growth is limited by other nutrients, commonly referred to as the carbon nutrient balance model (Bryant et al., 1983). Data compiled for a number of diatom species grown in chemostats under limiting conditions (other than silica) all show the same inverse relation between growth rate (μ) and silica content (S) (Pančić et al., 2019):

$$S \sim \mu^{-0.54} \tag{2}$$

This 'passive' physiological response implies that

$$\Delta S = 1 - (1 + \Delta\mu)^{-0.54} \tag{3}$$

thus, an average fractional decrease in growth rate ($\triangle\mu$) of 10 % would yield an average increase in silica content of just 5 %, less the observed 16 % average. Conversely, a 16 % increase in silica content would 'require' a 31 % decrease in growth rate if the

response was just 'passive'. The observed growth penalty is much less, suggesting that the response to copepod cues requires some additional adaptations.

One such adaptation is a decrease in cell volume. We observed in almost all treatments that cells shrink in response to grazer cues, on average by 3.2 % in volume (mean of all experiments). A decrease in cell size is one way of increasing the volume-specific silica contents of the cells. However, a 3.2 % decrease in cell volume is not accounting for the higher than expected silica content in induced cell. Thus, some further adaptation in silica kinetics is necessary to account for the elevated silica content of induced cells. The fact that even silica-limited cells increase their silica content is further evidence for additional modifications of the silica kinematics in response to grazer cues.

There may be further costs to silicification than what can be observed in laboratory experiments. Heavier silicification may imply faster settling velocity and lower net growth rates in surface water (Raven and Waite, 2004); or, alternatively, increase the cost for buoyancy control (Gemmell et al., 2016). Smaller cells suffer higher mortality rate due to other (smaller) grazers (Kiørboe, 2008). In addition, the obligate need for silica makes diatoms competitive inferior to most other phytoplankton whenever silica becomes limiting before other inorganic nutrients, which is typically the case in regions where diatoms dominate (Pančić and Kiørboe, 2018). Finally, heavier silicification of individual cells implies that a diatom bloom becomes silica limited earlier and at a lower cell concentration with consequently fewer resting stages formed at the end of a bloom. This represents a hidden cost of silicification. However, this cost is 'public' and shared among all individuals in the population and thus not favoured by natural selection unless the diatom bloom is monoclonal (i.e., all cells stem from the same mother cell and are genetically identical siblings). Blooms of diatoms and other phytoplankton are, however, typically not monoclonal but consist of many genotypes (Godhe et al., 2016; Tammilehto et al., 2017).

Trade-offs

Are the costs of increased silicification warranted by the benefits? That is, does silicification increase the fitness of the cells? The currency of fitness are specific mortality and growth rates, and a change in either of the two will lead to a change in fitness. Here, we have provided rough estimates of both. Commonly used proxies of fitness are the difference between growth and mortality (Abrams, 1990), or the ratio between the two (Gillam's rule) (Gilliam and Fraser, 1987). In either case, the fractional decrease in mortality rate has to exceed the fractional decrease in growth rate for silicification to be overall beneficial. On average, this appears to be the case for the scenarios examined here but the two changes are of similar magnitude; 10 % and 11 % respectively, at a nominal cue concentration of 10 nM. Obviously, predation mortality is expected to increase in proportion to the concentration of grazers, but so

does cue concentration and shell thickening. The resulting trade-off thus appears to be near 'neutral' and the fitness landscape therefore almost flat (Ostling, 2012; Purves and Turnbull, 2010). The flat fitness landscape may explain why the responses to copepod cues are very variable, both between and within species, and highly depending on the exact experimental conditions. Such "equalizing trade-offs" also allows for prolonged co-existence of many species (Ehrlich et al., 2017; Ehrlich and Gaedke, 2018; Våge et al., 2013). Diatoms are a particularly diverse group of photosynthetic protists in the ocean (Malviya et al., 2016) and a flat fitness landscape may help account for this high diversity of coexisting species.

6.5 Conclusions

The role of silicon in diatoms resembles its role in many terrestrial plants. It serves multiple purposes, both as a cheap structural element and as a defence agent (Raven, 1983; Strömberg et al., 2016), and plays many other possible roles, both in plants (Frew et al., 2018) and in diatoms (Mitchell et al., 2013; Romann et al., 2015). In terrestrial plants, silicification is inducible by grazers and highly variable, both within and between species (Hartley and DeGabriel, 2016; Strömberg et al., 2016), just as shown here for diatoms. While the cost of elevated silicification in plants in response to grazers remains unknown, we have here demonstrated a growth penalty in diatoms to heavier silicification. It is exactly such organismal trade-offs that together with environmental constraints govern the composition and function of biological communities (Chesson, 2000; Tilman, 1990; Vincent et al., 2012), including the pivotal role of diatoms in the global carbon budget (Tréguer et al., 2018).

Diatoms account for a large fraction of the biological carbon pump in the ocean (Smetacek, 1999). The defensive role of silicon in diatoms has implications to ocean biogeochemistry, not only for the fate of silicon, but also for the ability of diatoms to sequester carbon in the ocean (Assmy et al., 2013; Tréguer et al., 2018). Thus, highly defended diatoms may directly avoid grazing, while less defended thin-shelled species may escape predation by forming rapidly sinking aggregates that transport organic carbon to the ocean interior and constitute an important component of the biological pump. The silica content of like-sized diatoms varies by an order of magnitude (Pančić and Kiørboe, 2018) and unravelling the mechanisms that govern this variation is important for predicting the structure and function of phytoplankton communities, and the role of diatoms in the biological pump (Tréguer et al., 2018). Evolutionary adaptations and – as demonstrated here – plastic responses to grazers are two important factors governing the silica content of diatoms.

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Data availability statement

Data available from the Dryad Digital Repository:
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CHAPTER 7

Paper II

Thicker shells reduce copepod grazing on diatoms

Josephine Grønning*, Fredrik Ryderheim* & Thomas Kiørboe

Centre for Ocean Life, DTU Aqua, Technical University of Denmark, Kgs. Lyngby, Denmark

*Josephine Grønning and Fredrik Ryderheim contributed equally to this work.

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Abstract

The diatom frustule provides partial protection against copepod grazing. Whether the defense is due to the cells being de-selected or handled for so long that the grazers lose time for foraging is unknown. The mechanism has implications for the population dynamics of both defended and co-occurring, undefended nutrient competitors. We use video-observations to demonstrate that thick-shelled diatoms captured by the copepod *Temora longicornis* were rejected more frequently than thin-shelled diatoms, irrespective of cell size. The thick-shelled cells of the larger diatoms were handled for much longer, and the time spent handling these limits the consumption of phytoplankton. This may be why many diatoms, even in the presence of dense grazer populations, reach bloom concentrations, and thus, facilitate aggregation and mass sedimentation. This has implications for both carbon sequestration and for securing a large population of cells at depth ready to colonize the pelagic, when growth conditions again become favorable.

7.1 Introduction

Diatoms account for about 40 % of global ocean primary production (Falkowski et al. 1998), they are a significant source for the pelagic grazing food chain that supports higher trophic levels (Sommer et al. 2002), and they are a quantitatively important component of the biological carbon pump that sequesters carbon (Smetacek 1999; Benoiston et al. 2017). Their characteristic silica shell has many hypothesized functions that may govern both the production and the fate of diatoms and, hence, their important functions in marine ecosystems. For example, the siliceous shell may increase nutrient uptake (Mitchell et al. 2013), facilitate light harvesting (Romann et al. 2015), protect against UV radiation (Aguirre et al. 2018), and provide grazer defense (Smetacek 2001; Hamm et al. 2003). The latter role has been suggested based on the unparalleled strength of the diatom shell (Hamm et al. 2003; Aitken et al. 2016) and has recently been demonstrated experimentally: the grazing mortality due to copepods generally varies inversely with the silica content of the cell across and within diatom species (Liu et al. 2016; Pančić et al. 2019). Diatoms may further thicken their shell in the presence of grazer cues, thus harnessing the defense when needed albeit at the cost of a reduced cell division rate (Pondaven et al. 2007; Grønning and Kiørboe 2020). These properties of diatoms and their shell are key to their evolutionary success and prominent role in the global ocean (Benoiston et al. 2017).

Krill and copepods, probably the most important grazers on diatoms, have silicified 'teeth' that should be well suited to crack the diatom shell if not too thick and strong (Michels et al. 2012). Reduced grazing on thick-shelled cells has been recorded in black box incubation experiments (Liu et al. 2016; Pančić et al. 2019), but whether the reduction is due to the cells becoming un-breakable, are de-selected, or are handled for so long that the grazers lose time for foraging, is unknown. The defense mechanism has implications for the population dynamics, both for the defended diatoms and for their nutrient competitors and, hence, the structure of phytoplankton communities and the fate of primary production. For example, de-selection reduces grazing pressure on defended cells, while longer handling may decrease ingestion also of other cells. In this study, we open the black box through direct observations of individual copepod-diatom interactions to reveal the mechanism of defense. We demonstrate that cells with thicker shells are generally handled for a longer time and rejected with a greater likelihood than thin-shelled cells, accounting for the lower grazing on heavily armored diatoms.

