Why do coccolithophores calcify? Does the calcium carbonate shell serve as protection against viral infection and predation?

Dissertation

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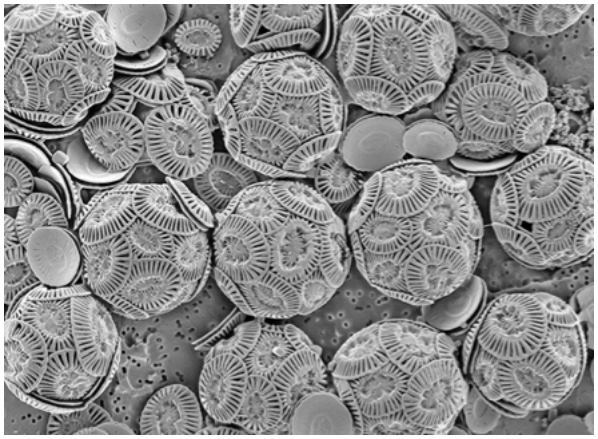

vorgelegt von

Mathias Haunost

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Photographer: Dr. Jan Michels

1. Gutachter:	Prof. Dr. Ulf Riebesell
2. Gutachter:	PD Dr. Frank Melzner
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Summary

Coccolithophores are an important group of marine phytoplankton. These tiny algae contribute significantly to the primary production in the pelagic ocean and have a major impact on the marine carbon cycle, including the uptake of carbon dioxide from the atmosphere and its long-term storage in the ocean. In this regard, their most important feature is the ability to precipitate calcium carbonate in the form of "coccoliths". These are small and intricately built calcite plates that cover the cell surface and form the so-called coccosphere, a shell composed of many individual coccoliths that surrounds the cell. Since their appearance in the Mesozoic, coccolithophores have generated a continuous rain of calcium carbonate to the bottom of the oceans for millions of years. A part of this material is preserved in the sediments and is stored in limestone rocks around the world until present.

As the formation, dissolution and storage of calcium carbonate plays an important role in the marine carbon cycle, the effects of ongoing human-induced ocean acidification on the performance of coccolithophores have been subject of thorough research over the past decades. However, it is still not clear why coccolithophores calcify and how their main property is related to their success in the natural environment. Consequently, it is presently not possible to predict their fate in the future ocean, in which calcification rates are thought to decline. There are many hypotheses regarding the question why coccolithophores calcify, e.g. that the precipitation of calcium carbonate could provide important products for photosynthesis, that the coccosphere could serve as a funnel to gather light energy, or that it protects the cells from harmful radiation. Another quite obvious theory is that the coccosphere serves as armor and protects the cells against natural predators.

Within the scope of my doctoral thesis, I investigated whether the coccosphere offers protection against virus infections and natural predators. I used some coccolithophore species commonly found in nature that can be kept in laboratory cultures and developed a method to artificially remove the coccosphere in order to test under controlled laboratory conditions whether cells without a calcite shell are increasingly eaten by a predator or increasingly infected by a certain virus.

The first study was aimed to investigate whether the coccosphere of the most widespread species *Emiliania huxleyi* provides a protection against infection by a specific *Coccolithovirus*. The results led to the conclusion that the coccosphere provides rather limited protection against the virus. More cells without than with a coccosphere died from the virus, however, cells from which the coccosphere was removed varied in their susceptibility to the virus over the course of

their daily growth cycle while calcified cells did not. The reason could have been that the susceptibility of *E. huxleyi* to the virus varies over the course of the cell cycle and that calcified cells were better protected than the uncovered cells at the respective time of infection. On the other hand, it is possible that the treatment to remove the coccosphere affected the cells sensitivity to the virus differently depending on the time in their cell cycle. Apart from that, the study showed that cells whose growth was stopped before infection occurred, and which were completely surrounded with coccoliths, died from the virus, indicating that the coccosphere cannot prevent the penetration of the virus. Recent studies have shown that the period between the infection and replication of the virus is variable and can depend on the stress level of the host cells. A further long-term experiment conducted as part of this work suggests that the virus-induced mortality is negatively correlated with the abundance of the host cells. Overall, the results lead me to the hypothesis that the viral particles do not necessarily have to penetrate the coccosphere, but may hijack a cellular uptake mechanism and are taken up actively by the host.

The second and third study were aimed to test whether the coccosphere protects against two different grazers. The major grazers of phytoplankton are small single-celled protozoa, a very diverse group of organisms often summarized under the term microzooplankton. In addition, phytoplankton is intensely consumed by larger multicellular organisms, the mesozooplankton, of which the copepods are one of the most important groups. Of both groups, one representative that is relatively easy to culture in the laboratory, was used to test the impact of the coccosphere on its food uptake. Somewhat surprisingly, copepods of the species *Acartia tonsa* ingested more calcified coccolithophore cells of *Gephyrocapsa oceanica* than cells of which the coccosphere was removed. This was presumably due to the fact that the prey organisms exhibit a size that is at the lower range of the predator's food size spectrum. The coccosphere enlarges the cell which probably made it easier for the copepods to detect and grasp the calcified cells.

The size effect of the coccosphere was also evident in the study with the protozoan predator *Oxyrrhis marina*. The heterotrophic dinoflagellate, which engulfs its prey items as a whole, was able to take up less cells with a coccosphere than without. Nevertheless, none of the coccolithophore species tested in this study fell out of the size range the predatory cells were able to ingest. However, ingested calcified cells occupied more of the space inside the predator and seemed to be digested less quickly. Moreover, predatory cells that were fed with calcified cells grew slower compared to individuals of *O. marina* that were offered cells without a coccosphere only. However, the results further show that *O. marina* did not actively select against calcified cells when both types were offered in a mixture. I concluded that these negative effects of calcification on the food uptake do not help against non-selective predators. Since the production of a coccosphere requires costs, competing phytoplankton species that do not invest energy in armor but in growth instead would likewise profit from the reduced grazing pressure, which in fact puts the coccolithophores on a competitive disadvantage in the face of non-selective predators. Nevertheless, the pelagic ocean comprises a huge diversity of protozoan predators, and it can be speculated whether some or many of these species would actively select against calcified cells in contrast to *O. marina*. Considering the sparsity of suitable prey in the dilute ocean, this raises the question whether most predators really have the choice of what to eat for most of the time, and if our current understanding of the arms race between predators and prey in the ocean is a too simplified view.

Zusammenfassung

Die Coccolithophoriden bilden eine bedeutende Gruppe innerhalb des marinen Phytoplanktons. Die kleinen Algen leisten einen signifikanten Beitrag zur Primärproduktion im Freiwasser der Meere und haben einen großen Einfluss auf den marinen Kohlenstoffkreislauf, da sie den Austausch von Kohlendioxid mit der Atmosphäre und dessen langfristige Speicherung im Ozean beeinflussen. Ihre besondere Rolle ist maßgeblich durch ihre Eigenschaft geprägt Kalziumkarbonat auszufällen und daraus „Coccolithen“ zu bilden. Das sind kleine, präzise geformte Kalkplättchen, die die Zelloberfläche bedecken und in ihrer Gesamtheit die Coccosphäre bilden, eine Schale aus vielen einzelnen Coccolithen, die die Zelle umgibt. Seit ihrem Erscheinen im Erdmittelalter generieren sie seit vielen Millionen Jahren einen kontinuierlichen Regen aus Kalziumkarbonat-Plättchen auf den Boden der Ozeane. Ein Teil dieses Materials blieb in den Sedimenten erhalten und bildet heute mächtige Schichten aus Kalkgestein. Da die Fällung, Lösung und Speicherung von Kalziumkarbonat eine wichtige Rolle im marinen Kohlenstoffkreislauf spielen, wurden die Auswirkungen der Ozeanversauerung durch den menschengemachten Klimawandel auf Coccolithophoriden in den letzten Jahrzehnten intensiv untersucht. Allerdings ist bis heute nicht bekannt, warum Coccolithophoriden überhaupt kalzifizieren und inwiefern diese Eigenschaft mit ihrem Erfolg auf dieser Welt zusammenhängt. Daher ist es auch nicht möglich, die weitere Entwicklung der Coccolithophoriden im zukünftigen Ozean einzuschätzen, in dem die Bildung einer Kalkschale kostenintensiver werden wird.

Es gibt mehrere Hypothesen, warum Coccolithophoriden kalzifizieren. Die Bildung von Kalziumkarbonat könnte zum einen wichtige Produkte für die Photosynthese liefern. Weiterhin könnte durch die Anordnung der Coccolithen um die Zellen die Lichtausbeute für die Photosynthese erhöht werden. Eine weitere Funktion der Coccolithen könnte sein, die Zelle vor schädlicher UV-Strahlung zu schützen. Die naheliegendste Theorie ist allerdings, dass die Coccosphäre als Panzerung dient und Schutz gegen natürliche Feinde bietet.

Im Rahmen dieser Arbeit wurde untersucht, ob die Coccosphäre Schutz gegen Fressfeinde oder vor Infektionen durch Viren bieten kann. Dafür wurde eine Methode entwickelt die Coccosphäre von einigen häufig vorkommenden Coccolithophoriden künstlich zu entfernen, um unter kontrollierten Bedingungen testen zu können, ob Zellen ohne Coccosphäre vermehrt gefressen oder von Viren infiziert werden.

In der ersten Studie wurde untersucht, ob die Coccosphäre der wohl am häufigsten vorkommenden Coccolithophoride *Emiliania huxleyi* Schutz gegen die Infektion mit einem spezifischen Coccolithovirus bieten kann. Die Ergebnisse dieser ersten Studie führten zu der Schlussfolge-

rung, dass die Coccosphäre eher einen geringen Schutz gegen das Virus bietet. Zwar starben mehr Zellen ohne als mit Coccosphäre, allerdings änderte sich die Infektions-Anfälligkeit der Zellen von denen die Coccospäre künstlich entfernt wurde im Verlauf ihres Zellzyklus. Das könnte einerseits dadurch erklärt werden, dass sich die Anfälligkeit dieser Alge gegenüber dem Virus im Laufe ihres Zellzyklus ändert und die Zellen mit Coccosphäre zu diesem Zeitpunkt besser geschützt waren als die Zellen ohne Schale. Andererseits könnte es aber auch der Fall gewesen sein, dass die Entfernung der Coccosphäre zu unterschiedlichen Zeiten im Zellzyklus die Zellen artifiziell empfindlicher gegenüber dem Virus gemacht hat. Unabhängig davon haben weitere Experimente gezeigt, dass Zellen die in ihrem Zellzyklus stagnieren und komplett von einer Kalkschale umgeben sind vom Virus infiziert werden. Dies deutet darauf hin, dass die Coccosphäre das Eindringen der Viren nicht verhindern kann. Neueste Studien zeigen, dass das Virus auch längerfristig in den Zellen verbleiben kann und der Zeitpunkt zwischen der eigentlichen Infektion und dem Tod der Zelle variabel ist. Die Infektionsrate könnte dabei vom Stresszustand der Zelle abhängig sein. Ein durchgeführtes Langzeitexperiment deutet darauf hin, dass die durch das Virus induzierte Mortalität negativ mit der Dichte der Wirtspopulation korreliert. Die Gesamtheit dieser Ergebnisse führen zu der Hypothese, dass das Virus vermutlich nicht passiv durch die Coccosphäre dringen muss, um in die Zelle zu gelangen, sondern durch einen bestimmten Mechanismus aktiv von der Zelle aufgenommen wird.

In der zweiten und dritten Studie wurde getestet, ob die Kalkschale bestimmte Fressfeinde abschreckt. Die bedeutendsten Fressfeinde vom Phytoplankton sind kleine einzellige Protozoen - eine diverse Gruppe von Organismen, die oft als Mikrozooplankton zusammengefasst werden. Weitere bedeutende Prädatoren des Phytoplanktons sind größere mehrzellige Organismen, das sogenannte Mesozooplankton, in dem die Copepoden eine der bedeutendsten Gruppen stellen. Im Rahmen der Studien wurde der Effekt der Coccosphäre auf die Nahrungsaufnahme je eines repräsentativen Fressfeindes aus diesen beiden Gruppen getestet.

Überraschenderweise fraßen Copepoden der Art *Acartia tonsa* mehr Zellen mit als ohne Kalkschale. Das lag vermutlich daran, dass die angebotenen Coccolithophoriden der Art *Gephyrocapsa oceanica* mit ihrer geringen Größe am unteren Ende des Nahrungsspektrums der Räuber lagen. Zellen mit Kalkschale waren größer und eventuell leichter aufzunehmen.

Ein Größeneffekt der Coccosphäre war auch in den Versuchen mit dem einzelligen Dinoflagellaten *Oxyrrhis marina* sichtbar. Die Protozoen konnten weniger kalzifizierte Zellen aufnehmen also solche bei denen die Kalkschale entfernt wurde. Zudem könnte durch die Auflösung des Kalks in den Nahrungsvakuolen des Räubers die Weiterverarbeitung der Nahrung verlangsamt werden. Die Kalkschale hatte einen signifikanten Effekt auf die Nahrungsaufnahme und das Wachstum von *O. marina*. Trotzdem haben die Dinoflagellaten nicht aktiv gegen kalzifizierte Zellen selektiert und nicht bevorzugt Zellen ohne Coccosphäre gefressen. Bei nicht selektiven Prädatoren bringt der negative Effekt der Coccosphäre auf ihr Wachstum allerdings nicht nur den Coccolithophoriden einen Vorteil, sondern auch konkurrierenden Arten im Phytoplankton, die nicht den Aufwand betreiben eine Kalkschale zu bilden.

Es gibt unzählige verschiedene Mikrozooplanktonarten und man kann spekulieren, ob im Gegensatz zu *O. marina*, ein bedeutender Teil dieser Organismen dazu fähig ist, den Kalk wahrzunehmen und das Fressen von Coccolithophoriden zu meiden. Allerdings ist es für solch klei-

ne Organismen in der Natur oft mühsam Nahrung zu finden, und es stellt sich die Frage ob die Mehrheit der Mikrozooplankter über den Großteil ihrer Lebensdauer wirklich eine Wahl haben, welche Nahrung sie aufnehmen können und ob die derzeitige Sichtweise über die Räuber-Beute Beziehungen im Ozean nicht zu einseitig ist.

Contents

1 | Introduction

1.1 General introduction to phytoplankton and the pelagic food web

Marine phytoplankton comprises a taxonomically diverse group of single-celled algae that drift with the currents and photosynthesize: They utilize sunlight to oxidize H_2O for energy production and thereby release O_2 as byproduct. The energy is used to reduce CO_2, which serves as carbon source for the buildup of organic material. Hence, phytoplankton forms the basis of aquatic food webs by converting inorganic carbon and nutrients to organic matter. Marine phytoplankton accounts for almost half of the global primary production (Field et al., 1998). However, phytoplankton constitutes only 1–2 % of the total photosynthetic biomass on the planet because it is quickly turned over, revolving every 2–6 days on average (Falkowski, 1994; Falkowski and Raven, 2007).

This fast turnover is partly due to rapid phytoplankton growth, which is controlled by light and nutrient availability and balanced by several loss processes that influence the build-up of phytoplankton biomass in the ocean. Cells may just sink out of the sunlit surface layer into depth, or perish in unfavorable conditions (e.g. nutrient limitation) and lyse (Agustí et al., 1998; Kirchman, 1999; Agustí and Duarte, 2013). A major cause of algal mortality is lysis due to viral infection (Suttle, 2005; Wigington et al., 2016), or as a consequence of programmed cell death induced by adverse biotic or abiotic factors (Bidle and Falkowski, 2004; Bidle, 2016). Moreover, phytoplankton is grazed intensely by unicellular protozoa and larger metazoan plankton. In this way, the carbon and assimilated nutrients that are fixed through photosynthesis are transferred from phytoplankton via zooplankton to fishes and larger predators (Fig. 1.1).

Phytoplankton further fuels a complex microbial food web (Pomeroy, 1974; Azam et al., 1983; Fenchel, 2008). Organic material in form of exudates or lysate (dissolved organic matter), as well as detritus (dead particulate organic matter) provides a resource for a great diversity and abundance of bacteria, which are responsible for the recycling of organic matter and the regeneration of nutrients that become available again for primary production. Bacteria in turn are eaten by ciliates and flagellates, which again are consumed by larger zooplankton, so that a part of the matter and energy is returned to higher trophic levels.

The microbial food web constitutes the predominant component of biological productivity in oligotrophic regions where nutrients are depleted, as well as in regions where primary production is limited by the scarcity of a specific nutrient (Sarmiento and Gruber, 2006). Small cells have a high nutrient affinity and are able to respond quickly to low level nutrient enrichments (Thingstad and Sakshaug, 1990), thus in oligotrophic regions primary production is dominated by small phytoplankton species of the picoplankton (<0.3 μm), which prosper from regenerated nutrients. Small cyanobacteria of the genera *Prochlorococcus* and *Synechococcus* account for a substantial proportion of marine primary production (Flombaum et al., 2013). However, the picoplankton further comprises a great diversity of photosynthetic eukaryotes (Moon-van der Staay et al., 2001; Massana et al., 2004; Not et al., 2007). The picophytoplankton constitutes an integral part of the microbial food web and is omnipresent throughout the sunlit layer of the oceans. It accounts for a large proportion of the phytoplankton biomass also in eutrophic regions with high nutrient supply of the higher latitudes (Ward and Waniek, 2007), where it is most prevalent during winter (Massana, 2011), but also contributes to the seasonal phytoplankton blooms

(Larsen et al., 2004; Hunter-Cevera et al., 2016). Eutrophic regions facilitate high biological production whereby a notable part of the organic matter is exported from the surface in form of aggregated cells and detritus (Smetacek, 1985; Briggs et al., 2011). A major fraction of the organic material is consumed, decomposed and remineralized back into inorganic components on its way to the ocean floor and within the sediments. The remineralized nutrients are welled up again into the sunlit layer through the ocean currents, where they promote new phytoplankton growth. Due to the advantageous uptake kinetics, the picophytoplankton benefits from the upwelling of remineralized nutrients, but their gain in biomass is immediately consumed and disappears in an increasingly efficient microbial food web (Barber and Hiscock, 2006). Most of the export production in nutrient rich regions is generated by three specific phytoplankton groups, whose evolutionary success story dates back to the Mesozoic era (Katz et al., 2004; Knoll et al., 2007). In the recent ocean these are: diatoms, dinoflagellates and coccolithophores, which account for the major proportion of phytoplankton biomass and export production.

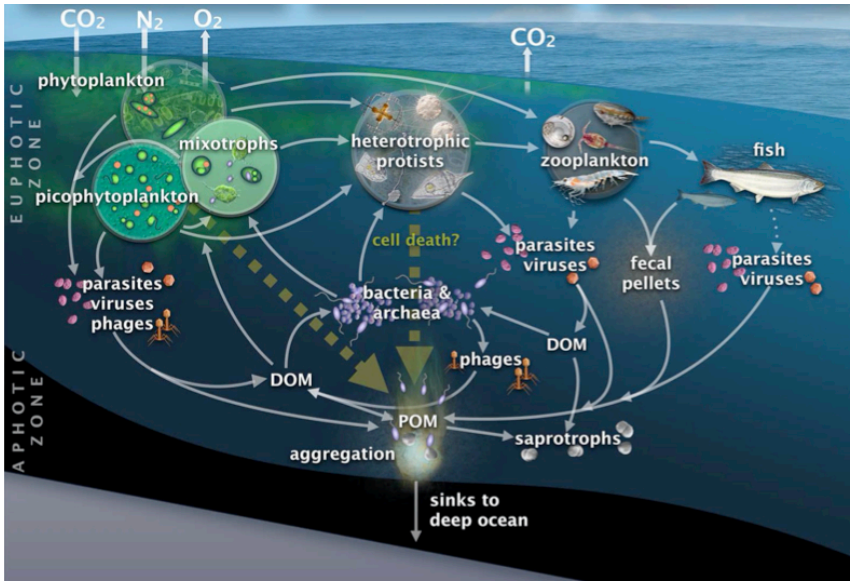

Figure 1.1: The figure modified from Worden et al. (2015) shows the interactions within the pelagic food web. In the sunlit layer (euphotic zone), phytoplankton converts CO_2 into organic matter and serves as food or resource for a variety of planktonic grazers, which release dissolved (DOM) and particulate organic matter (POM), thus providing resources for marine bacteria and archaea. It is thought that all organisms are subject to parasitic and viral attack (or phages that attack bacteria and archaea), which contributes to the cycling of organic material. A fraction of the organic biomass that is produced in the euphotic zone and not respired back to CO_2 in organisms sinks to the deep ocean.

The fixation of inorganic carbon and nutrients during photosynthesis, their modification and turnover in the marine food web, and their transport to the deep ocean is referred to as the biological pump (Eppley and Peterson, 1979; Volk and Hoffert, 1985; Ducklow et al., 2001). The biologically driven sequestration of atmospheric carbon to the ocean's interior is a key component of the global carbon cycle. The biological pump sequesters 5–15 Gt carbon per year in the deep ocean (Falkowski et al., 1998; Henson et al., 2011), where it is stored for decades to millennia (Guidi et al., 2015; Boyd et al., 2019). A small amount of the organic carbon that withstands remineralization in the sediments is stored for millions of years (Ridgwell and Zeebe, 2005; Estes et al., 2019). Albeit a small fraction of this long-lasting carbon storage, which has

been processed in the lithosphere and was pyrolyzed to petroleum, fuels the industrial world of today whereby it is released into the atmosphere and leads to drastic consequences for marine ecosystems through ocean warming and ocean acidification (Caldeira and Wickett, 2003; IPCC, 2021).

1.2 The role of coccolithophores in the carbon cycle

Coccolithophores are an integral part of the marine phytoplankton and play an important role in the biological carbon pump and the marine element cycling. Their main feature is the ability to precipitate calcium carbonate ($CaCO_3$) in form of small calcite plates. These so called coccoliths are assembled to a coating around the cell, termed coccosphere. Coccolithophores contribute $\sim$1–10% to the marine primary production (Poulton et al., 2007) and up to 40% of the carbon fixation during bloom events (Poulton et al., 2013).Together with Foraminifera and pteropods, coccolithophores are the main producers of $CaCO_3$ in the pelagic zone. They contribute $\sim$50% to the $CaCO_3$ sedimentation in the open ocean (Broecker and Clark, 2009) where approximately half of the marine net $CaCO_3$ production takes place (Milliman, 1993). The calcite produced by coccolithophores promotes the biological pump by ballasting detritus aggregates and fecal pellets of larger predators, which thus sink faster and deeper. Thus, coccoliths enhance the biological carbon pump in the open ocean by accelerating organic matter sinking, which leads to a reduction of the atmospheric CO_2 level (Francois et al., 2002; Klaas and Archer, 2002; Ziveri et al., 2007; Honjo et al., 2008). On the other hand, the process of calcification itself releases CO_2. During coccolith formation, a calcium ion (Ca^{2+}) is combined with 2 bicarbonate ions (HCO_3^-) to form $CaCO_3 + H_2O + CO_2$. The removal of HCO_3^- is expressed as a reduction in seawater alkalinity, which in turn affects its carbonate chemistry in a way that for each unit of $CaCO_3$ about 0.6 mols of CO_2 are formed per mol $CaCO_3$ precipitated, and the amount of CO_2 released per unit of precipitated $CaCO_3$ increases with rising atmospheric CO_2 concentration (Frankignoulle et al., 1994; Zondervan et al., 2001; Rost and Riebesell, 2004).

Most of the $CaCO_3$ produced in the open ocean dissolves again, but a minor fraction accumulates in the sediments and constitutes a long-term carbon sink (Milliman et al., 1999; Sulpis et al., 2021). The burial of $CaCO_3$ depletes the ocean's Ca^{2+} inventory, which is balanced by the chemical weathering of carbonate and silicate minerals in soils, which, simply put, react with atmospheric CO_2. Mineral weathering also delivers the 2 HCO_3^- required for calcification. The weathering of carbonate mineral returns Ca^{2+} and HCO_3^- in the same ratio back to the sea per unit CO_2 as they are used for the precipitation of calcite. However, the weathering of silicate mineral consumes additional atmospheric CO_2, so that the fraction of the buried $CaCO_3$ that is fueled by the weathering of silicate minerals constitutes a loss of carbon from the hydrosphere to the geologic reservoir (Ridgwell and Zeebe, 2005). The weathering rate increases with increasing CO_2, thus calcification acts as a negative feedback system that regulates the atmospheric CO_2 concentration over hundred thousands of years (Ridgwell and Zeebe, 2005).