7.2 Materials and methods

Phytoplankton cultures

The diatoms *Cyclotella cryptica* (CCMP336, NCMA Bigelow Laboratory, Maine, USA), *Ditylum brightwellii*, and *Thalassiosira weissflogii* (both unknown strains, DTU Aqua, Denmark) were grown in B1 medium with silica ($\sim$ 500 µM) at salinity 30 and temperature 16 °C. To acquire cells with different silica contents, we grew cultures in high light (HL, 120 µmol photons m^{-2} s^{-1}) or low light (LL, 15 µmol photons m^{-2} s^{-1}) at either 12:12 or 24:0 light:dark cycles because slower growing, light-limited cells grow thicker shells (Liu et al. 2016; Pančić et al. 2019). The cultures were kept in exponential growth phase by dilution with fresh media every few days.

Cell sizes and densities were monitored with a Multisizer 4e Coulter Counter (Beckman Coulter, California, USA), and exponential growth rates were calculated from temporal variations in biovolume (µm^3 mL^{-1}). The biogenic silica content of the diatoms was quantified as described in Grønning & Kiørboe (2020). Silica content is expressed per cell volume as the strength of the shell scales approximately with silica per volume, rather than with silica per surface area (Pančić et al. 2019).

Growth rates and silica contents of the diatoms used for experiments varied as expected: cells grown in the low light treatments had consistently lower growth rates and higher silica contents than cultures grown in high light (Table 1). Cell sizes were similar between treatments except for *C. cryptica*, which were smaller in the low light treatment (Table 1).

Copepod foraging behavior

We used the feeding-current feeding copepod *Temora longicornis* as model copepod grazer. These copepods vibrate their feeding appendages to generate a feeding current from which they harvest prey (Kiørboe 2011). Copepods were taken from a continuous culture, where they are maintained on a diet of *Rhodamonas salina*, *T. weissflogii*, *Heterocapsa triquetra*, and *Oxyrrhis marina*.

We filmed the foraging behavior of adult females that were glued to a human hair by their dorsal surface using cyanoacrylate-based super glue. The copepods were acclimated overnight in filtered seawater and in darkness. The following day, the tethered copepod was attached to a micromanipulator and submerged in a 10×10×10 cm^3 aquarium. Prey cells were added (300 cells mL^{-1}) and kept suspended by a slowly rotating magnetic stirrer. Four 3-minute sequences of copepod foraging were immediately recorded at 50 fps using a Phantom V210 High-speed camera (Vision Research, New Jersey, USA). The tethered copepod was then transferred to an aquarium with the alternate prey treatment (HL or LL) and another four sequences

Table 1: Summary of diatom prey used in the foraging and handling time (HT) experiments. Foraging experiments were carried out with multiple cell densities (noted in brackets as cells mL^{-1}). Cc: *C. cryptica*, Tw: *T. weissflogii*, Db: *D. brightwellii*. HL: high light, LL: low light. ESD: equivalent spherical diameter as measured by particle counter. Values for silica are means ± SD (n = 2–3).

Species	Exp. type	Treatment	L:D cycle	ESD (µm)	Growth (d^{-1})	Silica (fmol $µm^{-3}$)
Cc	Foraging (300 mL^{-1}), HT	HL	24:0	11.25	0.71	1.19 ± 0.09
Cc		LL		9.52	0.29	5.35 ± 0.21
Tw	Foraging (300 mL^{-1})	HL	12:12	14.66	0.41	0.62 ± 0.15
Tw		LL		15.12	0.29	0.84 ± 0.04
Tw	HT	HL	24:0	14.98	0.44	0.44 ± 0.10
Tw		LL		14.68	0.17	1.18 ± 0.01
Tw	Foraging (150 mL^{-1})	HL	12:12	13.08	0.29	0.46 ± 0.03
Tw		LL		13.56	0.17	0.66 ± 0.03
Tw	Foraging (1000 mL^{-1})	HL	12:12	13.12	0.45	0.44 ± 0.06
Tw		LL		13.25	0.32	0.71 ± 0.06
Db	Foraging (300 mL^{-1})	HL	12:12	22.99	0.20	0.69 ± 0.01
Db		LL		23.77	0.11	0.86 ± 0.01
Db	HT	HL	24:0	26.97	0.22	0.46 ± 0.01
Db		LL		28.05	0.06	0.74 ± 0.01

were recorded. The camera was equipped with lenses to provide a field of view of 1.3×1.0 mm^2. Collimated light was provided by an infrared lamp shining through the aquarium directly towards the camera. Four (*C. cryptica*) or five (*T. weissflogii*, *D. brightwellii*) copepods were used for each prey species. The videos were analyzed for quantification of the number of cell captures, handling times (only *D. brightwellii* in this specific set-up), and the fraction of captured cells that were rejected by the copepod. In addition, we recorded captures of individual cells at a higher frame rate (1000 fps) and magnification (1.7×1.3 mm^2) to better quantify handling time for ingested prey (see Video S1 for example). These handling time recordings were done either the day after the foraging experiments (*C. cryptica*) using the same copepods, or at a separate time (*D. brightwellii*, *T. weissflogii*) using freshly tethered copepods.

In an additional experiment we tested the effect of food saturation and prey density on the efficiency of the defense. Copepods were glued as described above and fed *T. weissflogii* grown in low light conditions overnight at a concentration of 1000 cells mL^{-1}. The next day we fed the copepods T. weissflogii grown in low- or high light at 150 or 1000 cells mL^{-1} while observing their behavior as described above. All raw data are available in Grønning et al. (2022).

Statistics

To analyze the effect of cellular silica content on the fraction of rejected cells, we fitted a mixed-effects logistic regression to the data using the R package *lme4* (Bates et al. 2015). 'Treatment' (HL and LL grown cells) and 'Sequence' (to examine whether there was a temporal effect) were fixed effects and the individual copepods the random effect. The model was weighted by the amount of captures. The error term has a binomial distribution, and the random copepod intercept was assumed to be normally distributed. The best fitting model according to the Akaike information criterion (AIC) was used and validated by visual inspection of the residual plots. The random effect-variance component was close to zero for *C. cryptica* and *T. weissflogii* data, but was kept in the model to incorporate the dependency of the response variable on the copepods.

7.3 Results

Foraging behavior

The copepods detected and captured cells irrespective of their silica content. However, the fraction of captured cells that were rejected by the copepods was significantly higher for thick-shelled compared to thin-shelled cells in all three diatom species (Fig. 1a–c). In addition, cells of larger species were rejected more frequently than those of smaller species, particularly evident for thick-shelled cells.

Rejection frequency of *T. weissflogii* was similarly higher for the high silica treatment in the two experiments, where copepods were offered food prior to filming (Fig. 1d–e). Overall, cells were more frequently rejected in the high cell-density treatment (Fig. 1d–e), but the increased chance of rejection compared to the low silica treatment was similar (Odds ratios = 3.27 and 3.20 in the 150 and 1000 cells mL^{-1} experiments, respectively). Thus, prey density and saturation had no impact on prey selectivity.

There was generally no temporal effect ('Sequence') on the fraction of rejected cells, but in the high concentration *T. weissflogii* experiments (1000 cells mL^{-1}), the chance of rejection slightly decreased over the course of the four video sequences (mixed effects logistic regression, z = -2.3, $p = 0.02$).

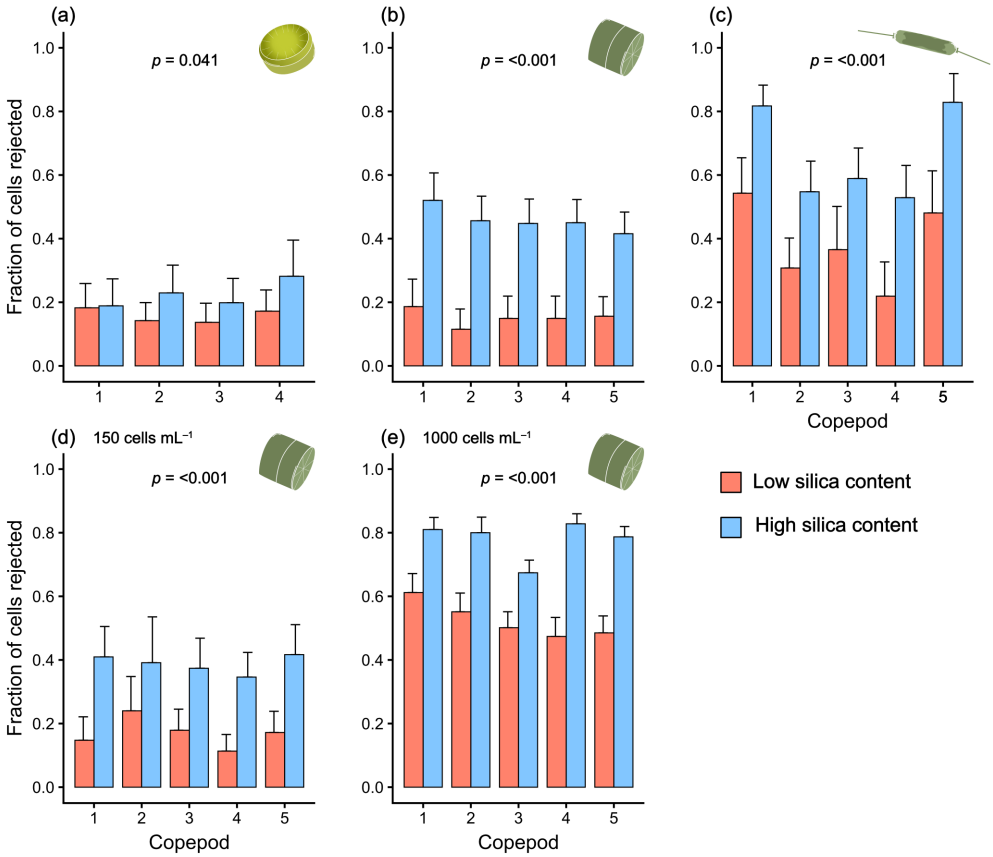

Figure 1: Fraction of caught cells that were rejected by the individual copepod in three diatom species (a) *C. cryptica*, (b, d–e) *T. weissflogii*, and (c) *D. brightwellii*. Data in (a–c) are from the experiments with starved copepods and 300 cells mL^{-1}, while (d) and (e) are the *T. weissflogii* experiments with pre-fed copepods and 150 and 1000 cells mL^{-1}, respectively. Red and blue bars denote diatom cultures with low and high silica content, respectively. P-values indicate significant effect of the treatment on rejection frequency. Odds ratios with 95 % confidence intervals: (a) Cc: 1.51 [1.10, 2.07], (b) Tw: 4.57 [3.57, 5.90], (c) Db: 3.26 [2.39, 4.47], (d) Tw150: 3.27 [2.50, 4.28], (e) Tw1000: 3.20 [2.76, 3.70]. Error bars show 95 % Wilson Score Interval (n = 35–535).

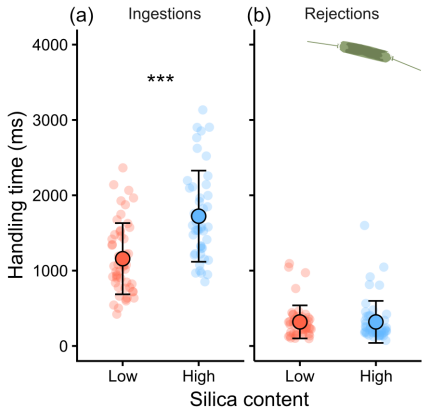

Figure 2: Prey-handling times derived from low-speed (50 fps) videos for (a) ingestions and (b) rejections of *D. brightwellii* with cells of low (red) and high (blue) silica content. The data are compiled from observations of several copepods per treatment. Asterisks indicate significant differences between treatments according to one-way ANOVA (Ingestions: $F_{1,92} = 25.70$, $p < 0.001$; Rejections: $F_{1,98} = 0.001$, $p > 0.05$).

Prey-handling time

Captured cells were rejected or ingested only after being handled – 'examined' – by the feeding appendages. Some rejected cells were actively pushed away by the feeding appendages (Video S2), but the majority were just let go (Video S3). These more passive rejections were distinguishable from the interactions where the cell was lost by the copepod during the capture process.

The handling times of rejected *D. brightwellii* cells were shorter than those of ingested cells and independent of silica content (Fig. 2). In contrast, handling times of ingested cells were significantly longer for thick-shelled than for thin-shelled cells in *D. brightwellii* and *T. weissflogii*, while in the small *C. cryptica*, handling times were short and independent of silica content (Fig. 3). In addition, handling times of ingested cells increase with cell size, particularly evident for thick-shelled cells (Fig. 3).

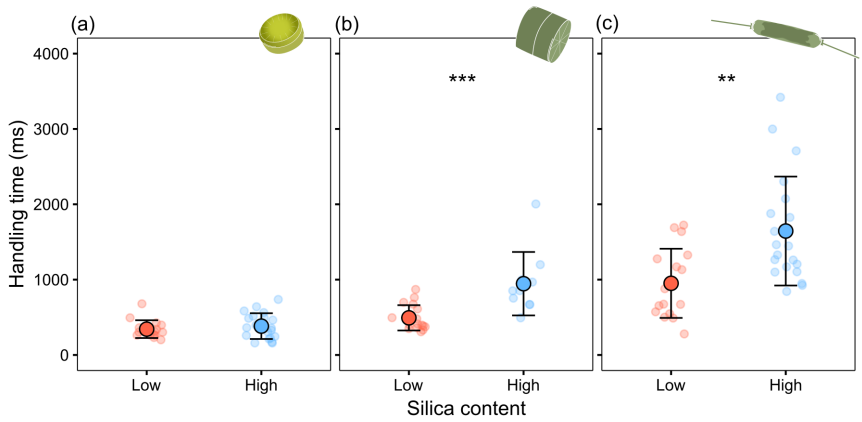

Figure 3: Figure 3. Copepod prey-handling time for ingested prey derived from high-speed videos (1000 fps) of (a) *C. cryptica*, (b) *T. weissflogii*, and (c) *D. brightwellii* for cells with low (red) and high (blue) silica content. Cell sizes increase from panel (a) to (c). The data are compiled from observations of several copepods per species and treatment. Large circles show means ± SD and small circles are the individual data points. Asterisks indicate significant differences between treatments according to one-way ANOVA. Cc: $F_{1,34} = 0.64$, $p = 0.43$; Tw: $F_{1,25} = 15.77$, $p < 0.001$; Db: $F_{1,35} = 11.67$, $p = 0.002$.

7.4 Discussion

Handling and selection of captured diatoms

Feeding-current feeding copepods perceive particles that arrive in the feeding current individually as the particles come near the mechanoreceptory setae on the feeding appendages, which elicits a capture response (Koehl and Strickler 1981; Videos S1–3). Copepods are known to be selective feeders, but prey selection appears to happen post capture while the prey is being handled by the feeding appendages, as demonstrated here for diatoms with different silica content, and elsewhere for dinoflagellates with different degrees of toxicity (Ryderheim et al. 2021) or bioluminescence (Prevett et al. 2019). The feeding appendages are equipped with both mechano- and chemosensors (Hallberg and Skog 2011) and both sensory modalities are probably involved in prey selection. However, it is unclear how the copepod assesses the silica content or strength of the shell of diatoms.

Cells with high silica content are actively rejected by the copepods, particularly the larger cells. Small cells may be swallowed intact, while larger cells may need to be crushed before they can be ingested. The mouth width of the copepod *Oithona davisae* is about 10 µm and it has a prey size spectrum similar to that of *T. longicornis*, despite its smaller size (Saiz et al. 2014; Gonçalves et al. 2014). Thus, the size-threshold for

swallowing/crushing cells before ingestion may be around 10 µm, hence accounting for the difference in the degree of selectivity and handling times between small and large prey in our experiments. This could also, at least in part, explain the recent findings of Xu et al. (2021) who reported that increased silica content in the diatom *Amphora coffeaefornis* had no defensive benefit, but rather the opposite, as copepods had higher ingestion rates on cells with increased silica content. The size of *A. coeffeaefornis* in their experiments was just ∼8 µm ESD (it is also elongated in shape, i.e., the actual width of the cell is <8 µm), and in our experiments we clearly see a decrease in the efficiency of the defense with decreasing cell size. Larger copepods than *T. longicornis* may be able to feed on larger cells without crushing them, but larger copepods are found in areas where phytoplankton cells are also generally larger, i.e., in sub-polar and polar regions (Barton et al. 2013; Brun et al. 2016), and hence our findings may apply more generally.