1.3 Calcification in coccolithophores

The specific structure of the coccoliths and the architecture of the coccosphere is species-specific (Siesser and Winter, 1994; Young and Henriksen, 2003; Frada et al., 2010) (Fig. 1.2). Coccoliths are formed inside the cell within a vesicle that is derived from the Golgi apparatus (Wilbur and Watabe, 1963). Within the coccolith vesicle, the $CaCO_3$ is precipitated onto an organic baseplate that serves as template (van der Wal et al., 1983; Westbroek et al., 1989; Young et al., 1999). The growth of the calcite crystals along this baseplate is tightly controlled by the cellular machinery (Henriksen et al., 2004; Young et al., 2009). Once the new coccolith is formed, the coccolith vesicle fuses with the cell membrane and the coccolith is released to the cell surface where it is mounted into the coccosphere (Young and Henriksen, 2003).

Coccolith production is associated with significant effort and costs. The controlled biomineralization of $CaCO_3$ requires the transport of Ca^{2+} and HCO_3^- to the point of precipitation inside the coccolith vesicle, while healthy concentrations of both substrates have to be maintained inside the remainder cell (Brownlee and Taylor, 2004; Mackinder et al., 2010). HCO_3^- has to be converted to carbonate (CO_3^{2-}) by the removal of a proton (H^+), so that Ca^{2+} and CO_3^{2-} can form $CaCO_3$. The reaction would cease when H^+ accumulates at the site of crystal formation, so it is directly removed from the cell in order to keep up pH homeostasis (Suffrian et al., 2011; Taylor et al., 2011), whereby the export of H^+ is probably energetically coupled with the import of Ca^{2+} (Mackinder et al., 2011; Holtz et al., 2013). The import of the substrates and export of side products are estimated to make up most of the costs for calcite production. Together with the expenses for the production of coccolith-associated polysaccharides and the metabolic costs for the cellular machinery, the energetic costs for calcification are estimated to make up around one third of the cell's photosynthetic energy budget (Monteiro et al., 2016).

1.4 Coccolithophore phylogeny

Coccolithophores are haptophytes, which is a clade of eukaryotes characterized by the haptonema, a flagellum-like organelle that serves for prey capture and attachment. Haptophytes are represented in great diversity within the picoplankton where they act as key mixotrophic grazers on bacteria (Liu et al., 2009; Unrein et al., 2014). Within the Haptophyta, the coccolithophores are included in the class Prymnesiophyceae, together with two other orders that include familiar bloom forming members: The Prymnesiales, which are mixotroph and potentially toxic (Edvardsen and Paasche, 1998), and the Phaeocystales, some of which can form large gelatinous colonies that occasionally cause the sea foam on beaches (Blauw et al., 2010). Besides these two, the Prymnesiophyceae include 4 additional orders that are pooled into the subclass Calcihaptophycideae and comprise 10 extant families (Jordan et al., 2004; de Vargas et al., 2007). Except of the members of one family (the Isochrysidaceae), all other species within the Calcihaptophycideae are called coccolithophores. They add up to 200 known and around 500 possibly existing species (Young et al., 2005).

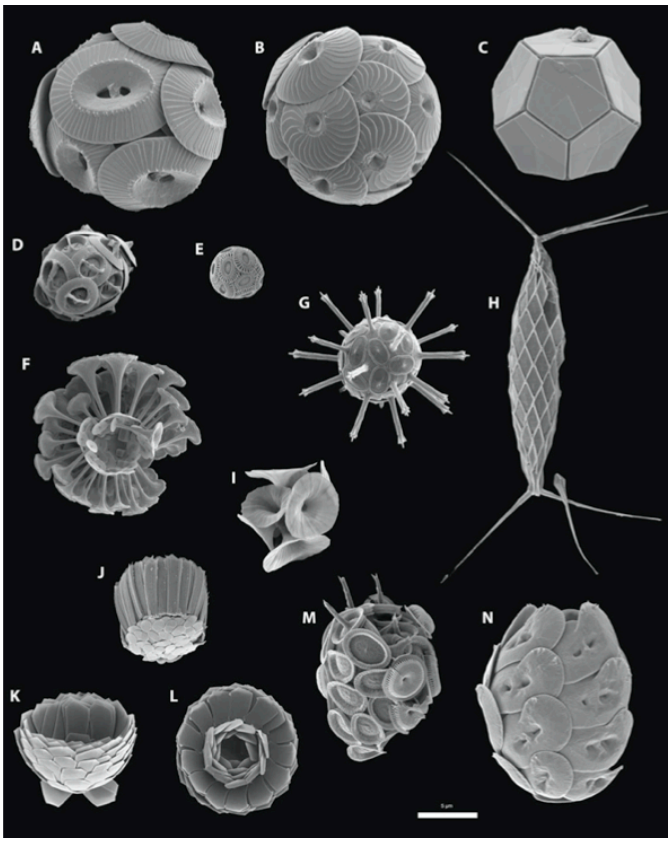

Figure 1.2: The figure adopted from Monteiro et al. (2016) shows scanning electron microscopic pictures of different coccolithophore species. Scale bar = 5 μm. The species names are: (A) *Coccolithus pelagicus*, (B) *Calcidiscus leptoporus*, (C) *Braarudosphaera bigelowii*, (D) *Gephyrocapsa oceanica*, (E) *Emiliania huxleyi*, (F) *Discosphaera tubifera*, (G) *Rhabdosphaera clavigera*, (H) *Calciosolenia muraayi*, (I) *Umbellosphaera irregularis*, (J) *Gladiolithus flabellatus*, (K, L) *Florisphaera profunda*, (M) *Syracosphaera pulchra*, (N) *Helicosphaera carteri*.

1.5 Trajectory of coccolithophores through deep time

Coccolithophores expanded in the Mesozoic and extended the site of the marine carbonate precipitation from the shelf to the open ocean (Hay, 2004), which created an important regulatory system of the ocean carbon chemistry and a sink for $CaCO_3$ in deep sea sediments (Hay, 2004; Ridgwell and Zeebe, 2005; Ridgwell, 2005).

Molecular clock methods suggest that calcifying haptophytes evolved between 329–291 million years ago (Liu et al., 2010). The first fossil evidence of coccolithophores dates back to 217–204 million years ago in the late Triassic (Bown et al., 2004; de Vargas et al., 2007; Gardin et al., 2012). Fossil coccoliths have been found in sedimentary rocks of the Alps that were located at the floor of the Tethys Ocean at that time. In the Jurassic, the coccolithophores increased in abundance and species diversity. In the subsequent Cretaceous, coccolithophores reached the highest global diversity ever (Bown et al., 2004). At that time, their huge abundance led to the generation of enormous calcareous sediment layers at the seafloor, which resulted in the formation of huge limestone and chalk deposits after which the period is named. Coccolithophores overcame several oceanic anoxic events during the Cretaceous (Erba, 2006). The warm climate, thus high

sea levels and enhanced weathering led on to increased plankton growth and export production, causing oxygen depletion in many ocean basins and the accumulation of rotting sludge at the sea floor that founded important petroleum deposits. The marine food web in the Cretaceous also facilitated the proliferation of the diatoms (Falkowski and Raven, 2007). At the end of the Cretaceous, the Earth was hit by an asteroid, and a severe mass extinction took place. The long-lasting dark period during the following impact winter was apparently dominated by phytoplankton as well as coccolithophore species that were capable of phagocytosis and fed on other organisms (Gibbs et al., 2020). According to the authors, the marine food webs in the following Paleogene were reestablished by mixotrophic phytoplankton species, those that are capable of both, photosynthesis and phagotrophy. Indeed, several extant coccolithophore species have been shown to be able to phagocytize bacteria (Avrahami and Frada, 2020). Coccolithophores reached again a high species diversity towards the Paleocene-Eocene Thermal Maximum (Bown et al., 2004; Monteiro et al., 2016), which represents a possible analog for the recently advancing climate change (Zeebe et al., 2009). Thereafter, their species richness declined continually and leveled off at recent values (Monteiro et al., 2016).

Since the thermal maximum in the Paleogene, coccolithophores faced increasing competition with diatoms (Falkowski et al., 2004), which contemporarily are the most successful group in terms of their contribution to global primary production and nutrient cycling (Smetacek, 1999; Kooistra et al., 2007). Their main distinguishable feature is the frustule, a cell covering made of silica. It has been suggested that diatoms were favored by the expansion of grasses (Falkowski et al., 2004), which contain a high proportion of silica in form of phytoliths that possibly have a protective function against herbivores (Hunt et al., 2008). The proliferation of grasses and grass eating mammals has thus enhanced the transport of silicon from the land to the sea where it stimulates the growth of diatoms (Falkowski et al., 2004).

1.6 The coccolithophore life cycle

Coccolithophores exhibit a heteromorphic life cycle (Young and Henriksen, 2003), which means that each species occurs as two different types of organisms. The coccolithophores shown in Fig.1.2 represent the diploid organism of each respective species. They possess two homologous sets of their genetic material and produce intricately built calcite scales termed heterococcoliths. The diploid cell undergoes meiosis, during which it splits into (potentially 4) smaller cells, each of which contains just one set of genetic material and is thus haploid. The haploid form is usually able to calcify, but produces more simply built coccoliths (termed holococcoliths and nannoliths), although in some species the haploid cells do not produce coccoliths at all (Young and Henriksen, 2003; Frada et al., 2010). Both, the haploid and the diploid cells grow asexually by cell division. Finally, two haploid cells can mate and recombine into a heterococcolith bearing diploid cell, which again contains two sets of genetic material, one of each parent cells.

The haplo-diplontic life cycle expands the niche of a coccolithophore species (de Vries et al., 2021) and the proportion of the diploid and haploid form can vary in time within an ecosystem, though the reasons for the life cycle switch within the single species are largely unknown (Houdan et al., 2006; Cros and Estrada, 2013; Šupraha et al., 2016). It has been found to occur

due to changes in temperature, trace metal or vitamin concentrations (Noël et al., 2004). In the coccolithophore *Emiliania huxleyi*, it was shown that viral infection favors the development of haploid cells, which, unlike diploid cells, are not attacked by the virus (Frada et al., 2008, 2017). However, traces of the viral genome were also found in haploid cells (Mordecai et al., 2017).

It has been speculated that the haploid forms of many coccolithophore species may contribute a large part to the enigmatic diversity of haptophytes that is found in the picoplankton (Taylor et al., 2017).

1.7 Distribution of coccolithophores

Coccolithophores occur in all major ocean basins, whereby the different species are distributed in different depths and latitudinal zones (Winter et al., 1994; Young, 1994; Boeckel and Baumann, 2008). The highest diversity is found in the stable oligotrophic environments of the tropical and sub-tropical latitudes, which are by far the most important sides of calcite sedimentation since they constitute the largest area of the oceans (McIntyre and Bé, 1967; Okada and Honjo, 1973; Baumann et al., 2004). In the lower latitudes, the species composition changes strongly over depth, some living far below the surface and are potentially mixotroph (Poulton et al., 2017). Other species are bound to relatively narrow temperature conditions (McIntyre and Bé, 1967), such as the cold water adapted *Coccolithus* spp. that proliferate at higher latitudes where they contribute a significant proportion to the global calcite production (Milliman, 1980; Tarran et al., 2001; Daniels et al., 2014, 2016). Most of the coccolithophore species are rare but omnipresent (Young, 1994; Poulton et al., 2017) and only a few are omnipresent and abundant. The best known and arguably most important one is *Emiliania huxleyi* (Paasche, 2002). It belongs to the order Isochrysidales, which likewise includes all members of the Calcihaptophycidae that do not calcify, and deviates in many respects from typical coccolithophores (de Vargas et al., 2007). *E. huxleyi* is the most abundant coccolithophore on Earth. It occurs in all oceans (Winter et al., 2014) and periodically forms extensive blooms (Tyrrell and Merico, 2004), which, are usually accompanied by other coccolithophore species in varying proportions (Mayers et al., 2019).

In the temperate zone, highest coccolithophore abundances are found during summer, subsequent to the diatom spring bloom (Margalef, 1978; Iglesias-Rodríguez et al., 2002; Balch, 2004). In the austral summer, high coccolithophore abundances occur in the Southern Ocean in an area near the polar front, which is termed the Great Calcite Belt (Balch et al., 2011, 2016). Coccolithophores seem to occur relatively common during winter and can dominate in the early spring bloom period (Dale et al., 1999; Schiebel et al., 2011; Smith et al., 2012, 2017). In the Mediterranean, coccolithophores usually reach high abundances during winter and then bloom again in the summer (Zingone et al., 2010; Cerino et al., 2017). However, the seasonal phytoplankton development in temperate and sub-polar regions is thought to follow a successive change of the phytoplankton composition from diatoms, which dominate the spring bloom until they are limited by a lack of dissolved silicate (Smetacek, 1998), to coccolithophores and dinoflagellates, which dominate during summer (Margalef, 1978; Lochte et al., 1993; Balch, 2004). Nevertheless, the clear seasonal succession from diatom dominated spring blooms to coccolithophores is no universal pattern. Coccolithophores and other phytoplankton groups can co-occur with di-

atom blooms (Hopkins et al., 2015) and coccolithophores may develop relatively independently compared to the dynamics of the overall phytoplankton community (Daniels et al., 2015).

1.8 Anthropogenic impact on the marine carbon cycle

Global economies largely rely on energy stored in fossil carbon that was fixated through photosynthesis and buried in the Earth crust during epochs long ago. The burning of fossil fuels has returned about 445 gigatons of buried carbon (Friedlingstein et al., 2020). Changes in land use have freed additional $\sim$ 210 gigatons that were formerly bound in terrestrial ecosystems. Anthropogenic CO_2 emissions have grown with increasing industrialization and presently amount to about 10.6 gigatons of carbon per year, which is equivalent to 39 gigatons CO_2 (Friedlingstein et al., 2020). The excess CO_2 molecules in the air absorb an additional share of the heat radiation that is emitted from the Earth's surface, which leads to global warming and thus increasing seawater temperature (IPCC, 2021). Most of the fossil CO_2 will eventually be taken up by the oceans where it reacts with water in a chemical equilibrium:

$$\mathrm{H_2O + CO_2 \rightleftharpoons H^+ + HCO_3^- \rightleftharpoons 2\,H^+ + CO_3^{2-}} \tag{1.1}$$

The increasing CO_2 concentration leads to higher H^+ concentrations in the seawater, which makes it more acidic. The buffer capacity of the seawater decreases due to a shift of the chemical equilibrium of the carbonate species towards a strong increase of CO_2, a slight increase of HCO_3^- and a strong decrease in the concentration of CO_3^{2-} (Wolf-Gladrow et al., 1999).

The current rise of the atmospheric CO_2 concentrations due to the continuous exploitation of fossil fuels has a strong impact on marine ecosystems (Riebesell et al., 2009). Most relevant changes for phytoplankton are the increasing sea-surface temperature and ocean acidification (Boyd and Doney, 2002; Monteiro et al., 2016). The energetic costs for the precipitation of $CaCO_3$ are thought to increase with decreasing pH and CO_3^{2-} concentration, which potentially impairs the competitive fitness of coccolithophores in the phytoplankton (Riebesell and Tortell, 2011). Furthermore, the decline in the CO_3^{2-} concentration leads to a decrease of the saturation concentration of $CaCO_3$, with important implications for the carbonate cycle and the chemical resistance of the cell covering of calcifying plankton (Riebesell et al., 2000). Declining calcification rates would weaken the ballast effect on the biological pump and thus attenuate the export of atmospheric CO_2 to the deep ocean (Riebesell et al., 2009). Increasing H^+ concentrations may affect the cellular cost for the maintenance of the pH homeostasis of phytoplankton, whereas photosynthesis could benefit from the rise in the concentration of CO_2, which can potentially limit phytoplankton growth (Riebesell et al., 1993). The increasing H^+ concentrations due to ocean acidification are thought to affect coccolithophore calcification in the near future up to the point when the dissolution of deep-sea $CaCO_3$ and terrestrial weathering catch up and the carbonate chemistry of the ocean again changes towards better conditions for calcifying plankton (Bach et al., 2015).

In addition to direct physiological effects of ocean acidification, climate change rapidly alters the physiochemical conditions for the phytoplankton. Increasing water temperatures alter

the currents that mix the ocean (Hu et al., 2020), and can influence the seasonal development and depth of the surface mixed layer (Somavilla et al., 2017), so the physicochemical conditions underlying the growth and distribution of the plankton. Recent studies indicate that the composition of the phytoplankton is changing. Coccolithophore blooms occur later and shorter and at the same time some coccolithophores expand poleward, thereby increasing their impact in the planktic community and on carbon cycling (Winter et al., 2014; Hopkins et al., 2015; Smith et al., 2017; Rigual Hernández et al., 2020).

1.9 Functions of coccoliths

The globally important role of coccolithophores is believed to be linked to their ability to calcify. However, the reason why coccolithophores calcify is still unknown. The production of coccoliths is associated with significant costs for the cell so that it can be assumed that corresponding benefits exist, and have been existent ever since coccolithophores appeared in the Triassic. It is impossible to assess the costs of calcification for these organisms if the corresponding benefits of this trait are unknown. Thus, it remains difficult to predict how the future changes will affect coccolithophore ecology. It is therefore an urgent need to answer why coccolithophores calcify.

There are several hypotheses about the function of calcification (Young, 1987, 1994; Raven and Crawfurd, 2012; Monteiro et al., 2016; Taylor et al., 2017). It has been proposed that calcification fuels photosynthesis, since the CO_2 that directly or indirectly originates as a byproduct in the $CaCO_3$ precipitation process can be taken up by the carbon fixing enzyme of the cells. This theory was intensely studied in *E. huxleyi*, the most abundant coccolithophore and found no support (Herfort et al., 2004; Trimborn et al., 2007; Bach et al., 2013; Monteiro et al., 2016; Taylor et al., 2017). Furthermore, coccoliths may serve as light funnels that channelize photons to the photosystem of deep dwelling species, and may also protect surface dwelling species from light stress and UV radiation by shedding excess radiation (Young, 1994; Gao et al., 2009; Monteiro et al., 2016). The coccosphere could also provide hydrodynamic advantage by stabilizing the viscous boundary layer around the cell, which could help nutrient uptake in patchy environments (Young, 1994; Bartal et al., 2015; Taylor et al., 2017). Indeed, calcification in coccolithophores could have developed several functions over the course of evolution and in view of the various niches in which the different coccolithophore species thrive (Monteiro et al., 2016).

Perhaps the most obvious theory about the function of the coccosphere is a protective role against predation. Coccospheres provide mechanical protection to a comparable extend as the frustules of diatoms (Jaya et al., 2016), which have been shown to protect the cells against grazing by copepods (Friedrichs et al., 2013; Pančić et al., 2019). It has been argued that, due to the universality of cell coverings in the phytoplankton, their protective function and the necessity of armor in phytoplankton evolution has just been overlooked (Hamm and Smetacek, 2007).

The ocean harbors a huge diversity and abundance of heterotrophic organisms (de Vargas et al., 2015), which rely on organic matter produced by other organisms. Moreover, many phytoplankton species are in fact mixotroph and eat other organisms although they are capable of photosynthesis (Unrein et al., 2007; Frias-Lopez et al., 2009; Hartmann et al., 2012). Mixotrophy

is generally widespread among planktonic eukaryotes and expected to be rather the norm than the exception (Flynn et al., 2013; Mitra et al., 2016). Various photosynthetic eukaryotes gather essential substances by eating other organisms, while others supplement their growth by, or survive through, phagotrophy when light is limited (Jones, 1997; Stukel et al., 2011). On the other side, protozoa that primarily subsist on phagotrophy often retain plastids of their prey or carry symbiotic algae (Stoecker et al., 2009, 2017). Thus, the clear distinction between phytoplankton and zooplankton is often inappropriate, especially within the smaller size classes of the food web. Unicellular protozoa, predominantly alveolates (particularly ciliates and dinoflagellates), but also Rhizaria (radiolarians, Foraminifera, and cercozoans), most of which also bear an extracellular skeleton, can be considered as the dominant predators within the smaller plankton size classes (Sherr and Sherr, 1994). Larger animals like copepods or krill are often referred to as the intermediate trophic level between phytoplankton and fish in the classical herbivorous food web, but they also feed on the smaller protozoa. The herbivorous and the microbial trophic pathways rather represent the two extremes of a continuous pelagic food web that vary in prominence in terms of location and time (Legendre and Rassoulzadegan, 1995). Viruses are omnipresent in the ocean and play an important role in the structure and function of aquatic food webs (Wilhelm and Suttle, 1999; Suttle, 2005; Wigington et al., 2016). The collectivity of viruses, prokaryotes, and picoeukaryotes has been described as the oceans microbiome (Sunagawa et al., 2015), or as the ocean's veil above which larger protists and metazoans might bloom (Massana, 2011).

Considering the wide variety of phytoplankton predators, it seems plausible to hypothesize that the coccosphere provides an effective armor, which facilitated the evolutionary success of coccolithophores.

1.10 Thesis content

This thesis addresses the hypothesis that the coccosphere offers protection against predators. The main predators of coccolithophores are viruses and microzooplankton (20–200 μm), mainly ciliates and phagotrophic dinoflagellates. Copepods are the dominant metazoan plankton predators in many parts of the ocean and can feed intensely on coccolithophore populations. I developed an approach to remove the coccosphere of cultured coccolithophore cells and to maintain them relatively free of coccoliths for days. Naked and calcified cells of the same cultured species were used to test the effect of the coccosphere on viral infection and on predation of a phagotrophic dinoflagellate as well as a copepod. The basic questions we aimed to address are:

- Does the coccosphere reduce the susceptibility of the cells to viral infection? (Chapter 1)
- Does the coccosphere serve as a defense against a phagotrophic dinoflagellate? Does it affect the nutritional uptake of the protozoan and does the grazer prefer naked over calcified coccolithophore cells? (Chapter 2)
- Does the coccosphere protect coccolithophores from being grazed by a calanoid copepod? (Chapter 3)

1.11 List of papers for thesis

The chapters of this doctoral thesis are based on the following three manuscripts:

I **Haunost, M.**, Riebsell, U., Bach, L.T. (2020): The calcium carbonate shell of *Emiliania huxleyi* provides limited protection against viral infection. Front. Mar. Sci. 7:530757, doi:10.3389/fmars.2020.530757

II **Haunost, M.**, Riebesell, U., D'Amore, F., Kelting, O., Bach, L.T (2021): Influence of the calcium carbonate shell of coccolithophores on ingestion and growth of a dinoflagellate predator. Front. Mar. Sci. 8:664269, doi:10.3389/fmars.2021.664269

III **Haunost, M.**, Riebesell, U., Taucher, J., Lischka, S., Bach, L.T., Riebesell *(to be submitted)*: The calcium carbonate shell of the coccolithophore *Gephyrocapsa oceanica* provides no protection against ingestion by the copepod *Acartia tonsa*.