The prey densities used in our study are within the natural range, e.g., as found during spring blooms in the North Sea (Riebesell 1991). However, prey density did not impact the selectivity of the copepods. While both cells with high and low silica were more frequently rejected in the high cell-density treatment, the increased chance of rejection due to thicker shells remained similar. Thus, while saturated prey conditions decreases the grazing mortality for diatoms that do not invest in the costly defense (Pančić et al. 2019; Grønning and Kiørboe 2020), it also increases the fitness of the ones that do. Hence, it will still be a worthwhile investment should grazing pressure be sufficiently high. The handling times of thick-shelled cells were up to 50 % longer than that of thin-shelled cells and therefore possibly limiting ingestion rates. Thus, estimating the maximum possible number of thick-shelled cells consumed per time as 1/handling time, and estimating cellular carbon contents from cell size (Menden-Deuer and Lessard 2000) yields maximum ingestion rates of 10–15 µg carbon female$_{-1}$ d^{-1} for the three prey species. This is similar to maximum consumption rates measured in incubation experiments for *T. longicornis* females (Franco-Santos et al. 2018). However, this estimate disregards time spent handling cells that are subsequently discarded, and since up to four out of five defended cells are discarded for every cell consumed, this may decrease the maximum possible number of cells consumed, even though discarded cells are handled faster than those consumed. Diatoms in nature typically grow at average light intensities similar to, or less than our low-light treatment and they may further thicken their shells if copepods are present (Grønning and Kiørboe 2020), thus reducing consumption rates. Limitation by other nutrients may also lead to slower growth and consequently thicker shells. Thus, some iron-limited Antarctic regions are dominated by particularly thick-shelled diatoms, such as *Fragilariopsis kerguelensis* that contain 3 times more silica than typical diatoms (Quéguiner et al. 1997; Smetacek 1999). Such thick-shelled, highly defended diatoms decreases the grazing pressure on not only defended diatoms, but also on undefended diatoms as well as on other phytoplankton due to the long handling times and time spent handling rejected cells. The effect is instantaneous but may be exaggerated in

the longer term due to reduced copepod consumption and consequent propagation rates.

The reduction in consumption rate in the presence of defended diatoms applies not only at saturating prey concentrations, but at all prey concentrations, as evident from the Holling type II functional response in consumption rate (I) to prey concentration (C)

$$I = \frac{\beta C}{1 + \beta C \tau} \tag{1}$$

where τ is the prey handling time and β the maximum clearance rate (i.e., the clearance rate at very low prey concentrations). At high prey concentration, the consumption rate is the inverse of the handling time (I = $1/\beta$), and the effect of defense on consumption rate the highest. At lower prey concentrations, the effect decreases, cf. eq. 1.

The evolutionary arms race

Diatoms evolved ∼250 million years ago more or less concurrent with the time that copepods evolved from benthic ancestors and colonized the pelagic. While the diatom frustule has many hypothesized advantages, ranging from viral protection to enhanced light harvesting and nutrient acquisition, the evolution of the frustule may have been partly the result of selection for grazer protection. Krill and copepods, in turn, have adapted to utilize this abundant food source by the evolution of siliceous 'teeth' that allows the grazer to crush diatoms that are not too thick-shelled. The silica-reinforced teeth of copepods may thus have evolved in an 'arms-race' response and may partly explain the dominance of copepods and krill in diatom grazing (Smetacek 2001). The need to crush larger diatom cells before ingestion may increase wear and tear on the copepods' teeth, which eventually may lead to an inability to forage on diatoms (Smetacek 2012; Michels et al. 2012). Both krill and copepods counteract this by frequent molting, where the exoskeleton including the teeth are renewed. Krill molt throughout their life cycle, while copepods stop molting when they reach adulthood. Thus, the ability of copepods, but not krill, to actively de-select heavily armored diatoms and reduce wear and tear may be an important component of this evolutionary arms race. Because late stage copepodites typically dominate the biomass of copepods, the selective feeding of copepods, in particular, may feed back on the evolution of diatom defenses.

Implications to the fate of diatoms

Diatoms play a key role in the biological carbon pump (Smetacek 1999) and the degree of defense by shell-thickening may have implications for the fate of diatoms. Weakly defended diatoms may be consumed by copepods and thus enter the grazing food chain. Copepod fecal pellets sink or are re-mineralized in the upper ocean, depending on their size, ballasting, and consequent sinking speed (Stamieszkin et al. 2015). Some species of diatoms form dense blooms, mainly during vernal temperature stratification in temperate and arctic seas or in other 'new', nutrient rich ephemeral habitats, and such blooms are typically terminated by mass sedimentation rather than by grazing (Assmy et al. 2013). This is often ascribed to the time-lag in the numerical response of copepod grazers in neritic temperate systems, but it also applies to diatom blooms in open water systems where overwintering Calanus spp. ascend en masse to the surface simultaneously with the developing bloom (Dünweber et al. 2010) or where krill are present year round. The limited copepod grazing pressure on phytoplankton due to the presence of defended diatoms may allow these blooms to develop, and the consequently high bloom concentrations may facilitate aggregation and subsequent mass sedimentation. This, in turn, secures a large seed population at depth or in the sediment from which cells may colonize the pelagic when growth conditions again are favorable.

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Data availability statement

The data is available through the Dryad data repository at:
https://doi.org/10.5061/dryad.c59zw3r8z

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CHAPTER 8

Paper III

Grazer-induced aggregation in diatoms

Josephine Grønning & Thomas Kiørboe

Centre for Ocean Life, DTU Aqua, Technical University of Denmark, Kgs. Lyngby, Denmark

This paper has not been submitted to any journal yet.

Abstract

Sinking is part of diatom life history. Typically, by the end of a bloom, diatoms form aggregates that sink rapidly to the bottom. This opportunistic lifestyle can be considered an adaptation to reduce predation losses, because predation pressure in the sediment is less that in the water column. In this study, we demonstrate that some diatoms become sticky and coagulate into aggregates when exposed to chemical copepod cues. Cell stickiness increases proportionally to the concentration of copepod cues and duration of exposure until saturation. Surprisingly, nutrient limitation (Si and N) did not increase stickiness. Furthermore, we demonstrate a chain length reduction of *S. marinoi*, when exposed to copepod cues. We argue that the increase in stickiness is adaptive when grazing mortality exceeds growth rate. This ensures that the maximum number of cells survive in the sediment, ready to utilize the next window of growth opportunity.

8.1 Introduction

Sinking is part of diatom life history. While flourishing in the surface when conditions are good, most diatoms spend the majority of their life in the sediment from where they can colonize the pelagic when conditions again become favorable (Ellegaard and Ribeiro, 2018; McQuoid and Hobson, 1996; Smetacek, 1985). This opportunistic life-style can be considered an adaptation to reduce predation losses, because predation pressure in the sediment is much less that in the water column. Thus, mortality rates of diatoms in the sediments of a coastal temperate area were on the order of 0.01 d-1 (Hansen and Josefson, 2004, 2001), order(s) of magnitude lower than in the pelagic, and cysts with a further thickened silica shell may survive for years (Hansen and Josefson, 2004, 2001; Itakura et al., 1997; McQuoid and Hobson, 1995).

Transport from the upper ocean to the sediment may be facilitated by the formation of aggregates. Typically, by the end of a bloom, diatoms form aggregates that sink rapidly to the bottom. These mass sedimentation events have been recognized as an important part of the succession of diatom blooms, enabling the transition from vegetative cells in the surface to resting stages that can overwinter at depth (McQuoid and Hobson, 1996; Passow and Alldredge, 1995; Riebesell, 1991; Smetacek, 1985). Aggregation and mass sedimentation also makes diatoms an important component of the biological carbon pump (Smetacek, 1999).

Aggregation may happen by physical coagulation (Burd and Jackson, 2009; Jackson, 1990; Kiørboe et al., 1990). Turbulence or other processes cause cells to collide; if 'sticky', the cells may adhere to one another. Two factors may enhance coagulation: (i) Collision frequency increases with the square of cell concentration and thus increases dramatically in the course of a bloom. (ii) Cells may become increasingly sticky when nutrient starved near the end of a bloom and thus further facilitate aggregation and subsequent sinking. The latter is often assumed (Smetacek, 1985; Thornton, 2002), but has in fact only been demonstrated in one species (*Thalassiosira pseudonana*, Kiørboe et al., 1990).

We recently discovered that some diatoms form aggregates in response to diffusible copepod cues, further emphasizing the defensive value of aggregate formation (Fig. 1). Here, we explore this phenomenon by quantifying the stickiness of six species of diatoms in response to copepod cues and for five of the species in both nutrient (Si, N) replete and deplete conditions. We demonstrate that some diatoms dramatically increase their stickiness in response to copepod cues, that the response is proportional to the concentration of cues (until saturation), and that the effect of nutrient limitation is variable but in most cases decrease rather than increase stickiness. Furthermore, we demonstrate a reduction in chain length of *S. marinoi*, when exposed to copepod cues.

Figure 1: Cultures of *Cyclotella cryptica* (10^4 cells mL^{-1}) in 250 mL bottles during repeated batch experiments of Grønning and Kiørboe, 2020. The photo is taken after 5 days of induction (left bottle: control, right bottle; induced with copepodamides to nominal concentration of 10 nM).

8.2 Materials and methods

We used copepodamides extracted from copepods as grazer cues (Selander et al., 2015). We first examined the effect of the duration of exposure to cues on the stickiness of a diatom, then explored the effect of cue concentration on the stickiness of six different species of diatoms, and finally we examined the combined effect of grazer cues and nutrient limitation (N, Si) on the stickiness of five species of diatoms.

Phytoplankton cultures and conditions

We used six species of diatoms: *Amphiprora paludosa* (CCMP125) and *Cyclotella cryptica* (CCMP331) obtained from NCMA at Bigelow Laboratory, Maine. *Phaeodactylum tricornutum*, *Skeletonema marinoi* and *Thalassiosira pseudonana* from Department of Marine Sciences, Gothenburg University, Sweden. *Thalassiosira weissflogii* (unknown strain), from DTU Aqua, Denmark. All cultures, except nutrient-limited cultures, were grown in B1 medium with saturated levels of silica ($\sim$ 500 µM) and prepared with pasteurized 0.2-µm filtered sea water. Cultures were grown at 16 °C in L:D cycles of 16:8 (120 µmol m^{-2} s^{-1}). All cultures were sonicated prior to experiments

to break down aggregates that may have been formed during cell cultivation. Cells used for experiments were in exponential growth, unless otherwise stated.

Estimating stickiness

Stickiness (α) is the probability of two particle sticking together upon collision. To measure stickiness of diatom cells, we followed the method of Kiørboe and Hansen (1993). Cells suspended in a fluid shear field will collide at a predictable rate that can be computed from classical coagulation theory. When two particles collide and combine, there is one particle less in suspension, and during the initial phase of the coagulation process the concentration of particles will decline according to (Kiørboe et al. 1990):

$$C_t = C_0 e^{-(7.824\alpha\phi G/\pi)t} \quad (1)$$

where C_t and C_0 are the particle concentrations at time t and *0*, respectively, ϕ is the volume concentration of suspended diatom cells, and G is the fluid shear rate. We generated a well-defined fluid shear field in a Cuette device, which consists of two cylinders: a fixed inner cylinder and a rotating outer cylinder. When rotating, laminar shear is generated in the gap between the two cylinders and the average fluid shear rate is (van Duuren 1968):

$$G = 4\pi N R_1 R_2 ({R_2}^2 - {R_1}^2)_{-1} \quad (2)$$

where N is the rotational speed (revs s^{-1}) of the outer cylinder, and R^1 and R^2 the radii of the inner and outer cylinder, respectively. Our Cuettes had a volume of 1.35 L and inner and outer radii of 4 cm and 5.75 cm, respectively. We used four parallel Cuettes mounted in a rack and all run by the same motor at a speed of $N = 0.6$ s^{-1}, resulting in a fluid shear rate of $G = 10.45$ s^{-1}.

To estimate the stickiness of the diatoms, we added suspensions with a volume concentration of $\sim$ 10 ppm to the Cuettes, started the rotation, and sampled the suspensions every 15 minutes for 2 hours. When sampling, the individual Cuette was removed from the rack, and a sample of 10 mL was carefully collected with a wide-mouthed pipette (opening $\sim$ 0.5 mm). The sample volume was replaced with diatom suspension and the Cuette was placed back on the rack. The samples were immediately counted on Coulter Counter (Beckman Coulter, Multisizer 3 or Multisizer 4e, USA) with a 100 or 200-µm orifice (depending on the diatom species) after appropriate dilution (max 10 % coincidence). Stickiness (α) $\pm$ SE was estimated from the exponential decline in particle concentration (equation 1).

Because the rack was limited to four Cuette cylinders only, replicates were only possible in the experiment testing development of stickiness over time. In the experi-

ments with varying concentrations and nutrient limitations, 'replicates' were obtained by repeating experiments on two consecutive days: after 3 and 4 days of induction.

Copepodamides

The copepod cues used were copepodamides purified from freeze dried *Calanus* copepods as described in Selander et al. (2015). Briefly, *Calanus* were extracted with methanol and crude extracts were subjected to liquid-liquid partitioning between methanol/water and heptane. Subsequently, the methanol-soluble fraction was fractionated on C18 SPE columns, and the impurities were eluted in 70 % methanol (aq) and copepodamides in 100 % methanol. Finally, the methanol extract was concentrated by rotary evaporation, dissolved in a small volume, and fractionated by reversed phase gradient elution HPLC. The copepodamide content of each fraction was determined through direct infusion mass spectrometry and positive fractions were pooled. The final concentration in this stock solution was determined by mass spectrometry.

When inducing diatoms with grazer cues, empty culture flasks were coated with copepodamides diluted in methanol and the methanol was next evaporated under a stream of nitrogen, leaving only the copepodamides. Diatom cultures were transferred immediately to the coated bottles and cues were released to the cultures during $\sim$ 24 hours. To ensure constant exposure to copepod cues, diatom cultures were transferred to newly coated bottles every day. For control treatments, the same procedure was followed with pure methanol.

Due to a short half-life time and unknown losses, the effective concentration of copepodamides were at least two orders of magnitude less the nominal concentration (Selander et al., 2019). Here, all copepodamide concentrations are nominal concentrations.

Temporal development of stickiness upon exposure to copepod cues

To test the development of stickiness when exposed to copepod cues over time, batch cultures of C. cryptica were induced with 10 nM of copepodamides for five days (starting on day 0), and Cuette-experiments were carried out on days 1-4.

Effects of copepod cue concentration

Diatoms were induced with copepodamide concentrations of either 0, 2, 5 and 10 or 0, 5, 10, and 20 nM and their stickiness was measured on day 3 and 4. One species, *S.*

marinoi, is chain forming and changes the chain length when exposed to copepodamides (Bergkvist et al. 2008). We determined responses in chain length by counting the number of cells per chain at days 3 and 4. Samples were fixed in acidic Lugol's solution (1 %) and a minimum of 400 chains were counted in a Sedgewick-Rafter chamber using an inverted microscope (Olympus, Tokyo, Japan).

Effect of nutrient limitation

Nutrient-limited cultures were either grown as batch cultures (all species) or in continuous cultures (*C. cryptica*). Batch cultures were divided into high- and low-nutrient treatments. For high- and low Si treatments, cells were grown at initial concentration of 500 µM and 93 µM Si, respectively. For high- and low-N, cells were grown in media with N-concentrations of $\sim$ 1200 µM or $\sim$ 80 µM. When the cultures in the low-nutrient treatment reached stationary phase, both high- and low-nutrient cultures were induced with copepodamides (5 nM nominal concentration). To produce replicates, cell stickiness was measured after 3 and 4 days of induction. Changes in chain length of *S. marinoi* were determined as above at days 3 and 4.

Continuous cultures were grown to $\sim$ steady state in chemostats with continuous inflow of B1 medium with modified Si or N concentrations of $\sim$ 93 and $\sim$ 80 µM, respectively. Two different dilution rates (0.25 and 0.80 d^{-1}) were used to achieve nutrient deplete and replete cells. Each treatment was run in duplicates. When reaching $\sim$ steady state, the cultures were induced with either methanol (control) or copepodamides before the stickiness of the cells were measured in Cuette-experiments after three and four days of induction.

Statistical analysis

To analyse the effect on copepodamide concentration on diatom stickiness, we used the Spearman's rank-order correlation. We assumed the stickiness data obtained after three and four days of induction to be replicates. In the nutrient-limited experiments, we used a linear model to test the individual and combined effect of nutrient concentration (Low or High) and presence of copepod cues (with or without) on diatoms stickiness. Day (3 or 4) and nutrient (Si or N) were included as additive effects in the model. Further, the weight was defined as 1/SE. A significance level of 5 % was used throughout and all statistical analyses were performed in *R* (version 1.4.1717).

8.3 Results

Temporal development of stickiness upon exposure to copepod cues

We tested how stickiness of *C. cryptica* changed with time of exposure to copepod cues (Fig. 2). Stickiness of the cells in the control treatment (pure methanol) did not change over time, whereas the stickiness of the copepodamide treated cells increased with duration of induction. From day 1 and 2 of induction to day 4, stickiness tripled from 0.07 to 0.20. Based on these results we used three and four days of incubation in all dose-response and nutrient-limited experiments.