1.12 Declaration of contribution

Manuscript I:

Idea: Mathias Haunost, Lennart Bach

Data acquisition: Mathias Haunost

Data interpretation and manuscript preparation: Mathias Haunost with contributions from Lennart Bach, and Ulf Riebesell

Manuscript II:

Idea: Mathias Haunost, Lennart Bach

Data acquisition: Mathias Haunost, Francesco D'Amore, Ole Kelting

Data interpretation and manuscript preparation: Mathias Haunost with contributions from Lennart Bach, Francesco D'Amore, Ole Kelting, and Ulf Riebesell

Manuscript III:

Idea: Mathias Haunost, Lennart Bach

Data acquisition: Mathias Haunost, Silke Lischka, Jan Taucher

Data interpretation and manuscript preparation: Mathias Haunost with contributions from Lennart Bach, Jan Taucher, Silke Lischka, and Ulf Riebesell

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2 | Manuscript I

The Calcium Carbonate Shell of *Emiliania huxleyi* Provides Limited Protection Against Viral Infection

M. Haunost[*,1], U. Riebesell[1], L.T. Bach[2]

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[1] GEOMAR Helmholtz Centre for Ocean Research Kiel, Kiel, Germany
[2] Institute for Marine and Antarctic Studies, University of Tasmania, Hobart, TAS, Australia

[*]corresponding author: mhaunost@geomar.de (M. Haunost)

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The Calcium Carbonate Shell of *Emiliania huxleyi* Provides Limited Protection Against Viral Infection

Mathias Haunost[1], Ulf Riebesell[1] and Lennart T. Bach[2]*

[1] GEOMAR Helmholtz Centre for Ocean Research Kiel, Kiel, Germany, [2] Institute for Marine and Antarctic Studies, University of Tasmania, Hobart, TAS, Australia

Coccolithophores are an important group of marine phytoplankton which cover themselves with the coccosphere – a shell composed of numerous calcium carbonate ($CaCO_3$) platelets. Despite more than a century of coccolithophore research, it remains speculative why coccolithophores calcify. Resolving this question is essential to assess the competitive fitness of coccolithophores in the future ocean where changes in calcification are expected. Here, we used the *Emiliania huxleyi* – *Emiliania huxleyi* Virus 86 host-virus model system to test the hypothesis that the coccosphere serves as a physical barrier reducing viral infection. Therefore, we removed the coccosphere from living *E. huxleyi* cells and compared the infection progress relative to calcified cells in a series of 6 experiments under different growth conditions. We found that the coccosphere does not constitute an effective physical barrier against viral penetration, since non-growing calcified cells were susceptible to viral infection and lysis (growth stopped by light limitation). However, we also found that protection against the virus may depend on the daily growth cycle. *E. huxleyi* reached higher peak abundances when decalcified cells were allowed to rebuild their coccosphere before entering cell division phase and being exposed to the virus, thereby suggesting that rates of viral infection could be reduced by the coccosphere during the critical phase in the cell cycle. However, the benefit of this potential protection is arguably of limited ecological significance since the concentrations of both, calcified and decalcified *E. huxleyi* approached similar values until the end of the bloom. We conclude that the coccosphere provides at best a limited protection against infection with the *Eh*V86.

Keywords: calcification, coccolithophores, *Emiliania huxleyi*, virus, phytoplankton, infection

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****Correspondence:***
Mathias Haunost
mhaunost@geomar.de

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INTRODUCTION

Coccolithophores are a group of marine planktonic algae which cover themselves with a shell (coccosphere) composed of multiple calcified platelets (coccoliths). They appear for the first time in the late Triassic and are present with variable diversity ever since (Bown et al., 2004). Coccolithophores contribute ∼1–10% to marine primary production (Poulton et al., 2007) and ∼50% to open ocean calcium carbonate ($CaCO_3$) sediments (Broecker and Clark, 2009).

Research on coccolithophore calcification has mostly focused on the intracellular mechanisms controlling calcification, the environmental factors influencing calcification, and the biogeochemical processes involving $CaCO_3$ (Riebesell et al., 2000; Brownlee and Taylor, 2004; Mackinder et al., 2010; Riebesell and Tortell, 2011). The question why coccolithophores calcify

has received much less attention so far but was recently highlighted as one of the most critical knowledge gaps in coccolithophore research (Monteiro et al., 2016). Without understanding the purpose of this key trait, it will be difficult to determine the relevance of projected changes in calcification rates for the competitive fitness of these organisms in the future ocean (Bach et al., 2015). Numerous hypotheses exist why coccolithophores calcify (Young, 1994; Raven and Crawfurd, 2012; Taylor et al., 2017; Müller, 2019). Recently, Monteiro et al. (2016) pointed out that the coccosphere may have different functions in different coccolithophore species but that the protection against grazing and/or virus and bacterial infection is potentially the one function that could be of universal benefit to most of them.

Emiliania huxleyi is the most abundant coccolithophore in the contemporary oceans (Tyrrell and Young, 2009) and regularly forms large blooms which are often terminated by viral infections (Bratbak et al., 1993; Brussaard et al., 1996; Wilson et al., 2002b; Schroeder et al., 2003). Some *E. huxleyi* viruses (*Eh*Vs) have been isolated from water samples during those algal blooms (Castberg et al., 2002; Schroeder et al., 2002; Wilson et al., 2002a) and the *E. huxleyi*-*Eh*V system has been frequently studied as a representative host-virus model within the eukaryotic phytoplankton.

The *Eh*Vs are coccolithoviruses which belong to the *Phycodnaviridae* and are nucleocytoplasmic large double-stranded DNA viruses (Schroeder et al., 2002). The genetic material in the *Eh*V virion is encased in a capsid of icosahedral shape and surrounded by a lipid membrane. The virion is thought to enter the host via membrane fusion (Mackinder et al., 2009) where the attachment takes place at distinct lipid-raft-microdomains in the cell membrane characterized by aggregations of specific glycosphingoplipids (Vardi et al., 2009, 2012; Bidle and Vardi, 2011). The virus possesses RNA polymerase genes and presumably replicates at least partly in the cytoplasm (Wilson et al., 2005). In the course of its replication cycle the virus modifies its host's lipid synthesis (Evans et al., 2009; Fulton et al., 2014; Rose et al., 2014; Rosenwasser et al., 2014; Hunter et al., 2015), makes use of its programed cell death pathway (Bidle et al., 2007; Vardi et al., 2009; Bidle and Vardi, 2011), and affects autophagy-like processes to generate viral progeny (Schatz et al., 2014). New virions are released by a budding mechanism (Mackinder et al., 2009) which ultimately leads to the lysis of the host cell.

It has been suggested that the coccoliths serve as a physical barrier which block the virus from entering the cell (Castberg et al., 2002; Mackinder et al., 2009). Indeed, microscopic observations indicated that viral particles were blocked by the coccosphere and detached again quickly from calcified cells of *E. huxleyi* (Mackinder et al., 2009). Johns et al. (2019) recently provided evidence that loose coccoliths in the water column protect against viral infection by binding free virions which then become unable to infect further hosts.

To further clarify the role of the coccosphere in viral infection we conducted several culture experiments to test whether the coccosphere could improve growth and resistance of *E. huxleyi* when exposed to the *E. huxleyi* Virus 86. We removed the coccospheres of the cells by a short acid-base "decalcification" treatment or by growing cells in medium with depleted calcium ion concentration. Naked and calcified cells were then exposed to the virus to test whether coccolith bearing cells are better protected against infection. Our hypothesis tested in this study is that the coccosphere of *E. huxleyi* reduces viral penetration into the host cell thereby increasing their survival.

MATERIALS AND METHODS

Overview

We conducted 6 experiments in which we exposed cultures of *E. huxleyi* (CCMP1516) to the *Eh*V86 and monitored the abundances of the host and the virus over time. In each experiment we compared the course of infection between calcified cells and naked cells, of which the coccospheres were removed by either an acid-base treatment or by growing them in Ca^{2+}-depleted medium (detailed description below). We then exposed naked and calcified cells at equal cell densities to the *Eh*V and measured the abundances of host cells and viral particles over the following days. At the same time, we monitored the abundance of *E. huxleyi* in cultures without virus to examine whether the respective coccosphere-removal procedure influenced the development of *E. huxleyi*. Consequently, each experiment was conducted in a 2 × 2 factorial design (calcified without virus, naked without virus, calcified with virus, naked with virus). The 6 experiments described in this paper differ (i) in the way the coccoliths were removed (acid-base treatment or low [Ca^{2+}] medium) and (ii) whether the virus was added to actively growing cultures in light conditions, or to cultures where growth was stopped by keeping them in the dark. Each of the two coccosphere-removal methods (acid-base treatment or low [Ca^{2+}] medium) was conducted once in a light-dark cycle and once in permanent darkness. Thereafter, we conducted a further acid-base experiment in a light-dark cycle, but modified the timing of the coccosphere removal and the virus addition. At the same time, we tested whether coccoliths that detached from the cells surface reduce the number of infective particles.

A detailed description of each of the 6 experiments is provided later in the "Materials and Methods" section after we have described the applied methodology. **Table 1** provides an overview of the experiments.

TABLE 1 | Overview of the experiments.

Experiment	Treatments	Light conditions
1	Calcified vs. acid-base treated cells	12:12 h light-dark cycle
2	Calcified vs. low [Ca^{2+}] treated cells	12:12 h light-dark cycle
3	Calcified vs. acid-base treated cells	Permanent darkness
4	Calcified vs. low [Ca^{2+}] treated cells	Permanent darkness
5	Calcified vs. acid-base treated cells; The difference to 1 is the timing of the virus addition	12:12 h light-dark cycle
6	Detached coccoliths only vs. dissolved coccoliths via acid-base treatment	12:12 h light-dark cycle

Basic Culturing Conditions

The culture medium was prepared with sterile filtered (0.2 μm) artificial seawater (Kester et al., 1967). We added sodium bicarbonate to gain a total alkalinity of 2350 μmol kg^{-1} and aerated the artificial seawater over night to ensure atmospheric equilibrium regarding the carbonate system. The artificial seawater was enriched with 64 μmol kg^{-1} $NaNO_3$, 4 μmol kg^{-1} NaH_2PO_4, 10 nmol kg^{-1} SeO_2, vitamins and trace metals according to the f/8 medium (Guillard and Ryther, 1962). Cultures of *E. huxleyi* were grown in a 12:12 h light-dark cycle with a photon flux density (PAR) of 230 μmol photons m^{-2} s^{-1} (measured with a LI-COR LI-250A light meter) at 15°C until they were used for the respective experiments.

Procedures to Remove the Coccospheres

In the low [Ca^{2+}] experiments, naked cells of *E. huxleyi* were obtained by using artificial seawater with a 100-fold lower Ca^{2+} concentration (0.1 mmol kg^{-1}) than in the usual recipe by Kester et al. (1967). Apart from the calcium concentration (and therefore a slightly lower salinity of 33.7) the culture medium and the culture conditions were the same as mentioned above.

For the decalcification experiments, we conducted previous tests to ascertain the gentlest way to dissolve the calcite shell with acid and base without causing too much harm to the cells. The best results, in terms of a complete removal of the coccosphere at a minimum number of cell death, were obtained by using 2.5 mL of 1N hydrochloric acid (HCl) per L of *E. huxleyi* culture with ∼50 × 10^3 cells mL^{-1}. After the addition of the acid, the culture bottle was mixed gently, but thoroughly for 1 min. Thereafter, the pH was brought back to the value before the acid addition by adding 1N sodium hydroxide (NaOH) solution. The culture bottle was gently mixed once more after the NaOH addition until all flocculation from the addition of NaOH was dissolved (∼2 min of mixing was required for this). The decalcification procedure was conducted with a large culture volume (2.3 L) to keep the headspace at a minimum relative to the volume of the culture. This was done to prevent elevated outgassing of CO_2 during the brief low pH/high CO_2 period. The addition of HCl and NaOH caused a decrease in the cell concentrations of 7–10%, likely because cells died in consequence of the direct contact with the concentrated acid and base during injections. To assure no further cell death after the treatment, we determined the cell concentration repeatedly by flow cytometry (see below) over a period of 30 min to ensure that it remained stable. To account for the fraction of dead cells in the decalcified cultures, the cell concentration in the calcified culture was diluted to the same level prior to the start of the experiments. This was done by filtering out the excess cells with a 0.2 μm syringe filter.

The absence of coccoliths after the acid base treatment was checked by flow cytometry and by cross polarized light microscopy using an inverted microscope (Carl Zeiss Axiovert 100). This microscopy method reveals calcium carbonate as bright shining crystals where calcite in and outside *E. huxleyi* can be easily seen (**Figure 1**).

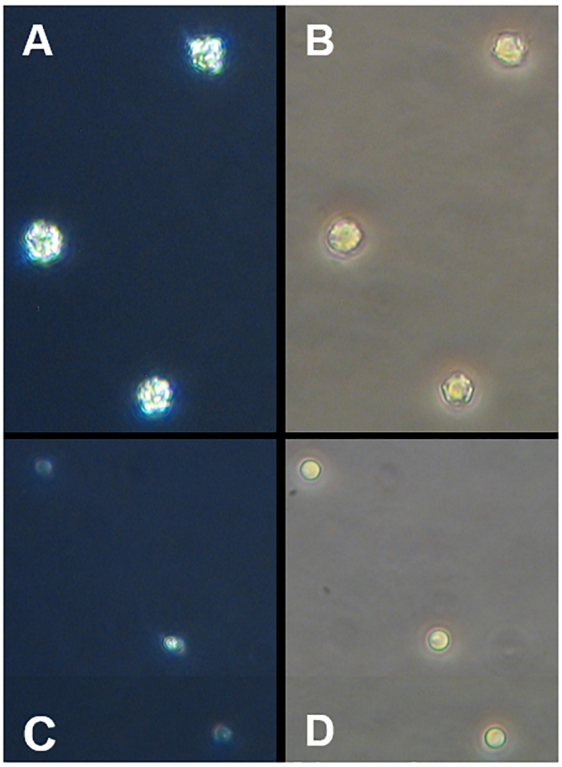

FIGURE 1 | Microscopic images of *E. huxleyi* (400x magnification). **(A)** Calcified cells under polarized light, **(B)** light microscopic picture from the same cells, **(C,D)** display cells after the treatment with acid and base.

Preparation of Virus Lysate

Fresh isolates of the *Eh*V86 were produced with *E. huxleyi* CCMP1516 cultures. Therefore, cultures of the host cells were grown to a concentration ∼ 250 × 10^3 cells mL^{-1} in 15°C and then inoculated with 0.5 mL viral lysate. The original virus lysate was kindly provided by Dr. Declan Schroeder. The *E. huxleyi* population crashed within 5 days due to viral infection. The lysate was 0.45 μm filtered and stored at 4°C in the dark until it was used for the experiments (the storage time of lysate was between 1–5 days).

Enumeration of Algal Cells and Viral Particles

Subsamples for cell counts were taken by transferring 1 mL of the cultures into Eppendorf tubes. Algal cell concentrations were measured at a flow rate of 66 μL min^{-1} in an Accuri C6 flow cytometer (Becton Dickinson). *E. huxleyi* could be determined based on the chlorophyll fluorescence and the side-scatter signal (SSC). The SSC is the light scattered at right angle when the cells pass the laser beam of the flow cytometer and it is expressed in values without a unit. Calcified cells scatter more light at right angle and thus induce higher SSC signals compared to naked cells (Olson et al., 1989).

Viral particles were quantified following Brussaard (2004). Briefly, 1 mL subsamples were transferred into Cryovials and

fixed with electron microscopy grade glutaraldehyde (0.25% final concentration). The samples were incubated at 4°C for 30 min then flash-frozen in liquid nitrogen and stored at -80°C. For the analysis the samples were diluted in TE (Tris-EDTA, pH 8) buffer, stained with SYBR Green I, heated for 10 min at 80°C and measured with a FACSCalibur flow cytometer (Becton Dickinson) at low flow rate of ca. 15 μL min^{-1}. The viral particles in the samples could be identified based on the SYBR Green labeled DNA fluorescence and the SSC.

Experimental Setup

Experiment 1 (Acid-Base/Light-Dark Cycle)

In Experiment 1 calcifying *E. huxleyi* (CCMP1516) were grown in a 12:12 h light-dark-cycle and naked cells were obtained with the acid-base treatment. *E. huxleyi* was grown in a volume of 5 L to a concentration of $\sim$35 $\times$ 10^3 cells mL^{-1}. Thereupon the culture was split into two 2.3 L polycarbonate bottles. One of the bottles was treated with acid and base as described above to remove the coccoliths, while the other bottle that contained calcified cells was only diluted with growth medium to adjust the cell concentrations. Both cultures were further split into six 250 mL glass bottles. Three of these smaller bottles were inoculated with viral lysate while no virus was added into the other three bottles. All these steps were conducted at the end of the dark period under low light conditions ($<$ 0.01 μmol photons m^{-2} s^{-1}). The virus was inoculated just minutes before the following light period began (please note that the timing of the coccoliths removal and subsequent virus addition is important as will be discussed later). The virus lysate was pipetted in equal amounts into the replicate bottles. The cultures were homogenized every 1.5 h over the day by gently turning the bottles and mounted on a plankton wheel (one round min^{-1}) during night time in order to prevent unequal sedimentation of the algae among the treatments.

We performed Experiment 1 also (and simultaneously) with a non-calcifying strain of *E. huxleyi* (RCC 1242). This was done to investigate the consequences of the acid-base treatment on a strain that does not form coccoliths and to reveal possible effects of the acid-base treatment on the interplay between the host and the virus irrespective of calcification.

Experiment 2 (Low [Ca^{2+}]/Light-Dark Cycle)

In Experiment 2 naked cells of *E. huxleyi* were obtained using low [Ca^{2+}] growth medium. Apart from this, the procedures were similar as in Experiment 1. *E. huxleyi* was grown in two bottles (2.3 L), one of which contained normal medium and the other contained medium with a low [Ca^{2+}]. Both cultures were grown in a 12:12 h light-dark cycle to 35 – 40 $\times$ 10^3 cells mL^{-1}. The *E. huxleyi* concentrations in both bottles were diluted to the same level with the respective medium and then each culture was further subdivided into six 250 mL glass bottles. These steps were performed at the end of the dark phase under very low light conditions. Virus lysate was added in equal amounts into three bottles with low [Ca^{2+}] and three bottles with normal growth medium. Thereafter, all 12 culture bottles were put back into the light.

Experiment 2 was also conducted simultaneously with the non-calcifying strain of *E. huxleyi* to test whether the low [Ca^{2+}] only prevented calcification, or had further impacts on the host-virus interaction.

Experiment 3 (Acid-Base/Permanent Darkness)

In Experiment 3 *E. huxleyi* was raised in a 5 L bottle up to $\sim$40 $\times$ 10^3 cells mL^{-1} in a 12:12 h light-dark-cycle. When this concentration was reached the culture was kept in the dark for the remainder of the experiment. After 36 h in darkness, the culture was split into two 2.3 L bottles and one was treated with acid and base as described above. The 36 h period in the dark ensured that all metabolic energy reserves for calcification were consumed before the cells were decalcified. Previous experiments had shown that the decalcified cells were able to rebuild their coccosphere in the dark when the decalcification treatment was applied after a regular 12 h dark phase.

Subsequently, both cultures were further split into the six replicate bottles and the virus was added to three of them.

Experiment 4 (Low [Ca^{2+}]/Permanent Darkness)

Experiment 4 was identical to Experiment 3 except that the naked cells were obtained by growth in low [Ca^{2+}] medium. *E. huxleyi* was grown in two cultures (2.3 L), one with low [Ca^{2+}] medium and the other with normal growth medium. *E. huxleyi* was grown to $\sim$70 $\times$ 10^3 cells mL^{-1} in a 12:12 h light-dark cycle whereupon the culture was kept in the dark from then onward. After 36 h the cell concentrations in both bottles were adjusted to the same level by diluting the calcified culture with the respective medium. Subsequently, both cultures were further split into the 6 replicate bottles and the virus was added to three of them.

Experiment 5 (Acid-Base/Light-Dark Cycle)

In Experiment 5 naked *E. huxleyi* were obtained with the acid-base treatment. *E. huxleyi* was grown to $\sim$100 $\times$ 10^3 cells mL^{-1} in a 12:12 h light-dark cycle. The culture was split into two bottles and one was treated with acid and base. Then, both cultures were brought to the same cell concentration and further split into the replicate bottles. Experiment 5 was conducted in the same way as Experiment 1 except of one difference. The dissolution of the calcite and the subsequent addition of the virus were carried out in the middle of the light period, instead of the end of the dark period. That way, *E. huxleyi* was able to calcify for half of the light period (6 h) before the experiment started.

Experiment 6 (Absorption of Viral Particles by Coccoliths)

In Experiment 6 we tested the influence of coccoliths that are lost from the coccosphere on the viral particle concentration. For this purpose, a fraction of the initial 5 L stock culture from Experiment 5 was gently filtered through a 5 μm syringe filter. The filtrate contained no *E. huxleyi* cells but detached coccoliths which are smaller than 5 μm. The suspension with detached coccoliths was split into two bottles one of which was treated with acid and base (see above) in order to dissolve the coccoliths. Both bottles were further separated into triplicates and virus stock culture was added in equal amounts. The number of viral particles was measured in both

treatments after 24 and 48 h to test whether the viral particle concentrations were lower in the replicates that contained detached coccoliths.

Data Analysis

In each experiment the concentrations of *E. huxleyi* in the four treatment groups were compared over time based on the sample mean ($\overline{X}$) ± 95% confidence interval (CI), which was calculated with the standard error (se) and the respective t-distribution ($\overline{X} \pm t^{n-1} * se$). Each treatment group contained n = 3 replicates. We calculated the mean logarithmic response ratio (L) ± 95% CI (Hedges et al., 1999) to measure the effect the virus caused within the treatments at a given sampling time point:

$$L = \ln\left(\frac{\overline{X_P}}{X_a}\right) \pm t_{n+n-2} * \sqrt{v},$$

with the vairance $v = \frac{SD_p^2}{n_p \overline{X}_p^2} + \frac{SD_a^2}{n_a \overline{X}_a^2}$, where the subscripts p and a describe whether the virus was present or absent and SD is the sample standard deviation. L standardizes the ratio of the mean cell concentration in the infected cultures and the mean cell concentration from the respective replicates without virus. This was done because both methods that were used to remove the coccosphere had the potential to affect the concentration of *E. huxleyi*. For example, when L = 0 = ln (1), there is no effect and the mean cell concentration of the replicates with virus did not differ from the mean host abundance of the respective replicates without virus. A value of L = −0.69 = ln (0.5) could illustrate that *E. huxleyi* reached only half the concentration in the cultures that were exposed to the virus than in the respective cultures without virus. The effect of the virus within the treatments, calcified vs. naked cells, was then compared based on the effect size and the corresponding 95% CI between the treatments. We consider the effect size to be significantly different between the treatments when the confidence intervals do not overlap (α = 0.05).

RESULTS

Experiment 1 (Acid-Base/Light-Dark Cycle)

When no virus was added, the concentrations of the calcified and decalcified cells did not differ (**Figure 2A**). In both groups the cells continued to grow exponentially and the acid-base treatment did not affect the growth of *E. huxleyi*. When the virus was present, the maximum abundances of *E. huxleyi* were measured 24 h post infection. Afterwards, the cell concentrations decreased due to infection and death in both treatments (**Figure 2B**). An effect of the virus was observed 24 h post infection in both, the calcified ($L_{control} = -0.12 \pm 0.05$) and the decalcified ($L_{acid-base} = -0.20 \pm 0.02$) cultures and the decalcified cells showed a marginally, but significantly lower mean abundance. After 2 days, however, the concentrations were equal again in both treatments and showed the same temporal development until the end of the experiment (**Figure 2B**). The number of viral particles increased drastically between day one and two post infection and did not differ between the treatments (**Figure 2C**).

The acid-base treatment had a positive effect on the growth of the non-calcifying *E. huxleyi strain* (RCC1242) (**Figures 2D,E**). The cells which were treated with acid and base showed higher abundances than the controls. This difference seemed to be more pronounced in the cultures that were exposed to the virus (**Figure 2E**) than in those without virus (**Figure 2D**). However, a significant difference in effect size between the treatments could be observed at no time. When the cell concentrations in the infected cultures were set in relation to the concentrations the cells reached without virus, the effect of the virus was similar in both treatments. The collapse of the cultures in consequence of viral infection was much slower compared to the calcifying *E. huxleyi* CCMP1516. The numbers of viral particles increased in both treatments after 2 days and it was slightly higher in the untreated control cultures (**Figure 2F**). On day 4 and 5 post infection, the concentration of viral particles was higher in the acid-base treatment. However, at this time there were also more *E. huxleyi* cells present. Thus, there were more hosts for viral replication.

Figure 3 shows the concentrations of the calcified *E. huxleyi* CCMP1516 within the first 24 h in Experiment 1, as well as the respective side scatter (SSC) of the cells from the flow cytometry measurements. The SSC is indicative for the degree of calcification of a cell (Hansen et al., 1996) and it increases with an increasing amount of coccoliths on the cells surfaces. The data show a typical pattern of the SSC in relation to the daily light cycle in all treatment groups. The values increased over the course of the light period and were highest at the beginning of the dark phase (**Figure 3A**). The cells divided in the night (**Figure 3B**) which caused a decrease of the SSC because the coccoliths were shared between two daughter cells. The mean SSC of the decalcified cells was about 68% lower right after the acid-base treatment compared to the calcified cells. In the course of the subsequent light period the decalcified cells reconstructed their coccospheres and their SSC increased again. They apparently formed a new coccosphere within 6 h, because at that time their SSC reached the same values as measured from the calcified cells at the start of the experiment (**Figure 3A**). After 24 h, the differences in the SSC between the decalcified and the calcified cells were almost compensated.

Experiment 2 (Low [Ca^{2+}]/Light-Dark Cycle)

E. huxleyi grew minimally slower in the low [Ca^{2+}] medium and reached lower maximum abundances than in normal growth medium (**Figure 4A**). The cells did not produce coccoliths under low [Ca^{2+}]. The virus stopped growth of the calcified cells after 24 h (**Figure 4B**) ($L_{control} = -0.39 \pm 0.09$, $L_{low[Ca2+]} = -0.02 \pm 0.26$) whereas it took 48 h in the low [Ca^{2+}] treatment ($L_{control} = -1.53 \pm 0.05$, $L_{low[Ca2+]} = -0.27 \pm 0.10$). *E. huxleyi* reached substantially higher abundances under viral infection in the low [Ca^{2+}] treatment (**Figure 4B**). Concomitantly, the production of viral particles was initially lower under low [Ca^{2+}], but toward the end of the experiment

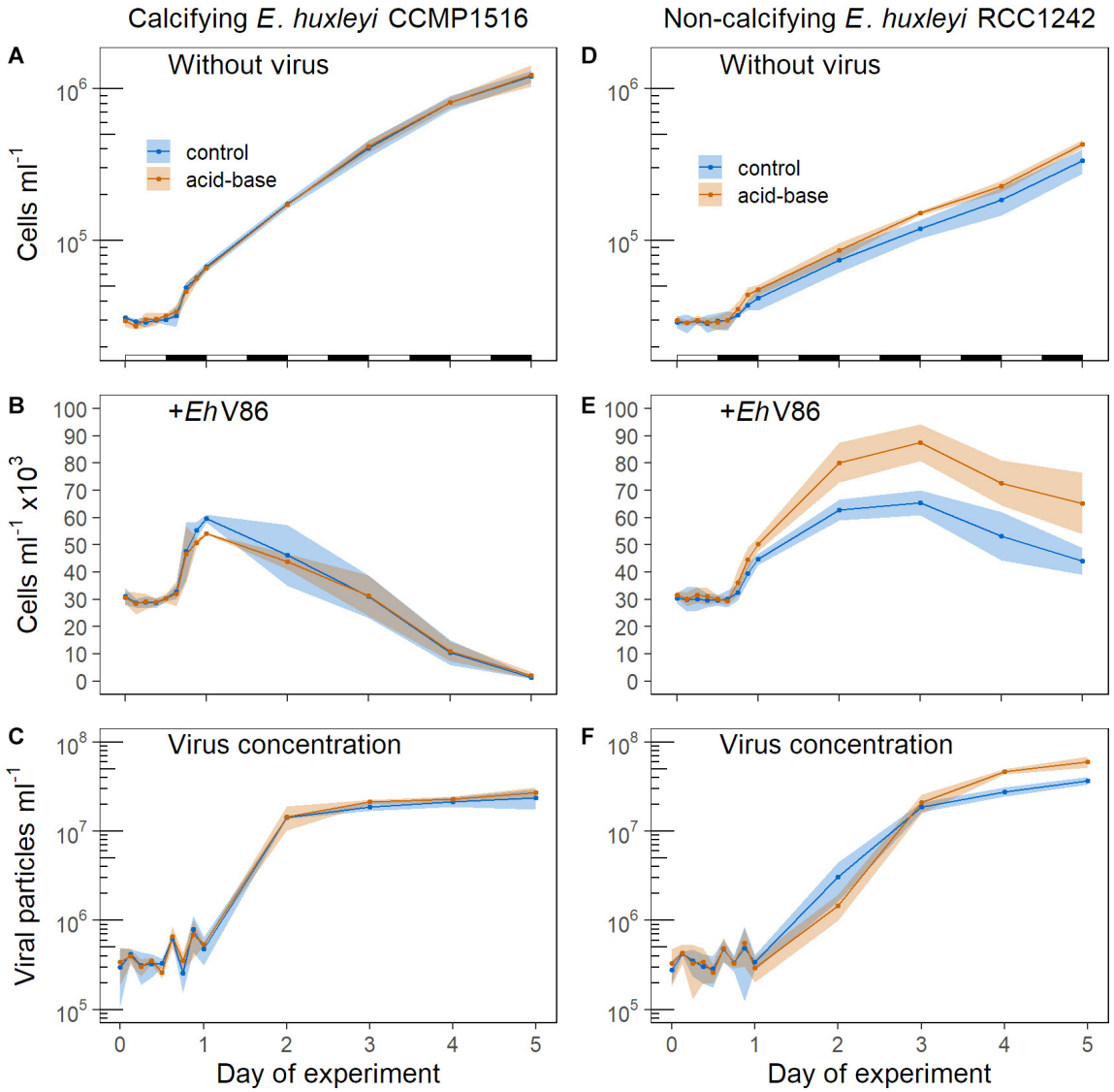

FIGURE 2 | Addition of virus to normal cells of *E. huxleyi* (controls = blue symbols) and to cells treated with acid and base (red symbols). Virus was added at the beginning of the light period (12:12 h light-dark cycle). Symbols represent the sample means and ribbons illustrate the 95% CI of the sample mean (n = 3). The acid-base treatment was conducted at the end of the dark phase before virus addition. **(A)** Concentration of calcifying *E. huxleyi* CCMP1516 without virus; **(B)** cell concentration when virus was added; **(C)** concentration of viral particles. **(D)** Concentrations of non-calcifying *E. huxleyi* RCC1242 without virus and **(E)** when virus was added; **(F)** concentration of viral particles. Light-dark cycle is indicated in **(A,D)**.

the concentration of viral particles was higher (**Figure 4C**) in line with a higher concentration of host cells.

The non-calcifying *E. huxleyi* (RCC1242) showed equal growth in both treatments when no virus was added (**Figure 4D**). When the virus was present, non-calcifying *E. huxleyi* reached higher abundances in the low [Ca^{2+}] treatment which is similar to the response observed in the calcifying strain (compare **Figures 4B,E**).

Overall, we observed that the effect of the virus was weakened under low [Ca^{2+}] in both, the calcifying and the non-calcifying strain of *E. huxleyi*. Thus, [Ca^{2+}] influenced the infection of *E. huxleyi* by *Eh*V86 irrespective of whether the cells possessed a coccosphere or not.

Experiment 3 (Acid-Base/Permanent Darkness)

Without virus, the concentrations of the calcified *E. huxleyi* remained stable throughout the prolonged darkness (**Figure 5A**). A significant decrease of the calcified cells due to viral infection could be observed from day 6 post infection ($L_{control} = -0.13 \pm 0.06$) (**Figure 5B**). Thus, the virus was able

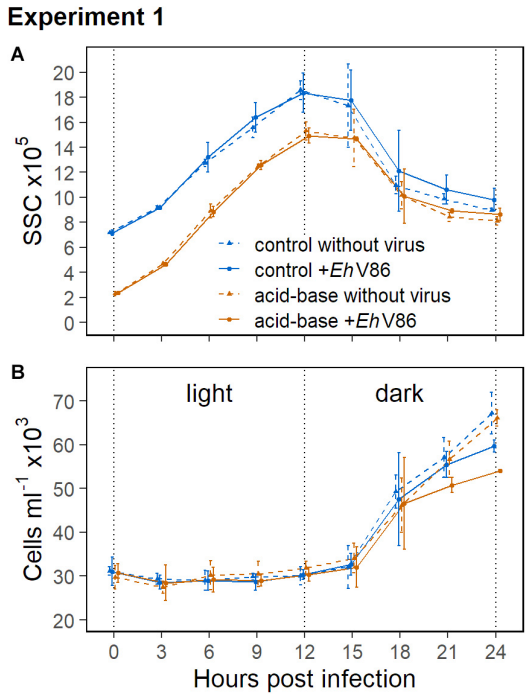

FIGURE 3 | Results of the calcifying *E. huxleyi* (CCMP1516) within the first 24 h of Experiment 1. Decalcified cells scatter less light (SSC) than calcified cells **(A)**. The SSC increased during the light period and cell division took place in the dark **(B)**. Mean ± 1 SD (n = 3).

to infect *E. huxleyi* through the coccosphere even though cell division did apparently not occur.

In the dark, *E. huxleyi* was not able to cope with the acid-base treatment as good as in the light (where growth was basically unaffected, **Figure 2A**), as can be seen in the declining cell numbers (**Figure 5A**). An effect of the virus on the decalcified cells could be measured on day 7 ($L_{acid-base} = -0.31 \pm 0.19$) and 8 ($L_{acid-base} = -0.47 \pm 0.22$), but on day 9 the variability of the cell concentrations in the cultures without virus increased considerably (**Figure 5A**). The decrease of the cell concentrations due to the virus did not differ between the treatments. However, the production of new viral particles was higher in the cultures that contained decalcified cells (**Figure 5C**).

Experiment 4 (Low [Ca^{2+}]/Permanent Darkness)

The concentrations of the calcified *E. huxleyi* remained stable for 6 days in the dark without virus, while the low [Ca^{2+}] cells decreased in concentration (**Figure 6A**). Experiment 4 confirmed that the virus was able to infect the calcified cells in the darkness when cell division ceased (**Figure 6B**). The concentrations showed a strong decrease on day 4 post infection ($L_{control} = -0.60 \pm 0.12$) and declined steadily thereafter. The concentration of viral particles increased (**Figure 6C**). Interestingly, from day 4 post infection onward we measured a positive effect of the virus on the algal concentration under low [Ca^{2+}] ($L = 0.20 \pm 0.04$, $L = 0.31 \pm 0.07$ on day 9). The *E. huxleyi* concentration was higher when the virus was present (compare **Figures 6A,B**). Thus, the treatment did not only prevent calcification, but in some way influenced the constitution of the host cells, or the interplay between the host and the virus. In the low [Ca^{2+}] treatment, the presence of the virus seemed to promote the survival of the host cells in permanent darkness. From day 4 until the end of the experiment the cell concentrations were consistently higher in the infected cultures than in those without virus ($L_{low[Ca2+]} = 0.20 \pm 0.04$, $L_{low[Ca2+]} = 0.31 \pm 0.07$ on day 9). The viral particle concentration remained stable under low [Ca^{2+}] over the course of the experiment.

Experiment 5 (Acid-Base/Light-Dark Cycle)

In Experiment 5 we tested whether the calcifying *E. huxleyi* were better protected against viral infection when the cells were able to calcify for 6 h in the light before the virus was added. Therefore viruses were not added directly at the beginning of the light period (as it was done in Experiment 1) when the population had just gone through cell division. Instead, the virus was added 6 h after the light phase had begun. During this period the cells were able to produce additional coccoliths.

Without virus, the concentrations of the decalcified and the calcified cells showed a similar development (**Figure 7A**). However, in the presence of the virus the decalcified cells reached substantially lower peak concentrations than the calcified cells (**Figure 7B**; but note that the mean cell concentration at the start of the experiment was about 3% lower in the acid-base treatment, because the balancing of the concentrations of both treaments did not work out precisely). Indeed, at the onset of the following light period (18 h post infection) the virus had a stronger effect on the decalcified cells ($L_{acid-base} = -0.56 \pm 0.03$) than on the calcified cells in the control treatment ($L_{control} = -0.20 \pm 0.03$). The differences between the treatments increased even further 24 h post infection ($L_{acid-base} = -0.76 \pm 0.09$, $L_{control} = -0.28 \pm 0.02$). Additionally, the initial production of viral particles was higher in the replicates which contained decalcified cells (**Figure 7C**). Please note that the samples for the quantification of viral particles taken at the start of this experiment (day 0) were lost. Thus, the value shown at day 0 is the mean of three subsamples taken from the initial virus lysate solution, which was pipetted in equal amounts into the replicates, as starting point for both treatments (**Figure 7C**). (Cytograms of Experiment 5 are provided as **Supplementary Material**).

Experiment 6 (Absorption of Viral Particles by Coccoliths)

In Experiment 6 we tested whether the difference in viral infection between calcified and decalcified cells was due to the adsorption of viral particles to detached coccoliths. Therefore, viral lysate was added to a suspension containing only detached coccoliths and the number of viral particles was compared to cultures in which the coccoliths in the suspension were dissolved with acid and base, before the virus was added. The coccolith suspension was obtained from the initial culture used

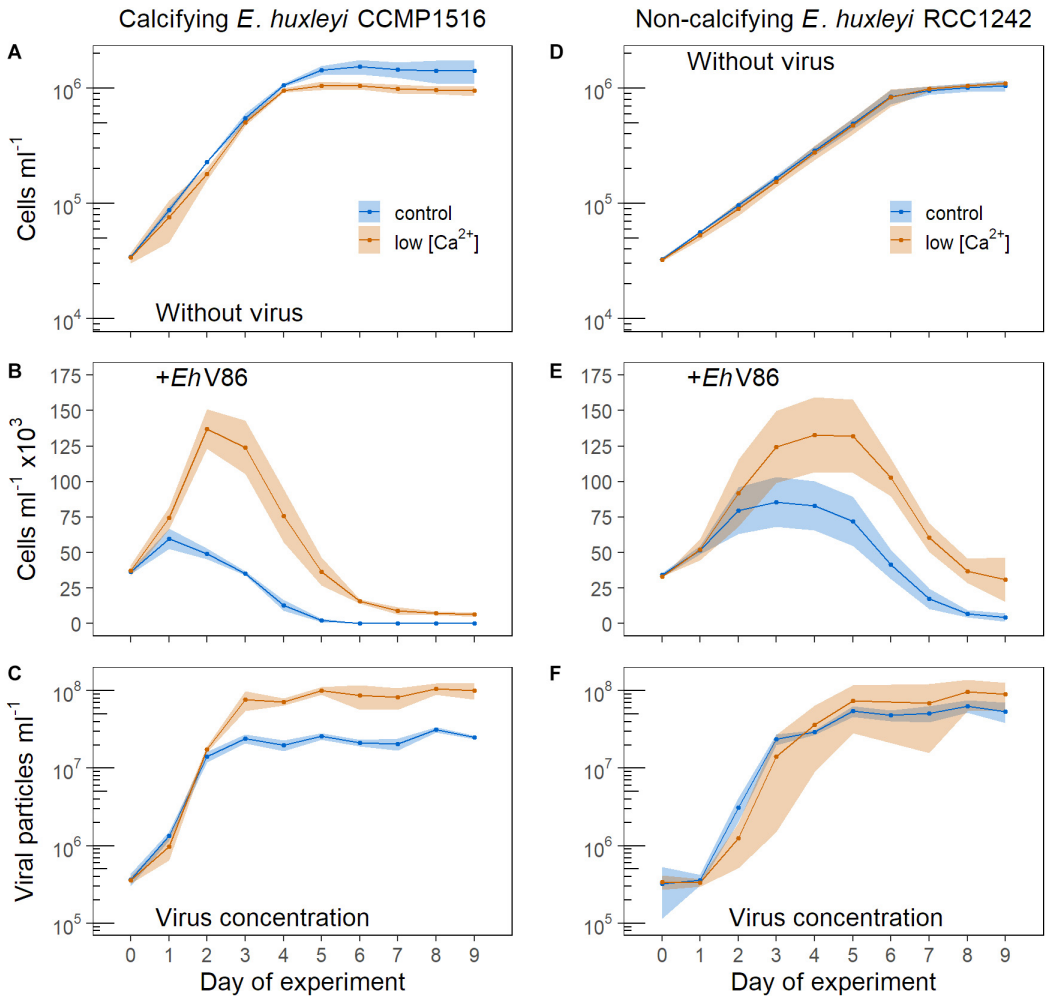

FIGURE 4 | Addition of virus to normal cells of *E. huxleyi* (controls = blue symbols) and to cells that were grown under low [Ca^{2+}] (red symbols). Virus was added at the beginning of the light period (12:12 h light-dark cycle). Symbols represent the sample means, ribbons the 95% confidence interval (n = 3). **(A)** Concentration of calcifying *E. huxleyi* CCMP1516 without virus and **(B)** when virus was added; **(C)** concentration of viral particles. **(D)** Concentration of non-calcifying *E. huxleyi* RCC1242 without virus and **(E)** when virus was added; **(F)** concentration of viral particles.

in Experiment 5, prior to its separation into the treatments, by filtering out the *E. huxleyi* cells. The idea was to set up the same ratio of detached coccoliths to viral particles as in Experiment 5, in which the protective effect of the coccosphere was tested. The number of viral particles did not decrease stronger in the replicates in which coccoliths were present (**Figure 8**). An effect of the coccoliths on the viral abundance could not be observed with this approach.

DISCUSSION

The Efficiency of the Coccosphere to Reduce Viral Infection

It has been hypothesized that the coccosphere can protect *E. huxleyi* from becoming infected with the virus and that the viral particles mainly attack the host during cell division (Castberg et al., 2002; Mackinder et al., 2009). When the cell divides, parts of the cell surface are not covered with coccoliths so that the virus should more easily reach and attach to the hosts plasma membrane to enter the cell. Nevertheless, infection can still occur through an intact coccosphere, probably due to gaps between the coccoliths (Mackinder et al., 2009). In both experiments that were conducted in permanent darkness, the virus was able to infect the calcified *E. huxleyi*, although cell division did not occur (**Figures 5**, **6**). These findings support the notion that the *Eh*V86 particles can penetrate the coccosphere to reach the cell membrane. However, the dark experiments did not conclusively show if the coccosphere could reduce viral infection rates because both, the acid-base as well as the low [Ca^{2+}] treatment also influenced the concentrations of the naked cells that were not exposed to the virus. In the light-dark cycle

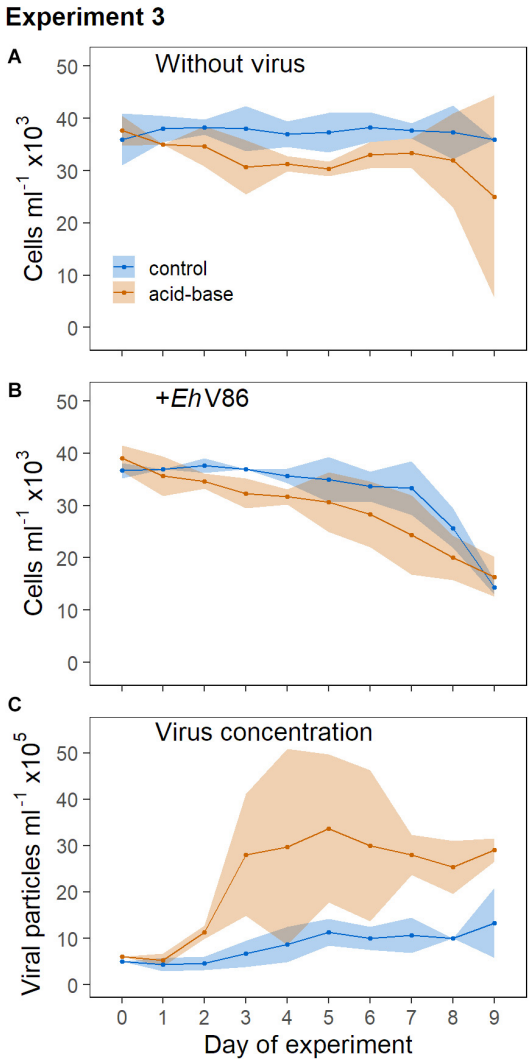

FIGURE 5 | Addition of virus to calcified and decalcified cells in permanent darkness. Sample mean ± 95% CI (n = 3). **(A)** Concentration of calcifying *E. huxleyi* CCMP1516 without virus and **(B)** when virus was added; **(C)** concentration of viral particles.

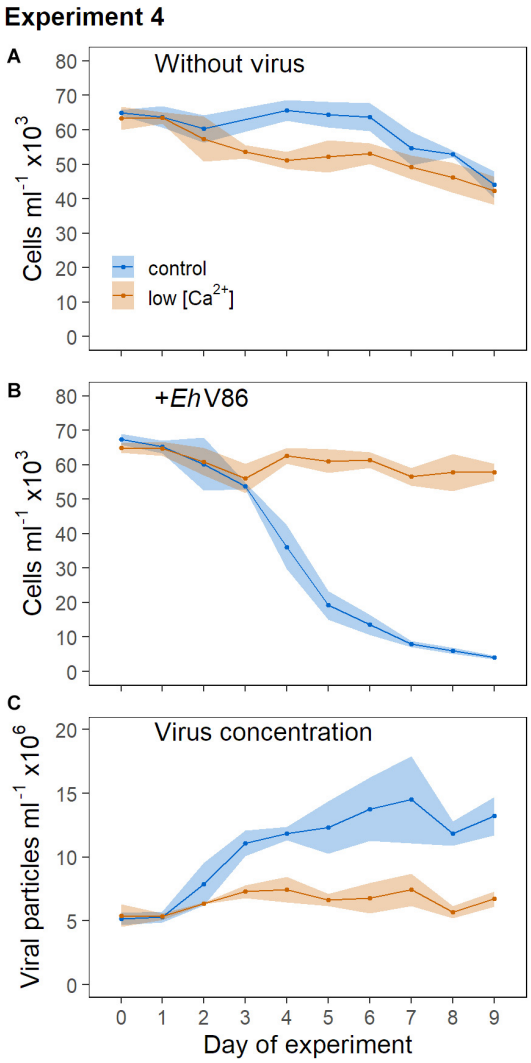

FIGURE 6 | Addition of virus to calcified cells and cells under low [Ca^{2+}] in permanent darkness. Sample mean ± 95% CI (n = 3). **(A)** Concentration of calcifying *E. huxleyi* CCMP1516 without virus and **(B)** when virus was added; **(C)** concentration of viral particles.

experiments, the low [Ca^{2+}] mitigated the course of infection in both strains of *E. huxleyi*, but the acid-base treatment did not alter the effect of the virus on non-calcifying *E. huxleyi*. We therefore had a closer look at the decalcification experiments in the light as will be discussed in the following.

Exponentially growing cells of *E. huxleyi* commonly show a synchronized cell cycle along with the light-dark-cycle, whereby the cells grow during the day and divide in the dark phase (Paasche, 1967; Jacquet et al., 2002; Müller et al., 2008). This was also the case in the light-dark cycle Experiment 1, where the virus was added to the growing culture at the onset of the light period and thus right after the majority of the population had just divided (**Figure 3**). At this point the coccospheres had just been distributed between the dividing cells. We hypothesized that viral particles could more easily reach the organic part of the cells in this phase because the coccosphere would have more gaps between the coccoliths directly after cell division. And indeed, the difference in peak abundance between the calcified and the decalcified *E. huxleyi* cells was relatively small (**Figure 2**). To understand if the assumed gaps influence infection we conducted Experiment 5, where the cells had 6 h in the light to calcify before the acid-base treatment was conducted and the virus was added. However, Experiment 5 did not confirm this hypothesis. The effect of the virus on the calcified cells was the same as in Experiment 1 (compare **Figures 2B**, **7B**). In both experiments, the calcified *E. huxleyi* approximately doubled in number before the concentrations declined. Thus, the infection of calcified cells does rather not depend on the length of time the cells have in the light to build the coccosphere. Paasche (2002) noted that cells of *E. huxleyi* are completely covered with coccoliths directly

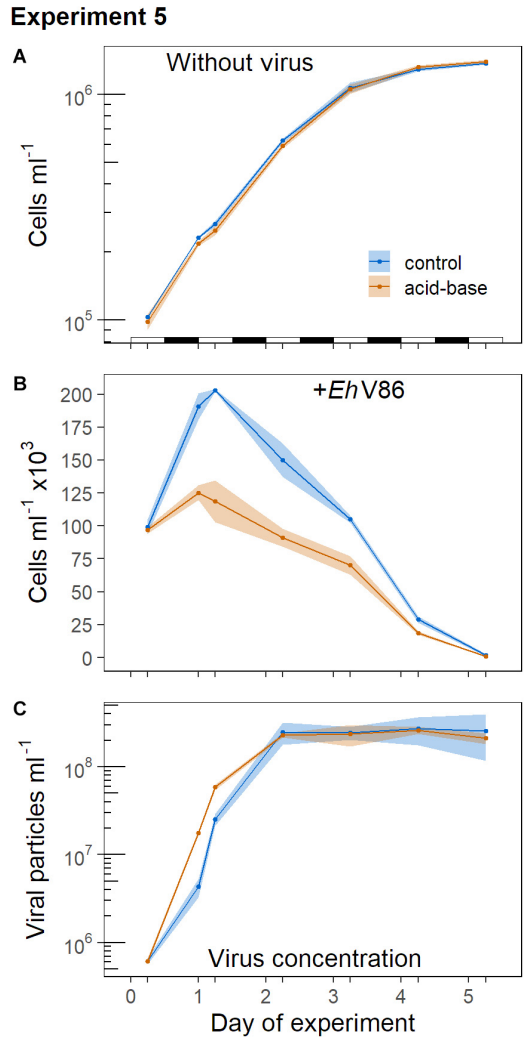

FIGURE 7 | Virus addition to calcified and decalcified cells in the middle of the light period, subsequent to the acid-base treatment. Sample means and 95% CI (n = 3). **(A)** Concentration of calcifying *E. huxleyi* CCMP1516 without virus and when virus was added **(B)**; **(C)** concentration of viral particles. The alteration of the light and dark phases is indicated in **(A)**.