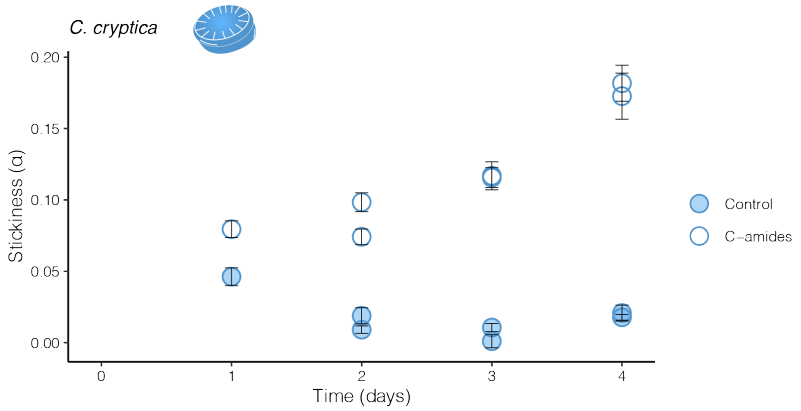

Figure 2: Change in the stickiness of *C. cryptica* during exposure to copepod cues. Open circles are control treatments (pure methanol) and closed circles are copepodamide treatments. Error bars are standard errors.

Effects of copepod cue concentration

In four of the six species examined, *A. paludosa*, *P. tricornutum*, *T. pseudonana* and *T. weissflogii*, the cell stickiness was independent of copepod cues (Fig. 3). Stickiness values were low and ranged between $\sim$ 0.003-0.08.

In two species, *C. cryptica* and *S. marinoi*, stickiness increased with the concentration of copepodamides (Fig. 3a-b). *C. cryptica* stickiness increased from 0.01 to 0.25-0.30 with increasing concentration of copepodamides until 5 nM and remained approximately constant at higher concentrations. For *S. marinoi* the stickiness similarly increased with increasing copepodamide concentration to 0.15, but with no signs of saturation within the range of concentrations tested.

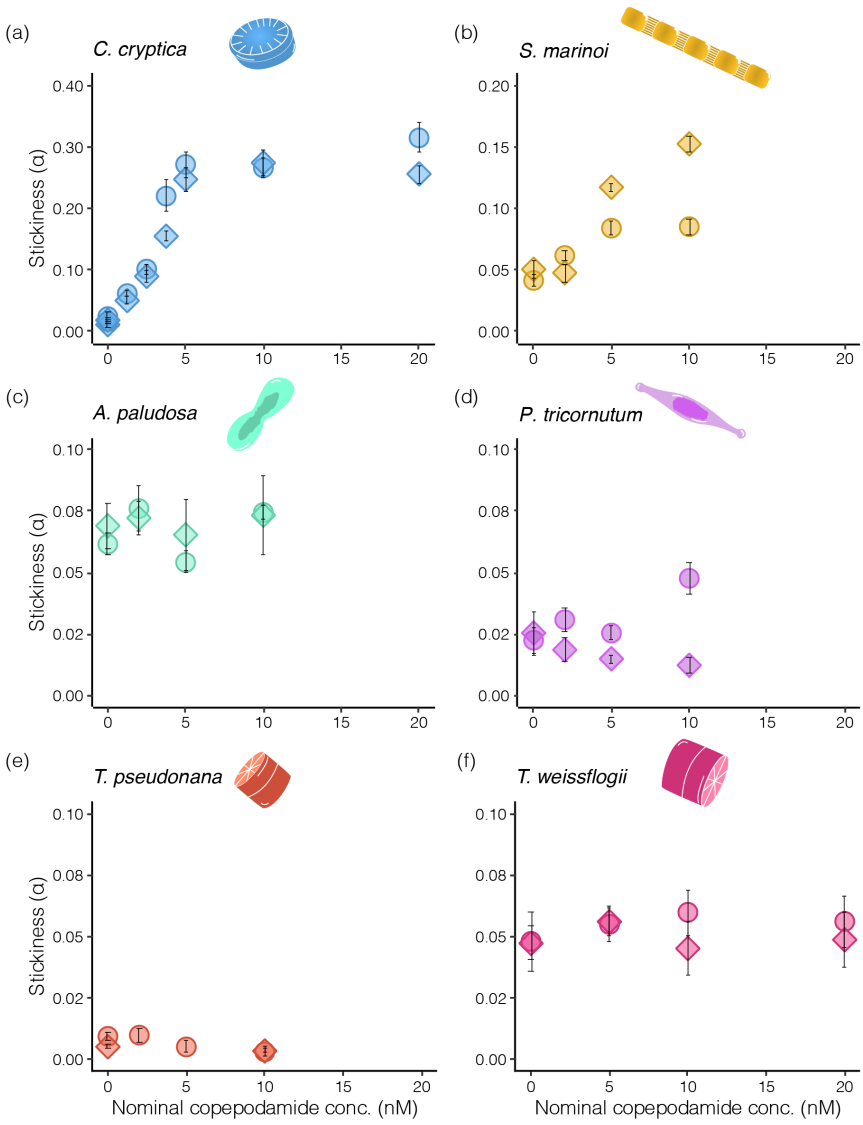

Figure 3: Relationship between stickiness and nominal concentration of copepodamides for six species of diatoms. Circles and diamonds denote stickiness after three and four days of induction, respectively. Spearman's rank-order correlation suggest a significant positive correlation between stickiness and copepodamide concentration in *C. cryptica* and *S. marinoi* ($p = 5.2$ x 10^{-9}, $\rho = 0.96$ and $p = 0.004$, $\rho = 0.88$). For the rest of the species the correlation was not significant ($p > 0.05$). Note the different scales of the y-axes. Error bars are standard errors.

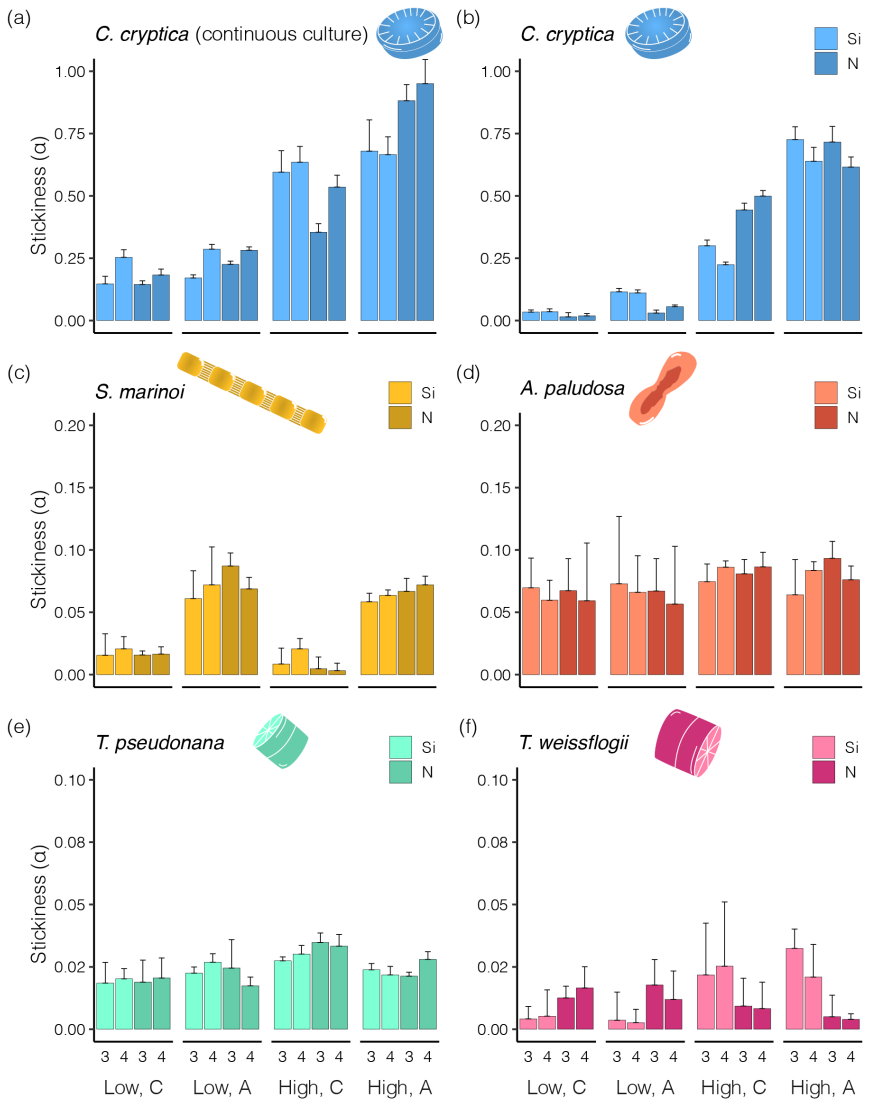

Figure 4: Effect of nutrient limitation and presence of copepod cues on stickiness of five species of diatoms. "Low" and "High" indicate nutrient-deplete and -replete conditions, and "A" and "C" the presence or absence of copepodamides. Number of days of induction (3 or 4) is indicated on the x-axis. Cultures were grown as continuous cultures (a) or as batch cultures (b-f). Note the different scales of y-axes. In the two *C. cryptica* experiments (a-b), stickiness of both continues and batch cultures were significantly affected by the nutrient level ($p = 0.0003$ and $p = 0.0001$, respectively) and the combination of nutrient level and presence/absence of copepodamides ($p = 0.02$ and $p = 0.008$. respectively). In the continuous culture (a), stickiness measured on day four was significantly higher than that measured on day three ($p = 0.04$). Stickiness of *S. marinoi* was significantly higher in cultures with copepodamides ($p = 1.8 \times 10^{-6}$), and stickiness of *A. paludosa* and *T. pseudonana* was significantly higher in nutrient-replete conditions ($p = 0.007$ and $p = 0.003$, respectively). Error bars are standard deviations.