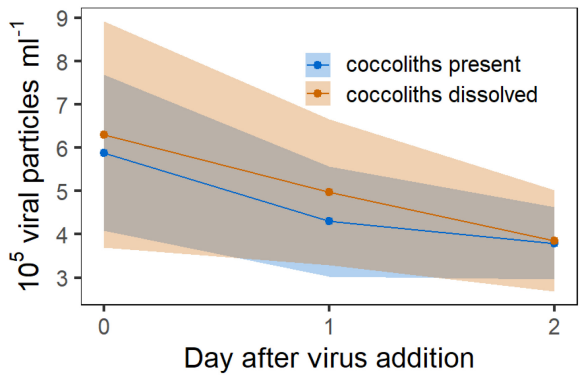

FIGURE 8 | Experiment 6. Counts of viral particles in a 5 μm filtered culture of *E. huxleyi* which contained no algal cells but detached coccoliths. Red symbols represent mean concentration of viral particles in the solution in which the coccoliths were dissolved with acid and base. Ribbons represent the 95% CI.

after cell division so that "no part of the cell surface is left exposed." This is confirmed by recent observations on dividing cells of *Coccolithus braarudii*, which show that the coccosphere is largely maintained throughout cell division with no obvious gaps being left when the cells divided (Walker et al., 2018). It was further shown that the cellular ratio of calcium carbonate to organic carbon remains relatively stable throughout the cell cycle of *E. huxleyi* (Kottmeier et al., 2020). The production of calcium carbonate during the light phase is closely linked with the increase in biomass and volume of exponentially growing cells (Müller et al., 2008; Kottmeier et al., 2020). Thus, the number of coccoliths remains relatively stable in relation to the volume of the cells throughout the cell cycle. Cells that had just divided are small, but fully covered with coccoliths. The protective effect of the coccosphere against viral infection was consequently either equally good, or equally poor in Experiment 1 and 5 where the virus was added in the beginning or in the middle of the light period, respectively. In contrast to the calcified cells, we observed a pronounced effect of the timing of virus addition on the decalcified cells. In Experiment 1, the decalcified cells nearly doubled whereas their concentrations increased only by about 25% in Experiment 5 (compare **Figures 2B**, **7B**). Either, the decalcified cells were more vulnerable to the virus in the middle of the light period due to the acid-base treatment. Alternatively, the extended time the decalcified cells had in the light when they were decalcified in the morning may have given them opportunity to reconstruct their coccosphere before the most vulnerable point in their cell cycle, e.g., during the dark phase when the cells replicate their DNA and divide (Müller et al., 2008; Kottmeier et al., 2020). In contrast, the cells that were decalcified in the middle of the light period were unable to fully reconstruct their coccosphere and thus poorly protected at this point. Clearly, our results from the dark experiments show that infection and viral lysis is not restricted to the cell division. Nevertheless, the results lend some support to the hypothesis that coccoliths can prevent viruses from reaching the organic part of the cell and the coccosphere can reduce infection. However, at the same time our results indicate that a potential protective effect of the coccosphere against the *Eh*V86 is probably of minor ecological relevance since the cell numbers of calcified and decalcified cells were almost identical at the end of the experiment (**Figures 2**, **7**).

The development of the *E. huxleyi* concentrations showed a characteristic pattern in all experiments in which actively growing cells in a light-dark cycle were exposed to the virus. The higher the maximum abundance of *E. huxleyi* was, the steeper was the subsequent decline of the cell concentrations. The results of the calcifying strain (CCMP1516) in particular suggests that, already after 2 days there should had been enough viruses present to infect the entire population. The net growth of *E. huxleyi* was stopped already within 2 days after virus addition and the numbers of viral particles increased drastically, but the concentrations of the host cells declined only

gradually. Thyrhaug et al. (2003) discovered a dynamic feedback mechanism in the *E. huxleyi-Eh*V system. The authors showed that the infection rate decreased when the abundance of the host cells declined in consequence of viral lysis. In our experiments the decline of the cell concentrations also seemed to be dependent on the relative abundance of *E. huxleyi* and the rate of cell lysis decreased the further the infection progressed. These observations suggest that calcification plays rather a minor protective role in the mutual succession of *E. huxleyi* and the *Eh*V.

Impacts of the Coccosphere Removal Procedures on Viral Infection

Our findings on the protective role of the coccosphere against viral infection critically depend on whether or not the methods that were applied to remove the coccosphere (acid-base treatment or low [Ca^{2+}] medium) affected the infection process as such. If these procedures somehow changed the susceptibility of the host to the virus, or the viral replication machinery it would be a confounding factor that is hard to distinguish from the protective role of the coccosphere.

The reduction of the [Ca^{2+}] in the growth medium clearly affected the interaction between the host and the virus. The cells of both, the calcifying (CCMP1516) and the non-calcifying strain (RCC1242) of *E. huxleyi* were less susceptible to infection when they were grown under low [Ca^{2+}] (Experiment 2, **Figure 4**). Furthermore, in the medium with low [Ca^{2+}] *E. huxleyi* was able to withstand the prolonged darkness even better when the virus was present (Experiment 4, **Figure 6**). Calcium plays important role in cell signaling and in certain structures of cell membranes (Verret et al., 2010) and there are various possibilities how a depletion in calcium ions may influence the biological interaction between the host and the virus. Regardless of the physiological mechanism, however, it is clear that the low [Ca^{2+}] had a confounding effect on the virus infection, which restricts us from using these experiments to interpret the role of the coccosphere in viral infection.

Johns et al. (2019) observed the contrary effect of a low [Ca^{2+}]. In their experiments, host cells were more susceptible to viral infection under low [Ca^{2+}] in most of the *E. huxleyi* strains the authors tested. It is therefore unclear whether calcium plays a direct role in the infection or replication process of the virus or whether the contradictory results attribute to the specific strain or other differences between the experiments, like the cell concentrations and related factors e.g., nutrient concentrations, carbonate chemistry etc. In general, the susceptibility of the host as well as the infectivity of the virus vary strongly depending on the examined strains of the host and the virus (Kegel et al., 2013; Nissimov et al., 2016). In this context, it is important to note that we examined only two *E. huxleyi* strains and a single strain of the *Eh*V, which does not allow us to generalize our results widely.

The acid-base treatment seemed to have a smaller effect on the cell physiology. Admittedly, the actual addition of acid and base caused a 10% decrease in the cell concentrations, but this decrease occurred within a short period after the procedure was conducted and it was likely due to the direct contact of the cells with the highly concentrated chemicals. The majority of the cells survived and their concentrations remained stable. The growth of the decalcified cells of *E. huxleyi* was equal to the calcified cells when light was supplied and no virus was added (Experiment 1, **Figure 2** and Experiment 5, **Figure 7**). However, in the dark experiment, the concentrations of the decalcified cells decreased also in absence of the virus (**Figure 5**). Without light, *E. huxleyi* was not able to compensate the acid-base treatment as effectively. However, the concentration of viral particles was higher in the cultures that contained decalcified cells. This raises the question whether the higher release of viral particles was due to an increased infection of the decalcified cells, or whether the treatment itself affected the replication of the virus. Strom et al. (2018) showed that the treatment of *E. huxleyi* with acid and base caused an elevated release of hydrogen peroxide (H_2O_2) of *E. huxleyi* into the surrounding medium. An enhanced excretion of H_2O_2 was also found during the lytic phase of infected *E. huxleyi*, concomitant with elevated intracellular concentrations of other reactive oxygen species (Evans et al., 2006). Reactive oxygen species play a role in the programed cell death pathway, which is linked to the replication cycle of the *Eh*V (Bidle et al., 2007; Sheyn et al., 2016). It is therefore possible that the acid-base treatment accelerated viral replication. However, when the acid-base method was tested with the non-calcifying strain (RCC1242), it led to enhanced the growth of the host cells, but the effect of the virus on the cell concentrations did not differ between the control and the acid-base treated cells (Experiment 1, **Figure 2**).

Another important aspect to consider is that the acid-base treatment did not only dissolve the coccospheres, but also loose coccoliths in the medium which detached from the cells. Typically, *E. huxleyi* produces more coccoliths than necessary to construct a single-layered coccosphere. The additional coccoliths are arranged in multiple layers around the cell, but also detach from the cell and spread into the surrounding medium (Paasche, 2002). Johns et al. (2019) found that free coccoliths can adsorb viral particles. Thus, the reduced infection observed in Experiments 1 and 5 could potentially be explained by the absorption of viruses by free coccoliths, which could have led to a reduced number of infective particles in the treatment with calcified cells.

To test if this mechanism shown by Johns et al. (2019) also occurred in our experiments, we exposed viral particles to a coccolith suspension and compared the development of the viral particle concentration relative to a suspension in which the detached coccoliths were dissolved prior to virus addition (Experiment 6, **Figure 8**). For Experiment 6, we used the *E. huxleyi* culture and the same virus stock solution from Experiment 5 to test for the adsorption of viral particles to free coccoliths. Thus, the number of detached coccoliths as well as the quantity of viral particles was equal in both experiments. Our measurements showed no difference in the virus concentration between the treatments, although we acknowledge the large variability in the results of this experiment. Nevertheless, these findings suggest that the absorption of viral particles by detached coccoliths was not the main mechanism explaining the large differences in the cell concentrations between calcified and decalcified *E. huxleyi* in Experiment 5.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

MH and LB designed the experiments. MH conducted the experiments and performed the measurements and data evaluation. All authors contributed to the data discussion and to the drafting of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2020.530757/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

SUPPLEMENTARY FIGURE

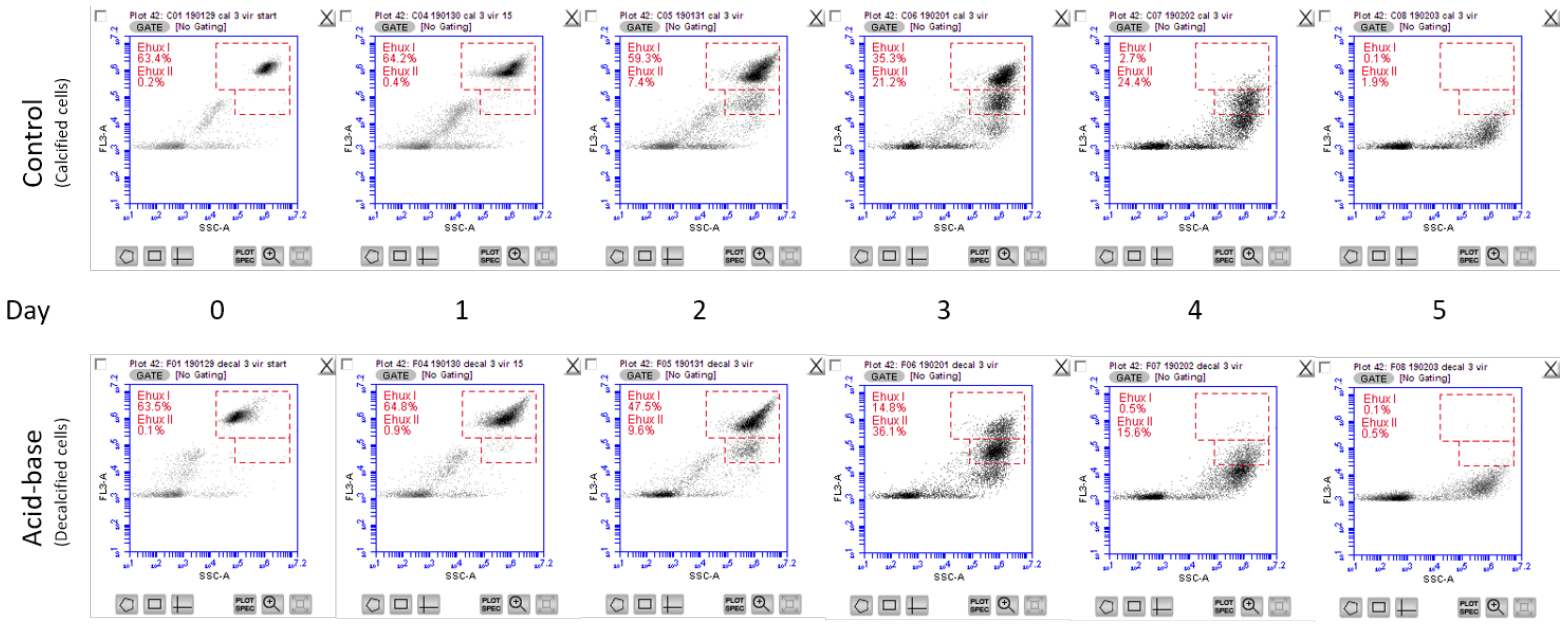

FIGURE 1. Decay of the *E. huxleyi* due to viral infection in two replicate cultures of Experiment 5. Each of the plots represent the results from an individual measurement with the Accuri C6 flow cytometer (Becton Dickibson) and were copied from the BD Accuri C6 analysis software (Becton Dickinson). The upper row shows the measurements from day 1 to 5 of a replicate bottle that contained calcified cells, the lower row shows the data from a replicate bottle that contained *E. huxleyi* which were treated with acid and base. Y axes: Red fluorescence signal. X axes: Sidescatter (right angle light scatter) signal.

3 | Manuscript II

Influence of the Calcium Carbonate Shell of Coccolithophores on Ingestion and Growth of a Dinoflagellate Predator

M. Haunost*,[1], U. Riebesell[1], F. D'Amore[1], O. Kelting[1], L.T. Bach[2]

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[1] GEOMAR Helmholtz Centre for Ocean Research Kiel, Kiel, Germany
[2] Institute for Marine and Antarctic Studies, Ecology & Biodiversity, University of Tasmania, Hobart, TAS, Australia

*corresponding author: mhaunost@geomar.de (M. Haunost)

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Influence of the Calcium Carbonate Shell of Coccolithophores on Ingestion and Growth of a Dinoflagellate Predator

Mathias Haunost[1], Ulf Riebesell[1], Francesco D'Amore[1], Ole Kelting[1] and Lennart T. Bach[2]*

[1] GEOMAR Helmholtz Centre for Ocean Research Kiel, Kiel, Germany, [2] Institute for Marine and Antarctic Studies, Ecology & Biodiversity, University of Tasmania, Hobart, TAS, Australia

Coccolithophores are an important group of ∼200 marine phytoplankton species which cover themselves with a calcium carbonate shell called "coccosphere." Coccolithophores are ecologically and biogeochemically important but the reason why they calcify remains elusive. One key function may be that the coccosphere offers protection against microzooplankton predation, which is one of the main causes of phytoplankton death in the ocean. Here, we investigated the effect of the coccosphere on ingestion and growth of the heterotrophic dinoflagellate *Oxyrrhis marina*. Calcified and decalcified cells of the coccolithophore species *Emiliania huxleyi*, *Pleurochrysis carterae*, and *Gephyrocapsa oceanica* were offered separately to the predator as well as in an initial ∼1:1 mixture. The decrease of the prey concentrations and predator abundances were monitored over a period of 48–72 h. We found that *O. marina* did not actively select against calcified cells, but rather showed a size selective feeding behavior. Thus, the coccosphere does not provide a direct protection against grazing by *O. marina*. However, *O. marina* showed slower growth when calcified coccolithophores were fed. This could be due to reduced digestion rates of calcified cells and/or increased swimming efforts when ballasted with heavy calcium carbonate. Furthermore, we show that the coccosphere reduces the ingestion capacity simply by occupying much of the intracellular space of the predator. We speculate that the slower growth of the grazer when feeding on calcified cells is of limited benefit to the coccolithophore population because other co-occurring phytoplankton species within the community that do not invest energy in the formation of a calcite shell could also benefit from the reduced growth of the predators. Altogether, these new insights constitute a step forward in our understanding of the ecological relevance of calcification in coccolithophores.

Keywords: calcification, phytoplankton, microzooplankton, grazing, *Oxyrrhis marina*

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Ocean University of China, China

****Correspondence:***
Mathias Haunost
mhaunost@geomar.de

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INTRODUCTION

Coccolithophores are single-celled phytoplankton that produce small calcium carbonate ($CaCO_3$) scales (coccoliths) which cover the cell surface in the form of a spherical coating, called coccosphere. They have been an integral part of marine plankton communities since the Jurassic (Bown et al., 2004; Hay, 2004). Today, coccolithophores contribute ∼1–10% to primary production in the surface

ocean (Poulton et al., 2007) and ∼50% to pelagic $CaCO_3$ sediments (Broecker and Clark, 2009). Their calcareous shell increases the sinking velocity of photosynthetically fixed CO_2 into the deep ocean by ballasting organic matter (Klaas and Archer, 2002; Honjo et al., 2008). At the same time, the biogenic precipitation of calcium carbonate during coccolith formation reduces the total alkalinity of seawater and releases CO_2 (Frankignoulle et al., 1994; Rost and Riebesell, 2004). Thus, coccolithophores play an important role in the marine carbon cycle by influencing the efficiency of the biological carbon pump and the oceanic uptake of atmospheric CO_2.

There are about 200 extant coccolithophore species in the oceans (Young et al., 2005). It is currently not known why coccolithophores calcify and how their ability to produce coccoliths is associated with their ecological success (Young, 1987, 1994; Raven and Crawfurd, 2012; Monteiro et al., 2016; Müller, 2019). The most plausible benefit of having a coccosphere seems to be a protection against predators or viruses (Hamm and Smetacek, 2007; Monteiro et al., 2016). Viral infection is an important cause of phytoplankton death in the oceans (Brussaard, 2004), and it has recently been shown that calcification can influence the interaction between a coccolithophore and its virus (Johns et al., 2019; Haunost et al., 2020). The major predators of marine phytoplankton are microzooplankton like ciliates and dinoflagellates. These are estimated to consume about two-thirds of the primary production in the ocean (Calbet and Landry, 2004) and microzooplankton can exert a strong grazing pressure on coccolithophore populations (Mayers et al., 2019). Although calcification does not prevent predation, it has been argued that the coccosphere reduces the grazing efficiency by making it more difficult for the predator to utilize the organic content of coccolithophores (Young, 1994). Heterotrophic protists are able to selectively choose prey on the basis of its size or shape and through chemical signals (Tillmann, 2004; Breckels et al., 2011) and may thus favor other prey that is available and not protected by coccoliths.

Currently, the evidence supporting or refuting a protective function of the coccosphere against predation is limited. Fileman et al. (2002) and Olson and Strom (2002) found that overall microzooplankton predation rates were reduced during blooms of the coccolithophore *Emiliania huxleyi*. In contrast, Mayers et al. (2019) found high microzooplankton grazing rates on natural coccolithophore communities. Recently, Mayers et al. (2020) showed that in situ ingestion rates of microzooplankton on *E. huxleyi* did not differ significantly from those on similar sized non-calcifying phytoplankton. In laboratory experiments the heterotrophic dinoflagellate *Oxyrrhis marina* preferred calcified over non-calcified cells of *E. huxleyi*, which was hypothesized to be due to size selective feeding behavior, since calcified cells are larger than non-calcified *E. huxleyi* (Hansen et al., 1996). Harvey et al. (2015) investigated predation by the dinoflagellate *O. marina* on different genotypes of non-calcifying *E. huxleyi* as well as calcified strains that differed in the degree of calcification. They found that the ingestion rate of *O. marina* was dependent on the genotype of *E. huxleyi* that was offered, rather than on their degree of calcification. In the same study, however, the authors found that predators which preyed on non-calcifying genotypes grew faster than those fed with calcified cells (Harvey et al., 2015). Strom et al. (2018) compared predation rates of the dinoflagellate *Amphidinium longum* on calcified relative to naked *E. huxleyi* prey and found no evidence that the coccosphere prevents ingestion by the grazer. Instead, ingestion rates were dependent on the offered genotype of *E. huxleyi* (Strom et al., 2018). Altogether, these two studies suggest that the genotype has a strong influence on ingestion by the microzooplankton species, but if and how calcification protects coccolithophores from microzooplankton predation could not be fully clarified.

In this study, we build upon these previous findings and expand the investigation of grazing protection of the coccosphere (which was so far focused on *E. huxleyi*) on two additional coccolithophore species (*Gephyrocapsa oceanica*, *Pleurochrysis carterae*) using the model species *Oxyrrhis marina*. Furthermore, we developed a protocol that enabled us to compare grazing rates on calcified, and de-calcified clones of the exact same genotypes for 2–3 days (as opposed to previous bioassays which were restricted to either ∼30 min or the use of different genotypes). We tested whether calcification (1) reduces prey ingestion rates, (2) influences the coccolithophore prey selection of *O. marina*, (3) affects *O. marina* growth post prey ingestion.

MATERIALS AND METHODS

The Model Organism *O. marina*

O. marina is a globally distributed heterotrophic dinoflagellate that inhabits coastal and shallow waters (Watts et al., 2011). It occurs in coastal habitats as well as intertidal pools and is a comparably well characterized model organism to study the trades of marine protozoa (Montagnes et al., 2011). *O. marina* is 20–30 μm in length (Lowe et al., 2011) and moves fast (90–179 μm s^{-1}) (Boakes et al., 2011). It feeds by phagocytosis and is able to engulf encountered prey items within <15 s (Öpik and Flynn, 1989; Höhfeld and Melkonian, 1998). We used this model organism because it is easy to keep in culture and can be raised to high concentrations. The ingestion rate of *O. marina* increases with increasing prey density and maximizes under sufficient food concentrations, when the ingestion rate is limited by the predators capability to process the food (Type II functional response) (Roberts et al., 2011).

Basic Culturing Conditions of *O. marina* and Prey Algae

A starting culture of *O. marina* (SAG 21.89) was kindly provided by Dr. Urban Tillmann (Alfred Wegener Institute, Bremerhaven). The dinoflagellates were fed with *Rhodomonas baltica*. The cryptophyte *R. baltica* as well as the coccolithophore species were cultured under the same basic conditions: Sterile filtered artificial seawater (Kester et al., 1967) was enriched with 256 μmol kg^{-1} $NaNO_3$, 16 μmol kg^{-1} NaH_2PO_4, 10 nmol kg^{-1} SeO_2, vitamins and trace metals according to the f/2 medium (Guillard and Ryther, 1962) and 2 mL kg^{-1} of natural North Sea water (autoclaved and 0.2 μm filtered) to prevent potential limitation by other micronutrients that are not included in the f/2 receipt

(Bach et al., 2011). Algal cultures were raised in a 12:12 h light-dark cycle with a photon flux density of 230 μmol photons m^{-2} s^{-1} (measured with a LI-COR LI-250A light meter) at 18°C. *O. marina* was inoculated into a growing culture of *R. baltica* and raised for a couple of days until high cell concentrations could be observed with the naked eye (The dinoflagellate cells appear in veil-like formations). Thereupon, the culture was placed dark at 18°C until all cells of *R. baltica* were eaten up, which was determined by flow cytometry (as described further below). The culture was further kept dark for about one week to starve the dinoflagellates. The concentration of starved cells was determined by flow cytometry. In this way, *O. marina* was raised to concentrations of $\sim 10 \times 10^3$ cells mL^{-1}.

Experimental Design

The coccolithophores *E. huxleyi* (B92/11), *G. oceanica* (RCC 1303) and *P. carterae* (unknown strain number) were grown separately in a large volume (5 L) of medium and raised to high cell densities for the 3 predation experiments ($\sim 275 \times 10^3$ *E. huxleyi* cells mL^{-1}, $\sim 220 \times 10^3$ *P. carterae* mL^{-1}, $\sim 130 \times 10^3$ *G. oceanica* mL^{-1}), in order to have a certain margin for the dilution steps that followed later, when the concentrations between the treatments were adjusted and the grazer cultures was added (described below) (**Figure 1**). Thereupon, the coccolithophore cultures were incubated in the dark for 36 h to stop the growth of the cells. The first 12 h of the dark incubation corresponded to the regular 12-h night cycle during which cell division took place. The goal of the subsequent 24 h of darkness was to deplete the energy reserves of the coccolithophore cells through respiration, in order to reduce the ability of the cells to build a new layer of coccoliths in the dark after the decalcification treatment was completed. All further steps during the dark incubation were conducted under low light conditions (<0.01 μmol photons m^{-2} s^{-1}) to keep energy inputs via photosynthesis at a minimum during handling.

After 36 h in the dark we conducted the "decalcification step," which was done with half of the population to remove the coccosphere of the treated cells (**Figure 1**). The large culture was split into two smaller bottles (2.3 L), one of which was treated with acid and base to remove the coccoliths. We added 2.5 mL 1M hydrochloric acid (HCl) L^{-1} to the cultures of *E. huxleyi* and *P. carterae* to dissolve the coccospheres. The cells of *G. oceanica* were decalcified by adding 3 mL of 1M HCl L^{-1} because this species is more heavily calcified. After the addition of the acid, the culture bottle was mixed for 1 min. Previous testing showed that the pH decreased to 4.3 during the brief acidification treatment. After this minute, the pH was brought back to the value before the acid addition by adding 1M sodium hydroxide (NaOH) solution. The bottles were rotated for 5 min to ensure that all flocculation from the addition of NaOH was dissolved. The decalcification procedure was conducted with a large culture volume (2.3 L) to keep the headspace at a minimum relative to the volume of the culture. In this way a potential degassing of CO_2 during the low pH/high pCO_2 period into the headspace of the volume was minimized. We tested this procedure in previous experiments to work out an appropriate volume of acid to be added to the cultures, which ensured the complete removal of the coccoliths (evaluated by microscopy) while keeping the decline of cell abundances at a minimum. However, cell concentrations always decreased to some extent due to the addition of the HCl and NaOH (by 6% for *E. huxleyi*, 18% for *P. carterae*, 7% for *G. oceanica*, of the target concentrations reported above). This was likely due to the contact with the highly concentrated acid and base at the time these were added. After the initial loss, the cell concentrations remained stable, which was determined by repeated flow cytometry measurements (as described below) over a period of 1 h. As the last step, the second (untreated) bottle with the calcified cells was diluted to an approximately equal concentration as in the acid-base treated bottles with 0.2 μm filtered artificial seawater. This facilitated the subsequent subdivision of both cultures into the replicate bottles of the individual treatments.

The content of both bottles containing either calcified or decalcified coccolithophores was split into eight replicate bottles (250 mL), respectively (**Figure 1**). Additionally, we established a mixed treatment by adding both, decalcified and calcified cells in a ∼1:1 mixture into eight replicate bottles. Thus, overall, we had a "decalcified" a "calcified" and a "mixed" treatment. Thereafter, an equal amount of the *O. marina* culture was added to the replicate bottles, some of which, however, were diluted with the same amount of 0.2 μm filtered artificial seawater instead and served as controls, to monitor the coccolithophore concentrations in the absence of the grazer. We took great care to keep the *O. marina* culture in a homogenous suspension during the addition by repeated mixing. All 8 bottles were mounted on a plankton wheel to prevent sedimentation of the algae. The experiments took place in complete darkness to minimize re-calcification of the decalcified cells and to prevent growth of the prey algae. Prey and predator abundances were sampled regularly, whereby the sampling took place under low light conditions as described above (**Figure 1**).

Enumeration of Prey and Predator Abundances

The coccolithophore concentrations were measured with a flow cytometer (Accuri C6, Becton Dickinson) at a flow rate of 66 μL min^{-1} from 1 mL subsamples that were taken from the replicate bottles and transferred into Eppendorf tubes. The coccolithophore population was identified based on the chlorophyll fluorescence signal (FL-3) versus the forward-scatter signal (FSC) using the BD Accuri C6 Software. Calcified cells scatter more light at right angles than decalcified cells so that both could be distinguished on the basis of their side-scatter signal (SSC) (Olson et al., 1989).

To enumerate the concentrations of *O. marina*, we took 20 mL subsamples, which were fixed with Lugol's iodine (1% final concentration). In the experiment that was conducted with *E. huxleyi* the cell number of *O. marina* was counted with an inverted microscope (Zeiss Axiovert 100) using 10 mL Utermoehl sedimentation chambers. The numbers of *O. marina* in the experiments with *G. oceanica* and *P. carterae* were counted with Sedgewick counting chambers using a light microscope (Carl Zeiss).

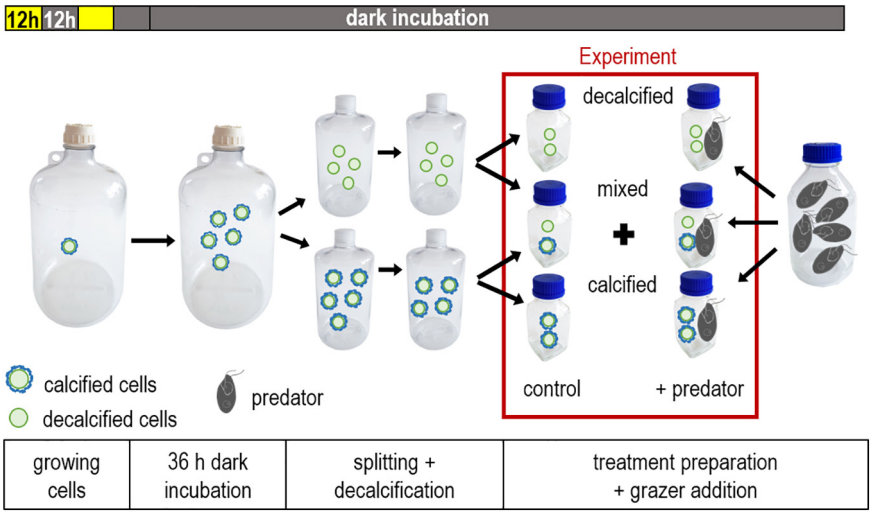

FIGURE 1 | Overview of key steps for preparing each experiment. The coccolithophore cultures were raised to high cell concentrations in a 12:12 hour light-dark cycle followed by a dark incubation of 36 h to terminate growth. The culture was split and decalcified, thereafter treatments (calcified, decalcified and mixed) with approximately equal cell concentrations were prepared. Each experiment started with the addition of the predator. The treatment combinations used in the experiments are highlighted in the red box.

Microscopic Observations

The cell size of the calcified and decalcified coccolithophores was measured via microscopy before the start of the experiment. 3.5 mL subsamples were taken from the initial cultures with calcified or decalcified cells before they were further split into the replicates. Subsamples were transferred onto slides for an inverted microscope (Zeiss Axiovert 100). Images were taken under 400-fold magnification with an Axiocam 105 color and cell diameters were measured with help of the ZEN 3.0 lite software (blue edition) (Carl Zeiss Microscopy GmbH). In addition, 3.5 mL subsamples were taken from single replicates that contained *O. marina* and either calcified or decalcified coccolithophores after 24, and 72 h, in order to observe grazer and prey interactions.

Data Analysis

The diameter of 20 calcified and decalcified cells of each species was determined and tested for significant differences in mean diameter with a Welch's unequal variances *t*-test. The counts of *O. marina* between the start (5 h after the start in case of the *E. huxleyi* experiment) and end of each experiment were used to calculate growth rates of *O. marina* for each replicate: $\mu = (\ln(x_t) - \ln(x_{t0}))/(t\text{-}t_0)$, where x = concentration of *O. marina*, t = time (in days). Differences in mean growth rates between the treatments were compared using an ANOVA. Data were tested for normal distribution and homogeneity of variances using Shapiro-Wilk and Levene's tests, respectively. In case the null hypothesis of the ANOVA (mean growth rates are equal) was rejected ($p < 0.05$), a Tukey's HSD test was used to compare the mean growth rates between treatments. Ingestion rates were calculated by dividing the difference in the prey cell concentration by the average predator concentration for a certain time interval, with the average predator concentration $\bar{x} = (x_t - x_{t0})/(\ln(x_t) - \ln(x_{t0}))$ (Heinbokel, 1978).

Predation preferences of *O. marina* on calcified vs. decalcified coccolithophores within the mixed treatments were analyzed using the Chesson's index α for depleting food densities (Manly et al., 1972; Chesson, 1983):

$$\alpha = \frac{\ln((n_{t0}^{cal} - i_{t-t0}^{cal})/n_{t0}^{cal})}{\ln((n_{t0}^{cal} - i_{t-t0}^{cal})/n_{t0}^{cal}) + \ln((n_{t0}^{decal} - i_{t-t0}^{decal})/n_{t0}^{decal})},$$

where n = cell concentration, i = ingested cells, and t = time. The index ranges from 0 to 1 and was calculated for every replicate. With only two prey types present, a value of $\alpha = 0.5$ implies no preference for one of the two prey types and $\alpha < > 0.5$ denotes that more cells of the respective type were consumed. A significant deviation of the mean α from 0.5 was tested with a Student's *t*-test. Statistics were done using R 3.6.1 (R Core Team, 2019), and the packages *car* (Fox and Weisberg, 2019), and *multcomp* (Hothorn et al., 2008). Graphs were done with *ggplot2* (Wickham, 2016).

RESULTS AND DISCUSSION

In the controls without predator, the concentrations of decalcified and calcified cells of all three coccolithophore species remained stable during the prolonged darkness (**Figures 2A–C**). Thus, the decrease of coccolithophores observed in the treatments where they were exposed to *O. marina* (**Figures 2D–F**) was mainly due to predation. In all three experiments, the decalcified coccolithophores were consumed considerably faster than the calcified cells.

The numbers of decalcified *E. huxleyi* decreased sharply within 12 h (**Figure 2D**) and fell below sufficient concentrations

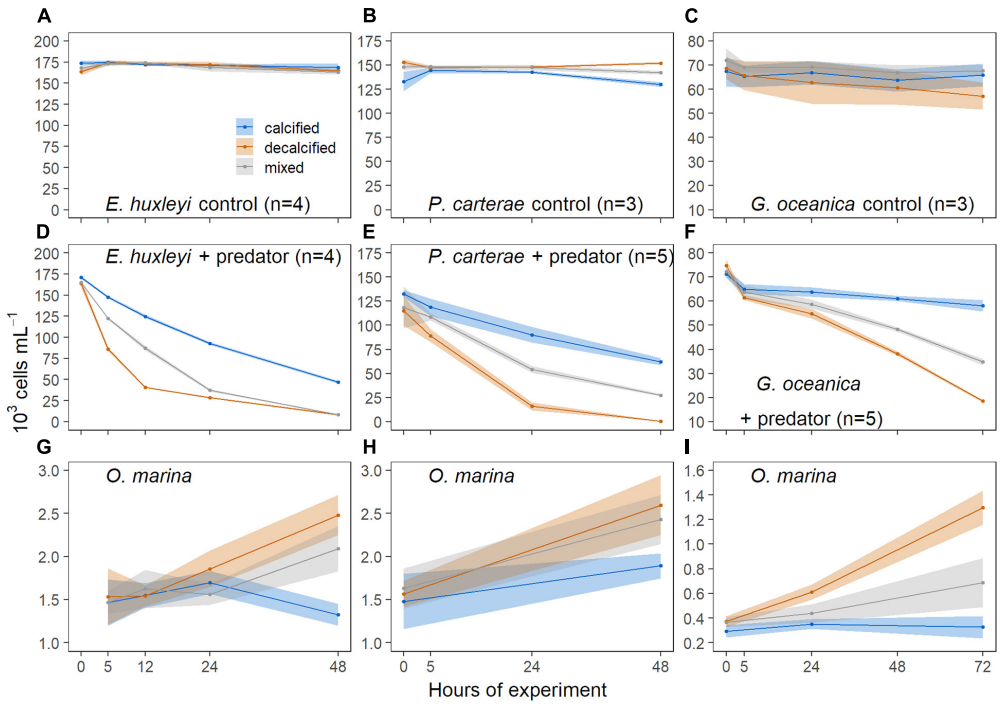

FIGURE 2 | Concentrations (mean ± SD) of *E. huxleyi* **(A,D)**, *P. carterae* **(B,E)**, *G. oceanica* **(C,F)** in the treatments without **(A–C)** and with **(D–F)** predators. Concentrations (mean ± SD) of *O. marina* in the respective treatments with *E. huxleyi* **(G)**, *P. carterae* **(H)**, *G. oceanica* **(I)**. Blue symbols = calcified, red = decalcified, gray = sum of calcified and decalcified cells in the mixed treatment.

to maintain feeding saturation of the predator cells (see also **Supplementary Figure 1**), so that the ingestion rate of *O. marina* was limited by the supply of food (whereas under feeding saturation it is thought that the ingestion rate is limited by the predator's capability to ingest and digest the prey items). The majority of the decalcified *P. carterae* was eaten up after 24 h (**Figure 2E**). The *G. oceanica* experiment was thereupon conducted with a higher prey to predator ratio, which ensured food supply for a longer period, so that the experiment could be continued for 72 h. The gray data points in **Figure 2** represent the sum of both, calcified and decalcified cells within the mixed treatments. The total concentrations (calcified + decalcified) always showed an intermediate decline compared to the treatments that contained either calcified or decalcified cells only. In each experiment, *O. marina* showed a higher increase in abundance when decalcified coccolithophores were fed, compared to the treatments that contained calcified cells only (**Figures 2G–I**). The predator concentrations in the mixed treatments were located in between those of the other two, and were closer to the predator concentrations in the decalcified treatment in the experiments with *E. huxleyi* and *P. carterae*, but rather in the middle, or even closer to the concentrations in the calcified treatment in the *G. oceanica* experiment (**Figures 2G–I**).

In the *E. huxleyi* experiment, *O. marina* reached a mean growth rate of -0.046 ± 0.118 (SD) day^{-1} in the calcified treatment, 0.248 ± 0.148 (SD) d^{-1} in the decalcified, and 0.175 ± 0.078 (SD) d^{-1} in the mixed treatment. The effect of the treatment on growth of *O. marina* was significant [F (2,9) = 6.648, $p = 0.0169$], and the Tukey HSD test revealed that the growth rate was significantly higher in the decalcified treatment, compared to the calcified ($p = 0.0164$), but did not differ from the mixed treatment ($p = 0.6735$). Furthermore, the mean growth rate of the predators did not differ significantly between the calcified and mixed treatment ($p = 0.0640$).

In the *P. carterae* experiment, *O. marina* grew with a mean rate of 0.131 ± 0.108 (SD) d^{-1} in the calcified, 0.251 ± 0.056 (SD) d^{-1} in the decalcified, and 0.200 ± 0.069 (SD) d^{-1} in the mixed treatment. There was no significant difference in the mean growth rates between the treatments [F (2,12) = 2.793, $p = 0.101$].

In the experiment with *G. oceanica*, the mean growth rates of *O. marina* were 0.029 ± 0.098 (SD) d^{-1} in the calcified, 0.417 ± 0.051 (SD) d^{-1} in the decalcified, and 0.197 ± 0.128 (SD) d^{-1} in the mixed treatment, and differed significantly between all three treatments [F (2,12) = 19.79, $p = 0.0002$]: calcified-decalcified ($p = < 0.001$), calcified-mixed ($p = 0.0455$), decalcified-mixed ($p = 0.0104$).

Comparison of Ingestion Rates Between Coccolithophore Species

O. marina showed no growth when fed with calcified *E. huxleyi* and *G. oceanica* and only marginal growth when calcified *P. carterae* served as prey. It can be assumed that at the end of all three experiments, the prey concentrations in the calcified treatments were still sufficient to ensure feeding saturation of

the grazers (**Figure 2**, compare **Supplementary Figure 1** for *E. huxleyi*). Hence, the ingestion rates of *O. marina* on the different calcified coccolithophore species can be compared.

Overall, the mean ingestion rates of *O. marina* on the calcified coccolithophores were lower when calculated for the entire period of 48 h compared to the respective ingestion rates calculated for the initial 24 h of the experiments, suggesting that the digestion of prey particles that had already been ingested limited further food uptake (unless in the second half of the experiments the predator cells just ate less for other reasons). An average of 57 ± 5.3 (SD) calcified *E. huxleyi* predator^{-1} day^{-1}, 26 ± 6.7 (SD) *P. carterae*, and 23 ± 7.0 (SD) *G. oceanica* pred. $^{-1}$ d^{-1} were consumed during the first 24 h of the experiments. Between 24 and 48 h, additional 33 ± 3.4 *E. huxleyi*, 17 ± 4.1 *P. carterae*, and 6 ± 6.7 (SD) calcified *G. oceanica* pred. $^{-1}$ d^{-1} were taken up. Ingestion rates calculated between 0 and 48 h were 21% lower in the *E. huxleyi* experiment, 18% lower for *P. carterae* and 36% lower for *G. oceanica* compared to the respective rates calculated for the period between 0 and 24 h. However, in the *G. oceanica* experiment, the decalcified cells were also still abundant after 48 h (**Figure 2F**), although the predator concentrations increased (**Figure 2I**). Here, *O. marina* ingested 35 ± 7.6 decalcified *G. oceanica* pred. $^{-1}$ d^{-1} during the first 24 h and 22 ± 1.4 cells pred. $^{-1}$ d^{-1} in the period between 24 to 48 h, when the ingestion rates were corrected with the calculated average predator concentrations during both intervals. Consequently, the ingestion rate of *O. marina* on decalcified *G. oceanica were* 37% lower in the second interval compared to the first 24 h.

Prey Preference of *O. marina* Within the Mixed Cultures

The decalcified cells of all three coccolithophore species could initially be distinguished from the calcified cells based on the strength of the side scatter signal measured with the flow cytometer. Both cell types appeared in two distinct clusters (**Figure 3**). However, a clear distinction between calcified and decalcified *E. huxleyi* was only possible until 24 h after the start of the experiment. Thereafter, the cluster of the decalcified cells overlapped with the cluster of the calcified cells, thereby impeding their differentiation in the flow cytograms (**Figure 3**). As a consequence, an increasing fraction of the initially decalcified cells was enumerated as calcified cells, which is why the concentrations of the latter increased between 24 to 48 h after the start (**Figure 4A**). More calcified than decalcified *E. huxleyi* cells were consumed during the first 24 h [$\alpha = 0.58 \pm 0.003$ (SD), t (3) = 53.29, $p < 0.0001$] and the calcified cells were eaten up completely after 48 h, whereas some decalcified were still left (**Figure 4B**).

Calcified and decalcified *P. carterae* could be distinguished from each other throughout the experiment in the mixed treatment based on the SSC and remained stable in concentration when predators were absent (**Figure 4C**). More decalcified than calcified *P. carterae* were consumed during the first 24 h of the experiment [$\alpha = 0.71 \pm 0.09$ (SD), $t(4) = 5.63$, $p = 0.0049$] and the decalcified cells were completely gone after 48 h (**Figure 4D**).

The decalcified *G. oceanica* were able to produce new coccoliths in the dark whereby the SSC of the cells increased. These re-calcified cells formed an intermediate cluster in the flow cytogram that was located in between those formed by the decalcified and the calcified cells, respectively (**Figures 3H,I**). Nevertheless, the cluster of calcified *G. oceanica* could be distinguished from the cluster of decalcified cells within the first 24 h of the experiment, during which *O. marina* ingested more decalcified than calcified *G. oceanica* [$\alpha = 0.73 \pm 0.05$ (SD), t (4) = 10.89, $p = 0.0004$] (**Figures 4E,F**). The intermediate cluster of re-calcified cells in the cytograms was more scattered when predators were present compared to the controls (**Figures 3H,I**). The mean SSC of the cluster of decalcified cells increased less over the course of the experiment when *O. marina* was present. This was also the case in the treatment that contained decalcified cells only, indicating that the grazers preferred the re-calcified cells (**Supplementary Figure 2**).

In the presence of *O. marina,* additional particle clusters could be observed on the cytograms, which were lower in red fluorescence than the coccolithophore cells, but showed similar FSC and SSC signals indicating they were of similar size (**Figure 3**). The additional particle cluster did not appear in the controls, but only in the presence of predators. Particles were more abundant in the cultures with calcified coccolithophores and likely represent egested food items.

Cell Size of Calcified and Decalcified Cells

The decalcified coccolithophores were smaller than the respective calcified cells: The mean diameter of decalcified *E. huxleyi* [3.77 ± 0.23 (SD) μm] was significantly smaller than the mean diameter of the calcified cells [4.81 ± 0.55 (SD) μm] [t (26) = 7.88, $p < 0.0001$]. This was also the case for *P. carterae* [decalcified = 8.50 ± 0.73 (SD) μm; calcified = 10.52 ± 1.02 (SD) μm; t (34) = 7.22, $p < 0.0001$] and *G. oceanica* [decalcified = 5.41 ± 0.74 (SD) μm; calcified = 7.01 ± 0.45 (SD) μm; t (31) = 8.27, $p < 0.0001$]. Calculations of spherical volumes ($V = 4/3\pi r^3$) from the measured diameters showed that the volume (organic cell + coccosphere) of all three calcified species was roughly twice as large as the volume of the decalcified cells (organic only) (∼58 vs. ∼28 μm^3 for *E. huxleyi*, ∼610 vs. ∼322 μm^3 for *P. carterae*, ∼180 vs. ∼83 μm^3 for *G. oceanica*). Thus, more decalcified cells could be taken up per individual predator simply due to their smaller size. The flow cytometry data suggest that the calcified *E. huxleyi* suffered less from the prolonged dark period than the decalcified cells (**Supplementary Figure 3**). The measurements showed that the chlorophyll fluorescence of the decalcified cells declined stronger and further indicate that the decalcified cells decreased slightly in size over the course of the experiment. However, this effect was small compared to the absolute difference between both treatments (**Supplementary Figure 3**).

Microscopic Observations

Observations that were done during (and after) the experiments from single replicates that contained either calcified or decalcified

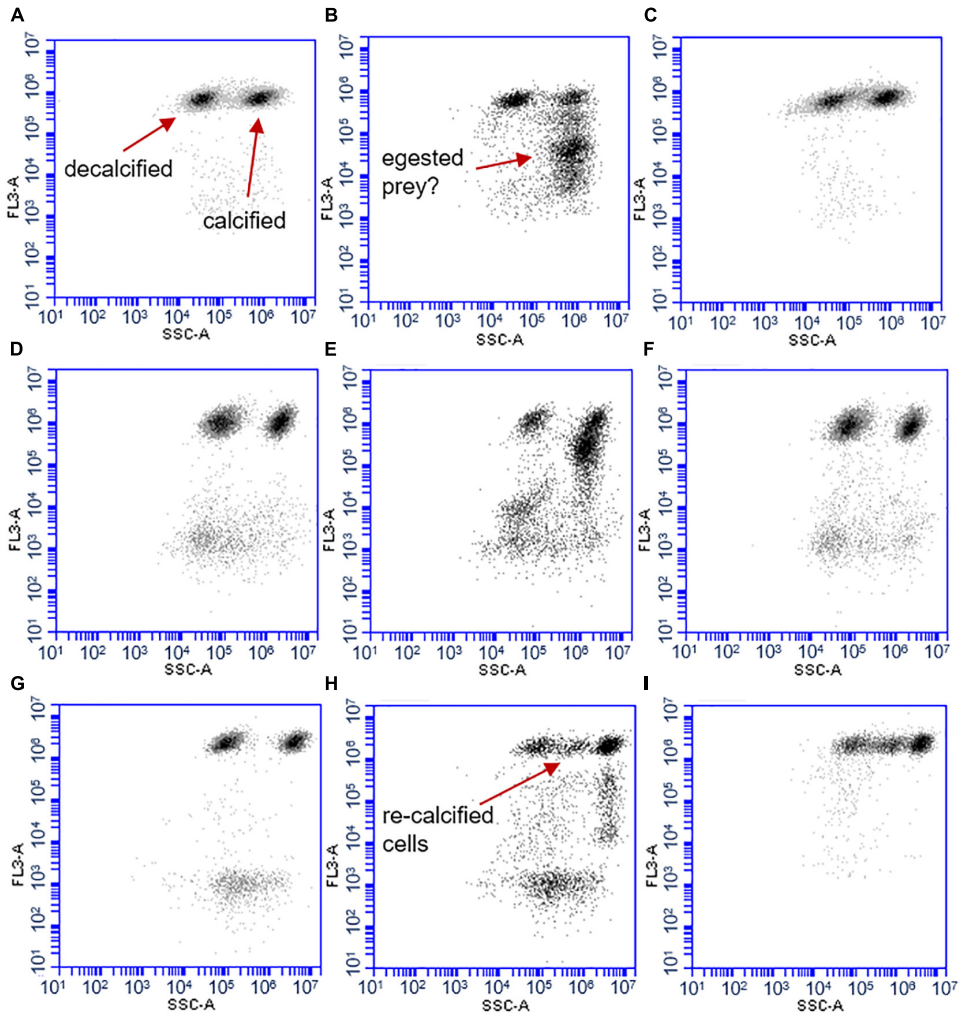

FIGURE 3 | Cytograms of mixed cultures that contained both, decalcified and calcified coccolithophores. Decalcified coccolithophores display lower side-scatter signals (SSC-A on the x-axes, strength of the signal without unit) than the calcified cells (FL3-A on y-axes = red fluorescence). Cytograms of an *E. huxleyi* culture with grazers **(A)** at the start, **(B)** after 24 h, **(C)** control without grazers after 24 h. **(D)** *P. carterae* at start, **(E)** after 24 h, **(F)**, control after 24 h. **(G)** *G. oceanica* start **(H)** after 72 h **(I)**, control without predators after 72 h.

cells showed that more decalcified than calcified cells fit into an individual *O. marina* (**Figure 5**). Moreover, calcified cells inside the predator kept their shape and many of the *O. marina* cells that had ingested several calcified coccolithophores looked deformed (**Figure 5A**). It also needs to be mentioned that *P. carterae* is a motile species. At the high concentrations at which *P. cartarae* was grown prior to the start of the experiment a large fraction of cells was not moving in both treatments. However, the decalcified cells became sticky and we could often observe pairs of cells moving stuck together. The potential reduction of mobility of these decalcified cells could facilitate the uptake by *O. marina*.