Effect of nutrient limitation

The stickiness of *C. cryptica* was higher in the copepodamide treatments compared to the control in both continuous culture and batch experiments, however this was not statistically significant (Fig. 4a-b). Surprisingly, nutrient replete cells had significantly higher stickiness than nutrient depleted cells, again in both continuous and batch cultures (Fig. 4a-b). Furthermore, the combined effect of predator cue presence and replete nutrient conditions were significantly elevating the stickiness of both continuous and batch cultures. In the continuous culture, the stickiness was significantly higher after four days of induction compared to after three days of induction. However, this was not the case in the batch culture. All stickiness-trends were the same regardless of the type of limiting nutrient.

Because the response patterns were consistent between continuous and batch cultures, the rest of the species were tested only in batch experiments. The responses were rather different between species. The stickiness of *S. marinoi* was not significantly affected by nutrient limitation but significantly by the presence of copepod cues (Fig. 4c). In *A. paludosa* and *T. pseudonana*, there was no effect of copepod cues, as above, but nutrient replete cells were significantly more sticky than nutrient deplete cells (Fig. 4 d-e). Finally, in *T. weissflogii* there were no effect of copepod cues, as above, but Si-replete cells were more sticky than deplete cells, while the opposite tended to be the case for N-replete and deplete cells, although this was not statistically significant (Fig. 4f).

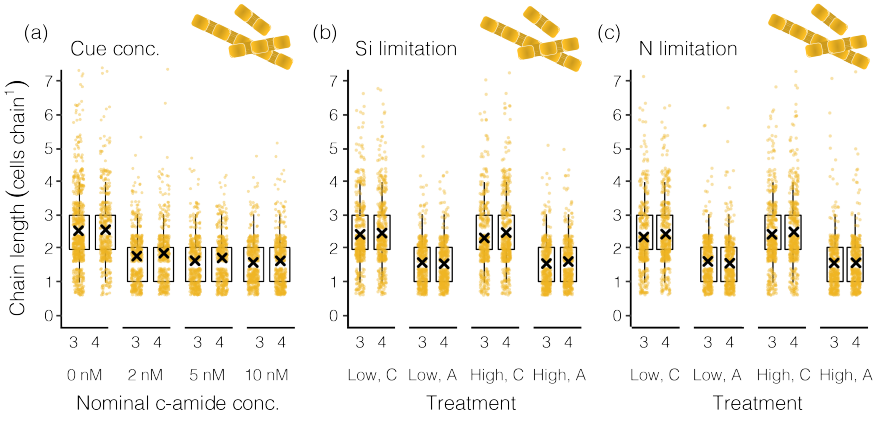

Figure 5: Boxplot showing the effect of copepod cues on chain length of *S. marinoi* after 3 and 4 days of induction (x-axes). (a): Relationship between chain length and nominal copepodamide concentration (0, 2, 5 or 10 nM). (b): Effect of low or high Si-concentration ("Low", "High") and the absence or presence of copepodamides ("C", "A") on chain length. (c): Effect of low or high N-concentration ("Low", "High") and the absence or presence of copepodamides ("C", "A") on chain length. Each point represents a chain, boxes contain the lower and upper quartile ranges, and X indicates the mean chain length. In all experiments, cells of a minimum of 400 chains were counted.

Chain length of *S. marinoi*

The chain length of *S. marinoi* was shortened when induced with copepodamides (Fig. 5), and the effect was the same in all treatments: average chain length in control treatments ranged between 2.3-2.6 cells per chain, and ranged between 1.5-1.9 cells per chain in all induced treatments independent of cue concentration (Fig. 5a). Concentrations of neither Si (Fig. 5b) nor N (Fig. 5c) were affecting chain length in *S. marinoi*.

8.4 Discussion

Diatoms have evolved a suit of defensive mechanisms that reduce their grazing mortality. The silica shell (frustule) is exceptionally strong and hard to crack (Aitken et al., 2016; Hamm et al., 2003; Smetacek, 2001) and the thicker the shell, the higher the chance that a copepod rejects a captured cell and the less is the grazing mortality (Liu et al., 2016; Pančić et al., 2019; Ryderheim and Grønning et al., 2022). Many diatoms may further harness this defence by thickening the shell in response to copepod cues (Grønning and Kiørboe, 2020; Pondaven et al., 2007). Other inducible defence mechanisms include the modification of colony size in some chain-forming diatoms – shorter chains reduce the risk of being consumed by a copepod (Rigby and Selander, 2021; Ryderheim et al., submitted) – and the production of toxins in *Pseudo-nitzschia* species that make copepods deselect toxin-producing cells (Olesen and Ryderheim et al., submitted; Zhang et al., 2021). It has similarly been described that a viral infection induces mass formation of resting spores that may subsequently germinate as healthy cells (Pelusi et al., 2021). Here, we have demonstrated yet another potential defence mechanism, the formation of aggregates in response to copepod cues. While small aggregates, like phytoplankton colonies, may be consumed by copepods at higher rates than solitary cells, aggregates will sink to the ocean interior or the seafloor where predation mortality is low (Hansen and Josefson, 2004, 2001; Ryderheim et al., submitted). Sinking aggregates may be colonized by copepods and other zooplankton that may graze on the aggregated cells (Kiørboe, 2000; Koski et al., 2017), but increased stickiness will speed up aggregation and lead to larger aggregates with high sinking speeds and, hence, short residence time in the water column. In that sense, aggregate formation may be considered an adaptation to reduce predation mortality.

We found elevated stickiness in response to grazer cues in two of six species. The other four species had grazer independent stickiness coefficient of magnitudes consistent with those reported earlier for overlapping species (Drapeau et al., 1994; Kiørboe et al., 1990). There is other evidence that diatoms may aggregate in response to grazers. Toullec et al. (2019) reported that *Skeletonema marinoi*, but not *Chaetoceros neogracili*, form aggregates in the presence of copepods, and ascribed this to the turbulence created by the feeding copepod. Irrespective of the mechanism, aggregation

in response to grazers is thus found in only some diatoms, and maybe not even consistently between strains of the same species, cf. the different finding for *S. marinoi* between Toullec et al. (2019) and the present study. All diatoms, thus, do not have all the described defence mechanisms. In fact, it is only Pseudo-nitzschia species that have the ability to produce grazer deterrent toxins, and not all species thicken their shell to the same extent or reduce colony sizes in response to grazers (Grønning and Kiørboe, 2020; Rigby and Selander, 2021). The ability to harness a particular defence is likely costly and so diatoms may only be able to afford a limited array of defences.

Thus, thickening the shell or producing toxins in diatoms comes with the cost of reduced cell division rate, and reducing chain-length in colony forming diatoms make the cells susceptible to microzooplankton grazers (Grønning and Kiørboe, 2020; Olesen and Ryderheim et al., submitted; Ryderheim et al., submitted). In the green alga *Scenedesmus subspicatus* flocculation has been demonstrated to be energy demanding (Roccuzzo et al., 2020). Aggregate formation in diatoms comes with the additional obvious cost that the cells loose growth opportunities in the sunlit surface layer when combining into rapidly sinking aggregates. One relevant fitness parameter for a diatom cell, however, is the number of daughter cells it can contribute to the overwintering seed population in the sediment or in the deep ocean. Thus, it may be beneficial for cells to become sticky when the grazing mortality exceeds the cell division rate, as this will maximize the number of overwintering cells at depth. This prediction is consistent with the fact that stickiness depends on the concentration of grazer cues and, hence, the predation risk.