Live *O. marina* were highly motile and thus difficult to observe and to photograph. However, they occasionally stopped their continuous spiral movement and remained motionless for a short period, during which some individuals sank to the bottom of the microscope slide and could be photographed. Pictures had to be taken quickly, which in part explains the lack of focus in some of them. The relatively few observations do not allow quantitative statements and, of course, can be challenged with respect to their representativeness regarding the bulk population of *O. marina*. Nevertheless, they provided important insights into prey digestion. After 72 h some individuals of *O. marina* that fed decalcified coccolithophores showed small inclusions (**Figures 5D,H**) that resembled coagulated prey items under progressive digestion as described by Öpik and Flynn (1989). We were not able to discover similar patterns in cells of *O. marina* that were fed with calcified coccolithophores, suggesting limited digestion of calcified cells. However, the lack of this observation may also have been due to the fact, however, that remnants of calcified cells were obscured by freshly engulfed cells,

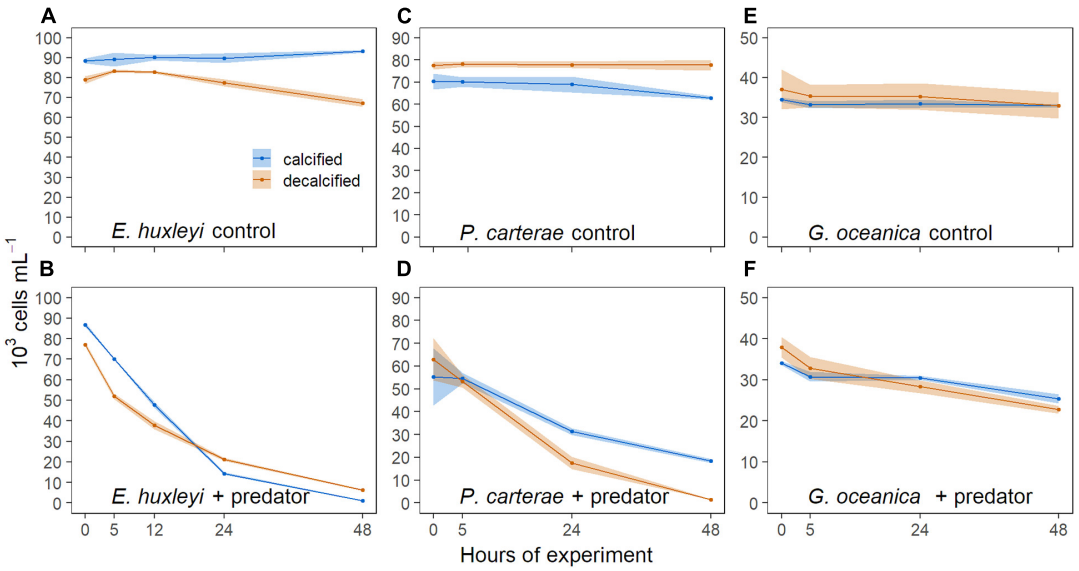

FIGURE 4 | Concentrations (mean ± SD) of calcified (blue) and decalcified (red) cells within the mixed treatment. **(A)** *E. huxleyi* without, and **(B)** with grazers. **(C)** *P. carterae* without and **(D)** with predators. **(E)** *G. oceanica* without and **(F)** in the presence of *O. marina*.

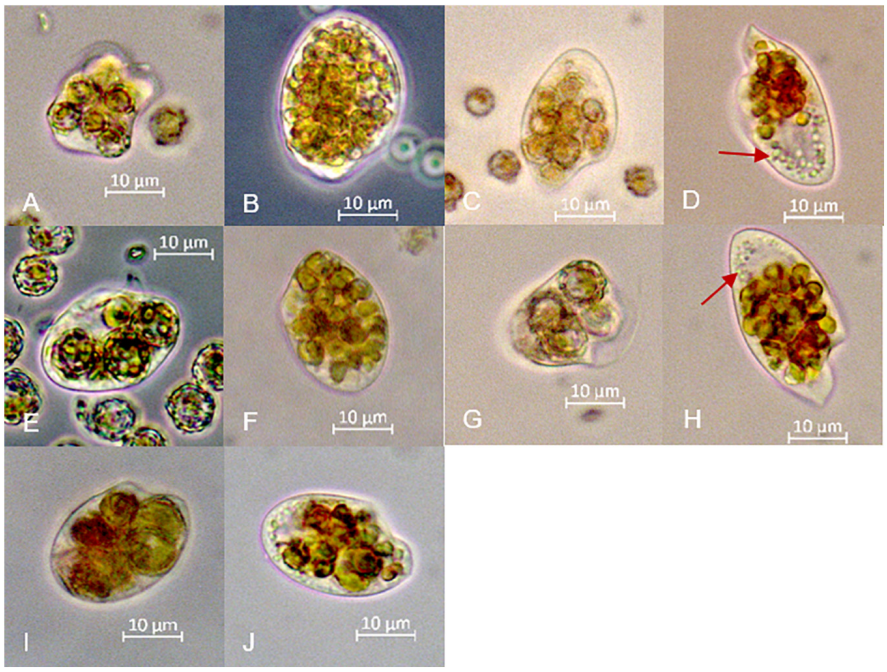

FIGURE 5 | Microscopic images of different individuals of *O. marina* with ingested prey. **(A)** calcified and **(B)** decalcified *E. huxleyi* after 24 h. **(C)** calcified and **(D)** decalcified *E. huxleyi* after 72 h. **(E)** calcified and **(F)** decalcified *G. oceanica* after 24 h and **(G)** calcified **(H)** decalcified cells after 72 h. **(I)** calcified and **(J)** decalcified *P. carterae* after 24 h. Red arrows point to the small spherical objects in the apical part of the predatory cells, which resemble prey cells in an advanced stage of digestion.

which were still abundant at the end of the experiments in contrast to the decalcified cells (**Figure 2**). We once observed an individual of *O. marina* egesting a relatively undigested calcified cell of *E. huxleyi* (**Figure 6**). The entire egestion process took about 15 min, during which the predator barely moved. In contrast, we observed another specimen that was packed with several calcified cells of *E. huxleyi* that rounded up its shape. It egested two virtually empty coccospheres within minutes and moved quickly out of sight (**Figure 7**).

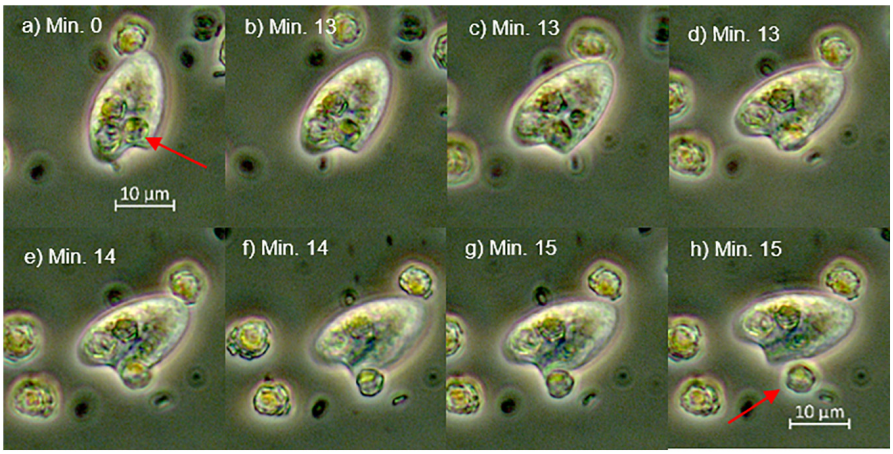

FIGURE 6 | This set of photos **(a–h)** show an *O. marina* specimen egesting a cell of *E. huxleyi* (red arrow). Prey egestion occurred within 3 min. Images were taken in a separate experiment where cells of *E. huxleyi* were not incubated for 36 h in the dark. The images vary slightly with respect to the focal plane.

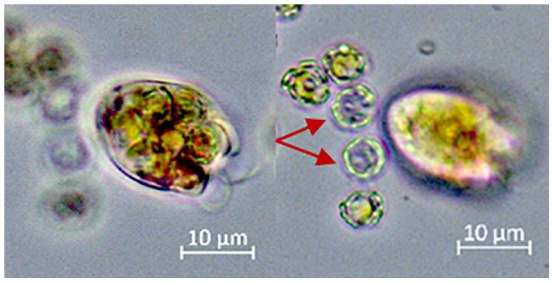

FIGURE 7 | The figure shows two images taken from the same cell of *O. marina* in different focal planes. The predator was packed with several *E. huxleyi* and egested visually empty coccospheres (red arrows) within a few seconds.

Effects of Calcification on Growth of *O. marina*

Öpik and Flynn (1989) described *O. marina* as voracious predator. It feeds by engulfing several prey particles one after another until there is no further space to take up more. Ingested food particles are then assimilated successively (Öpik and Flynn, 1989; Roberts et al., 2011). The microscopic observations showed that the coccolithophores *E. huxleyi*, *P. carterae* and *G. oceanica* fit well into the prey size spectrum of the predator. Within the pure cultures that contained either calcified or decalcified coccolithophores, the decalcified cells disappeared faster due to predation. Furthermore, *O. marina* was able to grow when feeding on decalcified coccolithophores but not, or at reduced rates, when feeding on calcified cells. Growth inhibition through calcification was most pronounced when *O. marina* was fed with the most heavily calcified species (*G. oceanica*) and least pronounced when fed with the least calcified one (*P. cartarae*). This suggests that growth inhibition due to calcification is positively correlated with the relative proportion of calcite to organic carbon in the prey (compare **Figure 8**). We propose 3 possible mechanisms to explain how the coccosphere could have reduced grazer growth. These will be discussed consecutively in the following but they are not mutually exclusive.

(I) Calcification of coccolithophores complicates digestion in phagotrophic protozoa: *O. marina* must cope with the coccosphere before reaching the nutritious organics of the cell. Our results are consistent with the findings of Harvey et al. (2015), who observed higher growth rates of *O. marina* when fed with naked *E. huxleyi* compared to calcified *E. huxleyi* strains. The coccosphere may impede the direct access of the digestive machinery to the valuable organics, thereby slowing down digestion. Harvey et al. (2015) hypothesized that the ingested $CaCO_3$ may impede the digestive process by buffering the acidic pH in the food vacuoles of the predator. Acidic digestion has been demonstrated in some protozoa and may be common in phagotrophic microzooplankton (Mast, 1947; González et al., 1993; Barbeau et al., 1996; Kodama and Fujishima, 2005). According to this hypothesis, the predatory *O. marina* cell has to counteract the pH increase in the food vacuoles due to carbonate dissolution to sustain an acidic environment for the optimal functioning of digestive enzymes. The microscopic observations of egested prey cells presented in **Figure 6** (and **Supplementary Figure 4**) lend some support for a dissolution of the $CaCO_3$ inside *O. marina* (compare the egested cell to the healthy *E. huxleyi* in **Figure 6**; but note that it remains to be tested whether the seemingly smaller coccosphere is due to corrosion or some coccoliths simply fell off). This specimen of *O. marina* was apparently not able to effectively digest the engulfed *E. huxleyi*. In contrast, the specimen of *O. marina* in **Figure 7** egested visually empty coccospheres and therefore seems capable to assimilate the organic material inside the coccosphere. The empty coccospheres shown in **Figure 7** may correspond to those flow

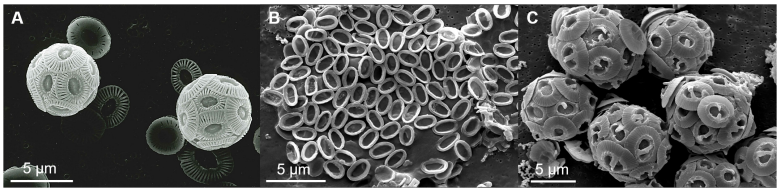

FIGURE 8 | Scanning electron micrographs of individual coccolithophore species from the respective strains used in the experiments (see methods). **(A)** Coccospheres of *E. huxleyi*. **(B)** collapsed coccosphere of *P. carterae*. **(C)** Coccospheres of *G. oceanica*. The pictures **(B,C)** were kindly provided by Dr. Giulia Faucher and were published previously in a study that compared physiological and morphological characters of four coccolithophore species under different abiotic conditions (Faucher et al., 2020).

cytometry populations that have a similar SSC value but a reduced fluorescence signal compared to healthy *E. huxleyi* (**Figure 3**; egested prey). The putative cluster of "egested cells" in **Figure 3** suggests that egestion of "empty" coccospheres is common. Thus, in addition to (or instead of) buffering a potentially acidic digestive environment, the coccosphere could also slow down digestion simply by impeding the access to the valuable organics of the prey cells.

(II) The coccosphere reduces the space to incorporate more cells: The coccosphere roughly doubles the total volume of the coccolithophore and *O. marina* is unable to destruct the coccospheres after ingestion. Thus, calcified cells require twice as much space within the digestive compartments of *O. marina*, which is likely to be detrimental for the turnover of organic material. The impact of this volume effect can be seen very clearly in **Figure 5** where much more de-calcified cells fit into *O. marina*. Furthermore, the volume effect would be even higher in those species which have spines or other coccolith extensions. Almost 50% of heterococcolith-bearing species have such extensions, suggesting that the volume effect on digestion is potentially relevant for many coccolithophores (Monteiro et al., 2016).

(III) The coccosphere adds ballast to the grazer thereby making it less agile: The ingestion of the coccosphere adds significant ballast to the predator since $CaCO_3$ is 2.7 times denser than organic material. The uptake of large amounts of $CaCO_3$ may increase the demand of energy needed for locomotion, counteracting gravitational sinking, and impact swimming speed and prey encounter rate.

Preference for Calcified or Decalcified Coccolithophores by *Oxyrrhis marina?*

It has been shown that *O. marina* is size-selective (Hansen et al., 1996; Strom et al., 2012). Our results show that *O. marina* consumed more of the calcified than decalcified *E. huxleyi*. In contrast, more decalcified *P. carterae* and *G. oceanica* were ingested. Furthermore, the results of the flow cytometry measurements indicate that re-calcified cells of *G. oceanica* were primarily eaten. The results suggest that the differences in ingestion rates between calcified and decalcified coccolithophores within the mixed treatments were more likely due to the size of the prey algae than due to calcification. With regard to the prey size, *O. marina* preferred the larger calcified *E. huxleyi* (∼4.8 µm), which was the smallest of the three coccolithophore species that was tested in our experiments, but the smaller decalcified cells of *P. carterae* (∼8.5 µm), which was the largest species (calcified ∼10.5 µm) (**Figure 8**). This is consistent with previous findings that *O. marina* feeds optimally on prey sizes > 4 µm (Roberts et al., 2011).

The results suggest that calcification does not prevent *O. marina* from ingesting coccolithophores and confirm previous findings that the coccosphere does not deter protozoan grazers (Harvey et al., 2015; Strom et al., 2018; Mayers et al., 2020). It remains a key question, however, whether other microzooplankton species that are better adapted to natural phytoplankton communities would be able to select against calcified species. Protozoa that are capable to avoid calcareous cells and to select for more easily digestible food items would potentially have a clear advantage as they likely grow faster. The ability of *O. marina* to chemoreception is well developed. It has been shown that the grazer is able to select prey items based on their food quality (Meunier et al., 2012). Moreover, *O. marina* has been shown to feed on artificial particles that are flavored with organic molecules (Hammer et al., 2001), and is able to distinguish between artificial particles that are coated with different carbohydrate-binding proteins (Wootton et al., 2007).

Prey concentrations and encounter rates are much lower under natural conditions than in our experiments. In nature, protozoa usually need to browse large volumes of seawater to be able to cover their nutritional demand (Kiørboe, 2011), suggesting that they rarely have a choice to select their food. It has been argued that selection likely favors the evolution of chemosensory receptors in protozoa in order to seek for prey along chemical gradients (Breckels et al., 2011). The question is whether selection favors the evolution of appropriate receptors to detect an inorganic shell surrounding an otherwise attractive prey item in such a dilute environment.

Our results indicate that coccolithophore calcification can reduce the feeding efficiency and growth of phagotrophic protozoa. The benefit of this effect for coccolithophores is likely depending on their dominance within the bulk phytoplankton community. In a scenario where the calcified coccolithophore is a minor component of the phytoplankton

community accessible to a non-selective protozoan, the decrease in predation pressure due to ingested calcite will primarily benefit the non-calcareous phytoplankton in the community. This is because competing non-calcifying species do not contribute to the substantial energetic burden associated with calcification (Monteiro et al., 2016), whereas they still benefit from the reduced predation pressure. However, reduced growth of the grazer through calcification will benefit coccolithophores that dominate the phytoplankton community and attenuate the top-down control on their proliferation (Harvey et al., 2015).

CONCLUSION

Our results show that coccolithophore calcification influences prey ingestion by *O. marina* primarily by altering the prey size. This can increase or decrease ingestion rates depending on the preferred size spectrum of the grazer. In contrast to ingestion, prey digestion is reduced by calcification. We concur with Harvey et al. (2015) that reduced digestion rates could be due to detrimental buffering of acidic digestion via intracellular calcium carbonate dissolution. Additionally, the coccosphere may simply impede the access to the organic part of the cell and/or handicap the swimming abilities of the predator by ballasting. We could show that the coccosphere strongly reduces the intracellular space for prey items inside the predator. This constitutes a simple, yet effective mechanism to impede digestion rates. However, we found that the coccosphere provides no direct protection against *O. marina* since it does not motivate the predator to select against calcified cells.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://doi.pangaea.de/10.1594/PANGAEA.932273.

AUTHOR CONTRIBUTIONS

MH and LB designed the study. MH, FD'A, and OK performed the experiments and conducted the measurements. MH, LB, and UR wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.664269/full#supplementary-material

Supplementary Figure 1 | Functional response of *O. marina* to different prey concentrations of calcified (blue) and decalcified (red) *E. huxleyi*. In a separate experiment, both cell types were kept separately in different concentrations in 20 bottles, that contained ∼2000 *O. marina* mL^{-1} each. **(A–D)** Measurements took place 2.5–48 h after the start of the experiment.

Supplementary Figure 2 | Decalcified *G. oceanica* produced new coccoliths over the course of the experiments, which caused an increase in their side scatter signal (SSC) measured via flow cytometry. The increase in SSC was less pronounced when predators were present, suggesting that the re-calcified cells were eaten preferentially.

Supplementary Figure 3 | Development of the forward scatter (FSC) as a measure of size, and the red (chlorophyll) fluorescence (FL-3) of calcified and decalcified coccolithophores in the controls over the course of the experiments.

Supplementary Figure 4 | The set of images shows an individual *O. marina* specimen that ingested a *Coccolithus braarudii* cell. The predatory cell was moving quickly and difficult to photograph. We were not able to adjust the white balance in the first photo in the upper left, which shows a cell of *C. braarudii* inside *O. marina* **(a)**. The microscopy slide was kept at 18°C in the dark for about 18 h and we were able to rediscover the same individual on the following day. We found it slowly moving at the bottom of the slide **(b–f)**. After some time, it started to rotate intensely whereby it egested the prey item **(g–o)** and moved on **(p)**.