A similar argument might predict that cells should become sticky when nutrients become limiting. Many diatoms increase the production of carbon-rich exopolymeric material when nutrient limited (Thornton, 2002), and exopolymeric material that coalesce into transparent exopolymeric particles (TEP) may enhance coagulation and subsequent aggregate formation and sinking (Alldredge and Passow, 1993; Gärdes et al., 2011). However, to our knowledge, increased stickiness of individual cells with increased nutrient depletion has been demonstrated in only one species, Thalassiosira pseudonana (Kiørboe et al., 1990), and that finding could not be reproduced here (Fig 4). In fact, we rather found the opposite pattern in several species: nutrient replete cells are more – not less – sticky than nutrient depleted cells. While this may be surprising, it is consistent with the development of phytoplankton stickiness observed during diatom blooms in both the sea (Kiørboe et al., 1994) and in mesocosm experiments (Dam and Drapeau, 1995). This may appear inconsistent with the fact that diatom blooms often terminate by mass sedimentation. However, collision rate between suspended cells increases with the square of cell concentration and thus increases rapidly during an exponentially developing bloom. One may speculate that the declining stickiness during bloom development is an adaptation to prevent premature aggregation and sinking before growth conditions have been fully utilized.

In addition to increase its stickiness in response to copepod cues, we also found the chain forming *Skeletonema marinoi* to reduce its chain length. These observations

are consistent with previous studies (Bergkvist et al., 2012; Toullec et al., 2019). Both of these two responses to copepod cues may be adaptation to reduce decrease grazing mortality, however they act in opposite ways: breaking up chains reduce the size, whereas aggregation increase the size. We argue that the defensive value of these two responses depend on the cell density: Although cells become more sticky, aggregation rate is low when cell density is low and breaking up chains is thus an effective way of reducing grazing mortality. Contrary, when cell density is high, stickiness becomes of higher importance and aggregation and sinking is a way to escape grazing. Therefore, despite having opposite effects on particle size, chain length reduction and aggregation may both act as defences but in different phases of a bloom.

Copepods are maybe the quantitatively most important grazers of pelagic diatoms in the ocean. Copepods colonized the pelagic at about the same time (2-400 million years ago) as diatoms evolved ($\sim$ 250 million years ago) (Behrenfeld et al., 2021; Bradford-Grieve, 2002) and copepods have evolved counter-measures to partly overcome the various diatom defences in a co-evolutionary arms race (Smetacek, 2001). Thus copepods have silicious 'teeth' suitable to crack diatom shells, many – but not all - copepods have adapted to consume toxic *Pseudo-nitzschia* species without any harm to themselves (Cook et al., 2022), and other copepods have specialized on colonizing and feeding on (diatom) aggregates (Koski et al., 2021). The evolution of costly defence mechanisms and the subsequent arms race between predators and prey in a 'red queen' dynamics promotes species diversity (Nair et al., 2019) and thus likely one reason that diatoms are one of the most diverse phytoplankton groups in the ocean.

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CHAPTER A

Appendix

Supplementary information

Paper I

Table 1: Statistics of linear regressions of dose-response experiments.

Species	Parameter	p-value	Slope	Intercept	Adjusted R^2
T. weissflogii	Silica	5.6 ± 10^{-1}	2.5 ± 10^{-3}	9.3 ± 10^{-1}	-0.06
	Growth (24h)	1.1 ± 10^{-2}	-6.8 ± 10^{-3}	5.5 ± 10^{-1}	0.44
	Growth (48h)	5.6 ± 10^{-2}	-7.1 ± 10^{-3}	6.4 ± 10^{-1}	0.25
	Volume (24h)	6.6 ± 10^{-1}	6.2 ± 10^{-1}	1.4 ± 10^{3}	-0.08
	Volume (48h)	3.8 ± 10^{-1}	-2.9	1.3 ± 10^{3}	-0.01
A. paludosa	Silica	1.4 ± 10^{-4}	1.1 ± 10^{-1}	4.2	0.76
	Growth (24h)	4.7 ± 10^{-2}	-3.1 ± 10^{-2}	3.6 ± 10^{-1}	0.27
	Growth (48h)	5.1 ± 10^{-2}	3.8 ± 10^{-2}	8.3 ± 10^{-1}	0.26
	Volume (24h)	4.7 ± 10^{-2}	-6.7 ± 10^{-1}	2.0 ± 10^{2}	0.27
	Volume (48h)	2.3 ± 10^{-2}	-2.2	1.9 ± 10^{2}	0.36
D. brightwellii	Silica	4.8 ± 10^{-4}	5.5 ± 10^{-2}	2.4	0.69
	Growth (24h)	4.9 ± 10^{-1}	2.8 ± 10^{-3}	2.8 ± 10^{-1}	-0.05
	Growth (48h)	7.9 ± 10^{-1}	9.6 ± 10^{-4}	3.1 ± 10^{-1}	-0.09
	Volume (24h)	5.8 ± 10^{-1}	-9.3	6.0 ± 10^{3}	-0.07
	Volume (48h)	6.0 ± 10^{-5}	-2.2 ± 10^{1}	6.6 ± 10^{3}	0.80
N. laevis	Silica	1.8 ± 10^{-4}	9.5 ± 10^{-2}	2.7	0.77
	Growth (24h)	4.1 ± 10^{-1}	-1.4 ± 10^{-2}	-2.1 ± 10^{-1}	-0.03
	Growth (48h)	2.4 ± 10^{-1}	-1.7 ± 10^{-2}	5.3 ± 10^{-1}	0.05
	Volume (24h)	3.5 ± 10^{-1}	-2.0	2.2 ± 10^{2}	-0.00

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Table 1 – continued from previous page

Species	Parameter	p-value	Slope	Intercept	Adjusted R^2
	Volume (48h)	7.7 ± 10^{-1}	6.8 ± 10^{-1}	2.4 ± 10^{2}	-0.09
N. incerta	Silica	2.6 ± 10^{-2}	1.9 ± 10^{-2}	2.0	0.35
	Growth (24h)	9.6 ± 10^{-3}	-3.3 ± 10^{-2}	3.7 ± 10^{-1}	0.46
	Growth (48h)	7.1 ± 10^{-1}	-4.1 ± 10^{-3}	7.3 ± 10^{-1}	-0.08
	Volume (24h)	5.5 ± 10^{-1}	-2.0 ± 10^{-1}	2.1 ± 10^{2}	-0.06
	Volume (48h)	2.5 ± 10^{-3}	2.2	2.0 ± 10^{-2}	0.58
C. cryptica	Silica	3.3 ± 10^{-2}	3.1 ± 10^{-2}	1.8	0.32
	Growth (24h)	5.6 ± 10^{-1}	-3.4 ± 10^{-3}	8.6 ± 10^{-1}	-0.06
	Growth (48h)	6.7 ± 10^{-2}	1.0 ± 10^{-2}	7.5 ± 10^{-1}	0.23
	Volume (24h)	6.7 ± 10^{-2}	-8.5	5.9 ± 10^{2}	0.23
	Volume (48h)	4.9 ± 10^{-2}	-5.7	5.9 ± 10^{2}	0.27
C. meneghiniana	Silica	1.3 ± 10^{-2}	1.4 ± 10^{-2}	9.1 ± 10^{-1}	0.43
	Growth (24h)	5.6 ± 10^{-1}	-1.7 ± 10^{-3}	7.9 ± 10^{-1}	-0.06
	Growth (48h)	2.3 ± 10^{-1}	-3.9 ± 10^{-3}	9.9 ± 10^{-1}	0.05
	Volume (24h)	4.2 ± 10^{-1}	-1.6	7.6 ± 10^{2}	-0.03
	Volume (48h)	1.9 ± 10^{-2}	-5.7	7.7 ± 10^{2}	0.38

In this project, we significantly improved the knowledge on defence mechanisms in marine diatoms. We examined the mechanisms, trade-offs and inducibility of silicification of the diatom shell, and inducibility of aggregation and its potential as a defence. We experimentally demonstrated that different species of diatoms increase their shell-thickness when exposed to chemical cues from copepods, which are the main grazers of diatoms. This is traded off against reduced growth and cell size of the diatoms, however the response is variable. Through direct video observations of predator-prey interactions, we demonstrated how increased shell thickness is indeed an effective defence against copepod grazing. This was manifested as a two-fold benefit: cells have an increased chance of being rejected by copepods, but also takes longer to ingest and thereby reducing the actual grazing time of the copepod. We further explored the potential of aggregation as a defence mechanism in diatoms. We demonstrated how some species react to copepod cues by increasing the stickiness of the cell and thus form aggregates that can sink and potentially escape predation.

Cover illustration: Shells of different species of diatoms collected, cleaned, and arranged under the microscope by British biologist Klaus Kemp.

Technical
University of
Denmark

DTU Aqua
Kemitorvet
DK-2800 Kgs. Lyngby

www.aqua.dtu.dk