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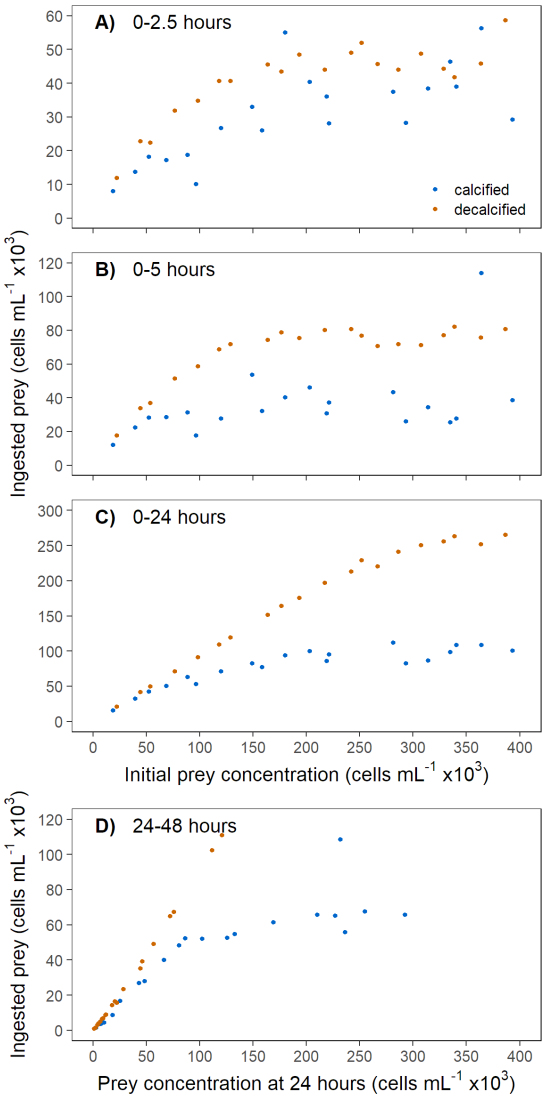
Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

SUPPLEMENTARY FIGURES



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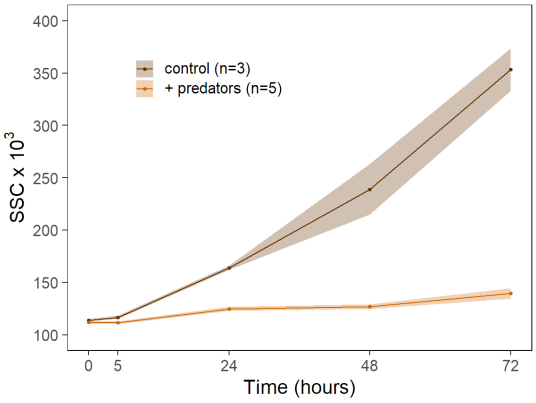

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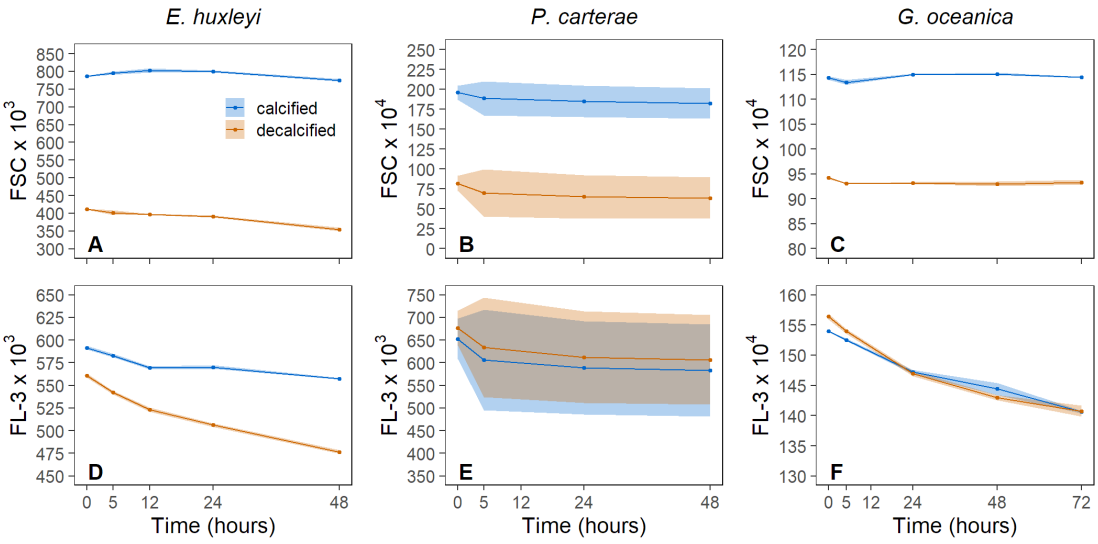

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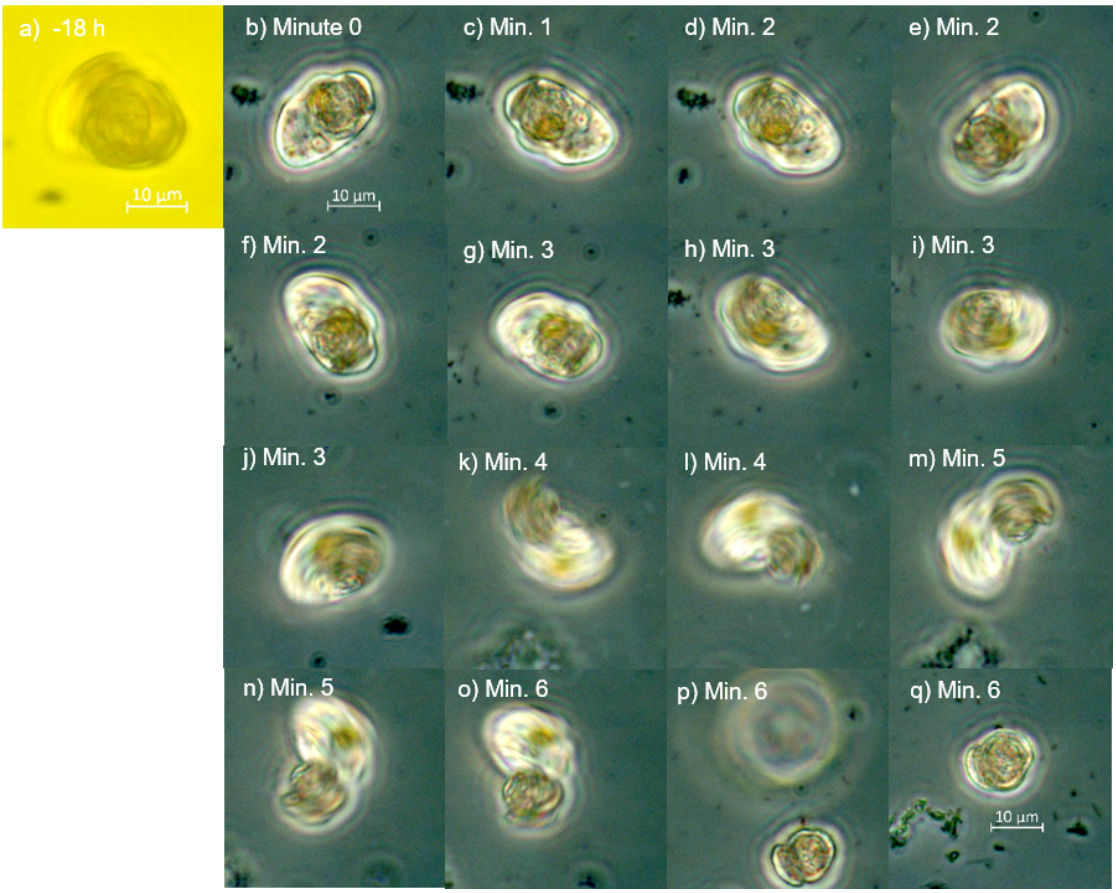

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4 | Manuscript III

The calcium carbonate shell of the coccolithophore *Gephyrocapsa oceanica* provides no protection against ingestion by the copepod *Acartia tonsa*

M. Haunost[*,1], U. Riebesell[1], J. Taucher[1], S. Lischka[1], L.T. Bach[2]

to be submitted

[1] GEOMAR Helmholtz Centre for Ocean Research Kiel, Kiel, Germany
[2] Institute for Marine and Antarctic Studies, Ecology & Biodiversity, University of Tasmania, Hobart, TAS, Australia

[*]corresponding author: mhaunost@geomar.de (M. Haunost)

[**]manuscript formatted for submission

The calcium carbonate shell of the coccolithophore *Gephyrocapsa oceanica* provides no protection against ingestion by the copepod *Acartia tonsa*

M. Haunost[1], U. Riebesell[1], J. Taucher[1], S. Lischka[1], L. T. Bach[2]

[1]GEOMAR Helmholtz Centre for Ocean Research Kiel, Kiel, Germany
[2]Institute for Marine and Antarctic Studies, Ecology & Biodiversity, University of Tasmania, Hobart, TAS, Australia

Abstract

Coccolithophores are a widespread group of marine phytoplankton, which form calcium carbonate scales (coccoliths) to cover themselves with a calcareous shell (coccosphere). The question why coccolithophores calcify is currently still unanswered but has been coined as one of the priorities in coccolithophore research. Here we tested whether the coccosphere of the cosmopolitan species *Gephyrocapsa oceanica* provides protection against ingestion by the calanoid copepod *Acartia tonsa*. Therefore, calcified and decalcified *G. oceanica* cells of the same population (decalcification achieved with a brief acid-base treatment) were provided as prey to adult females of *A. tonsa* for 48 hours. We found no difference in ingestion rates of calcified and decalcified *G. oceanica*. Furthermore, when *A. tonsa* was offered a mixture of calcified and decalcified cells, the copepod did not actively select against calcified *G. oceanica*. On the contrary, ingestion rates of calcified cells tended to be higher in this situation when both prey types were present. These findings suggest that the coccosphere of *G. oceanica* does not provide protection against ingestion by the globally relevant copepod species *A. tonsa*.

Introduction

Coccolithophores are an important group of phytoplankton, mainly characterized by their ability to calcify. The single-celled algae produce small scales (coccoliths) made of calcium carbonate ($CaCO_3$) to form a coating around the cell called coccosphere.

Coccolithophores are a substantial component of pelagic food webs by contributing 1 – 10% to the global oceanic primary production (Poulton et al. 2007). Since the emergence of coccolithophores in the Mesozoic, they have generated vast amounts of carbonate deposits (Bown et al. 2004; Hay 2004) and are responsible for a major proportion of the recent calcium carbonate production in the open ocean (Milliman 1993; Broecker and Clark 2009). Their coccoliths provide ballast mineral that enhances the sinking of particle aggregates and thereby promote the sequestration of atmospheric CO_2 into the ocean's interior (Klaas and Archer 2002; Honjo et al. 2008). On the other hand, the precipitation of $CaCO_3$ during coccolith formation reduces seawater alkalinity and diminishes the potential for atmospheric CO_2 uptake (Frankignoulle et al. 1994; Rost and Riebesell 2004).

Coccolithophores have been a major subject in planktological research over the past century. Nevertheless, the reason(s) why coccolithophores calcify are still poorly understood. Various hypotheses exist for potential benefits of coccospheres with the most widespread being that they provide protection against predation (Hamm and Smetacek 2007; Monteiro et al. 2016). The predominant predators of phytoplankton in the ocean are microzooplankton (Calbet and Landry 2004), mainly ciliates and dinoflagellates, and mesozooplankton composed of metazoan predators, among which copepods represent the most numerous group (Calbet 2001). So far, the protection of calcification against microzooplankton grazing has been investigated in a few studies, which found no evidence for selection against coccolithophores compared to non-calcified prey algae (Hansen et al. 1996; Harvey et al. 2015; Strom et al. 2018; Mayers et al. 2020; Haunost et al. 2021). However, some evidence supports the notion that the coccosphere reduces the food uptake and growth of unicellular predators (Harvey et al. 2015; Haunost et al. 2021).

Mesozooplankton is usually dominated by copepods (Verity and Smetacek 1996; Thompson et al. 2013) and accounts for the consumption of ~12% of the global oceanic primary production (Calbet 2001). Most pelagic copepods are omnivorous predators and can exert strong predation pressure on microzooplankton (Nejstgaard et al. 1997; Irigoien et al. 2005). They either act as ambush predators that actively trace their prey and conduct attack jumps to catch it, or they generate a feeding current from which non-

moving prey particles are perceived and captured individually (Kiørboe 2011a; b). Both feeding modes require a sophisticated sensory system that enables prey recognition and detection. Copepods actively select prey based on its size, motility or its chemical and nutritional quality (DeMott 1988; Tiselius and Jonsson 1990; Kiørboe et al. 1996; Schultz and Kiørboe 2009; Meunier et al. 2016). Copepods have been observed to feed intensively on coccolithophores, thus being responsible for the vertical transport of a large fraction of coccolithophore calcite to the deeper ocean (Holligan et al. 1983; Van der Wal et al. 1995). The coccoliths of ingested cells remain largely intact during gut passage, thus ballast and accelerate the sinking of the fecal pellets produced by copepods (Honjo 1975; Holligan et al. 1993; Steinmetz 1994; Van der Wal et al. 1995). However, a fraction of the ingested calcite dissolves, which could affect the food uptake efficiency of the grazers (Harris 1994; Pond et al. 1995; Jansen and Wolf-Gladrow 2001). The silicified cell walls of diatoms have been shown to provide an effective defense against copepod grazing as the ingestion rate (Pančić et al. 2019), and likewise the growth and egg production (Liu et al. 2016) of copepods decreased with an increasing silica content of the diatom prey. Calcification could potentially lead to similar effects on copepod grazing. In this study, we investigate the effect of the coccosphere on the feeding rate of the copepod *Acartia tonsa*. We offered calcified and naked cells of the coccolithophore *Gephyrocapsa oceanica* to adult females of *A. tonsa*. *G. oceanica* was chosen as prey species as it is a highly calcified species so that an effect of calcification should be more easily detectable. Naked cells of *G. oceanica* were acquired by use of a short treatment with acid and base to dissolve the coccoliths. Both, calcified and naked *G. oceanica* were offered separately as well as in mixed cultures to the copepod. We followed the decrease of the coccolithophore cell concentrations due to predation of the copepods over an incubation period of 48 hours to examine whether (1) calcification affects the ingestion rate and (2) *A. tonsa* shows a preference for naked or calcified cells.

Methods

Cultivation of *A. tonsa*

Eggs of *A. tonsa* were incubated in 0.2 µm filtered artificial seawater (Kester et al. 1967) at 18°C. Hatched nauplii were fed initially with *Rhodomonas baltica* and later on with a

mixture of *R. baltica*, *Isochrysis galbana*, *Emiliania huxleyi* and *G. oceanica* (preparation of growth medium is described below) when nauplii reached the copepodite larval stage. After 25 days 200 adult female copepods were picked from the culture and kept in cohorts of 50 individuals in 2 L of 0.2 µm filtered artificial seawater for 2 days (without any food provided). Thereafter, the starved adult female copepods were transferred randomly to 12 smaller glass bottles (250 mL Schott Duran) each containing 15 individuals.

Preparation of *G. oceanica* prey cultures

A culture of *G. oceanica* (RCC 1303) was raised in artificial seawater (Kester et al. 1967) with vitamin and trace metal concentrations according to the f/2 medium (Guillard and Ryther 1962) plus 256 µmol kg^{-1} $NaNO_3$, 16 µmol kg^{-1} NaH_2PO_4, 10 nmol kg^{-1} SeO_2 (Danbara and Shiraiwa 1999) and 2 mL kg^{-1} of filtered North Sea water (Bach et al. 2011). *G. oceanica* was raised in a large volume of 5 L in a 12:12 hours light-dark cycle with a photon flux density of 230 µmol photons m^{-2} s^{-1} (measured with a LI-COR LI-250A light meter) at 18°C up to a concentration of ~160.000 cell mL^{-1}. During the last 36 hours before this final concentration was reached, the *G. oceanica* culture was incubated in the dark to stop cell division and deplete the energy reserves of the cells. This procedure has been shown to reduce the ability of the cells to rebuild a coccosphere after removal with a short acid base treatment, which is described in the following.

After the dark incubation, the *G. oceanica* culture was split into two equal parts in 2.3 L polycarbonate bottles, which was done under low light conditions (<0.01 µmol photons m^{-2} s^{-1}). In one of the bottles the coccospheres were dissolved by adding 250 µl hydrochloric acid (1 M) kg^{-1} of culture. Right after acid addition the culture bottle was rotated by hand for one minute and thereafter the pH was brought back to the value before the acid addition by adding 1 M sodium hydroxide solution. The bottle was again rotated for 5 minutes until all flocculation that was caused by the addition of the alkaline solution was dissolved. The decalcification treatment was carried out with a large volume of culture and the headspace was kept minimal in order to minimize outgassing of CO_2 during the low pH treatment. The cell abundance of *G. oceanica* decreased by 7% due to the treatment, but the majority of the cells survived and the cell number remained stable as

indicated by repeated flow cytometry measurements conducted up to one hour after the treatment.

Predation experiment

The grazing bioassays with *A. tonsa* were initiated within 1.5 hours after the acid-base treatment of *G. oceanica*. At the start of the grazing experiment, calcified and decalcified cells of *G. oceanica* were added separately into four of the replicate bottles that contained *A. tonsa*, respectively. The remaining four bottles with *A. tonsa* were filled up with a 1:1 mixture of calcified and decalcified cells. In this way we set up a “calcified”, a “decalcified” and a “mixed” treatment. The purpose of the separate “calcified” and “decalcified” treatments was to test for differences in ingestion rates due to the coccosphere. The purpose of the “mixed” treatment was to examine whether copepods actively select for prey without a coccosphere. Additionally, four control bottles were set up for each treatment (calcified, decalcified, mixed), respectively. These controls contained no predators but only the respective *G. oceanica* cells to account for potential losses of food cells for other reasons than predation. All bottles were filled to the top and closed without headspace to avoid disturbance in predation behavior of *A. tonsa* due to bubbles. The bottles were mounted on a plankton wheel and rotated with one round per minute.

1 mL subsamples were taken on a regular basis to enumerate the concentrations of *G. oceanica* by flow cytometry (as described in next section). The volume removed during each sampling was replaced with 0.2 µm filtered artificial seawater and care was taken that no air became trapped inside the bottles after closing.

The experiment was terminated after 48 hours when each bottle was gently drained over a 150 µm mesh to separate the copepods, which were counted under a binocular microscope and observed for vital signs. All other particles < 150 µm were collected with nylon filters of 10 µm mesh size. Filters were stained with Lugol’s iodine solution and collected eggs of *A. tonsa* were counted with a binocular microscope.

Initial prey concentrations were estimated in order to assure adequate food availability for the 15 copepods in each replicate bottle, but also to allow the detection of an effect of predation on the food concentration (guided by Berggreen et al. 1988; Thor and Wendt 2010). We targeted for an initial prey concentration of 18 x 10^3 cells mL^{-1}, which

corresponds to 216 µg organic carbon L^{-1} with 12 pg organic C per *G. oceanica* (Sett et al. 2014) and 4.6 µg C of copepod biomass for adult female *A. tonsa* (Berggreen et al. 1988).

Flow cytometry

Cell numbers of calcified and naked *G. oceanica* were quantified with a BD Accuri C6 flow cytometer based on the forward scattered light (FSC) and the red fluorescence of the cells (FL-3). Calcified cells were distinguished from decalcified cells on the basis of the side scatter signal, since calcified cells scatter more light in a 90° angle than decalcified cells (Olson et al. 1989).

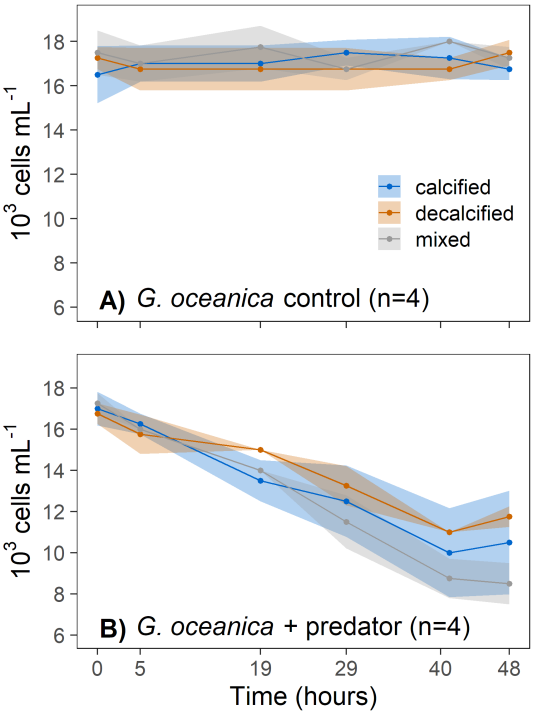

Figure 1 | Mean concentrations of calcified (blue) and decalcified *G. oceanica* (red) over the course of the experiment in A) the controls without predators and B) in the presence of *A. tonsa*. The ribbons display 1SD of the mean. Grey symbols represent the sum of calcified and decalcified cells within the mixed treatment.

Results

Ingestion of calcified and decalcified cells

In the pure cultures that contained either calcified or decalcified *G. oceanica*, the cell concentrations remained stable over the course of the experiment when predators were absent ("control", Figure 1 A). This was also the case for the sum of decalcified and calcified cells within the mixed cultures. In the presence of *A. tonsa*, the cell concentrations showed a similar decline in all three treatments (Figure 1 B). We observed a continuous decrease of the prey concentrations over the course of the experiment in all replicate bottles except of one: The prey concentrations in one of the replicates that contained calcified cells remained stagnant from the third sampling at 19 hours after the start until the end. The copepods within this replicate consumed 3060 cells $ind.^{-1}$ h^{-1} during the first 19 hours of the experiment (similar to the other replicates), whereas only about 5 cells $ind.^{-1}$ h^{-1} were consumed during the

Table 1 | Ingestion and clearance rates of *A. tonsa* on calcified vs. decalcified cells of *G. oceanica*. Sample means with standard deviation in brackets.

		calcified	decalcified
Ingestion rate (IR)	cells ind.$^{-1}$ hour^{-1}	3085 (320)	2021 (320)
	µg C µg C^{-1} day^{-1}	0.19 (0.02)	0.13 (0.02)
Clearance rate	mL ind.$^{-1}$ day^{-1}	4.40 (0.60)	2.87 (0.37)
Statistics	Student's t-test (IR cal = decal)	t(5) = 4.360, P = 0.0073	
No. of copepods	initial	15	15
Ingestion rate (IR)	cells ind.$^{-1}$ hour^{-1}	3085 (320)	2125 (295)
	µg C µg C^{-1} day^{-1}	0.19 (0.02)	0.13 (0.02)
Clearance rate	mL ind.$^{-1}$ day^{-1}	4.40 (0.60)	3.02 (0.33)
Statistics	Student's t-test (IR cal = decal)	t(5) = 4.116, P = 0.0092	
No. of copepods	recollected	14.5 (1.0)	14.25 (0.96)

remaining experiment. This strong deviance in one of the four replicates explains the large standard deviation in the calcified treatment in Fig. 1 B. We decided to exclude this deviating replicate from the calculation of the mean ingestion rates and for the statistical comparison of the ingestions rates of *A. tonsa* on calcified and decalcified cells. The influence of this decision is discussed at the end of the section.

Almost all copepods survived the experiment. Two individuals got lost in one replicate bottle of the calcified treatment. In the decalcified treatment two individuals were lost in one replicate, and in a second replicate another individual was missing at the end of the experiment. In the mixed treatment, one copepod was lost in two replicates each, and in a third replicate one copepod was found dead and another one was missing at the end of the experiment. It is not possible to say whether the missing individuals got lost during the final sampling of the copepods, or died over the course of the experiment.

The mean ingestion rate was significantly higher in the calcified than in the decalcified treatment, regardless of whether the initial number of copepods or the number of the recollected copepods at the end of the experiment were used in the calculation (Table 1). In the first option it is assumed that the copepods missing survived the experiment and got lost during the final sampling. The second option assumes the other extreme where the missing copepods got lost already in the beginning of the experiment. The difference between both options is only marginal (Table 1).

The mean clearance rate was lowest in the decalcified treatment (Table 1). Under the assumption that an adult female *A. tonsa* contains about 4.6 µg of organic carbon and a *G. oceanica* cell about 12 pg C, the specific ingestion rates were about 0.19 ± 0.07 (SD) µg C µg C^{-1} d^{-1} on calcified cells and 0.13 ± 0.02 (SD) µg C µg C^{-1} d^{-1} on decalcified cells.

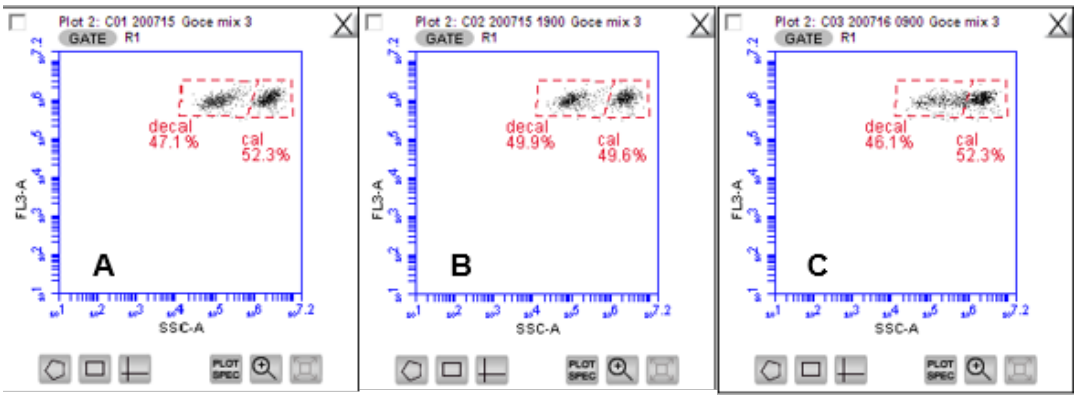

Figure 2 | Cytograms of one of the cultures that contained a mixture of calcified and decalcified *G. oceanica*. Both cell types could be distinguished based on their sidescatter signals, which are lower for decalcified cells. However, the decalcified cells produced new coccoliths even in the dark and their sidescatter increased. A) measurement at the start, B) after 5 hours and C) after 19 hours.

As noted above, we decided to exclude one replicate of the calcified treatment for statistical analysis because ingestion had basically stopped after 19 hours in this replicate. It is important to note that this exclusion affects the statistical significance of the results. Ingestion rates of *A. tonsa* on calcified and decalcified cells are significantly different when excluding this replicate ($t(5) = 4.360$, $P = 0.0073$) whereas they are not when it is included into the calculation ($t(4) = 1.216$, $P = 0.2910$).

Egg production

The overall food clearance over the course of the experiment was between 30 – 50% of the available prey cells whereby the copepods ingested about 15 – 26% of their own body weight day^{-1}. While these numbers suggest that incubated copepods were vital, egg production was too low to allow the assessment of the effect of calcification on the reproduction *of A. tonsa*. Only 0 to 5 eggs could be counted in each of the replicate bottles at the end of the experiment. 9 eggs in total were found in all bottles of the calcified treatment, 7 in the decalcified, and 11 in the mixed treatment. The nutritional input during the 48-hour experiment was probably not sufficient to compensate for the starvation period of two days prior to the start and to simultaneously support egg production.

Prey preference of *A. tonsa*

The “mixed” treatment displayed some measurement issues with separating calcified and decalcified prey types over the course of the experiment. At the start of the incubations,

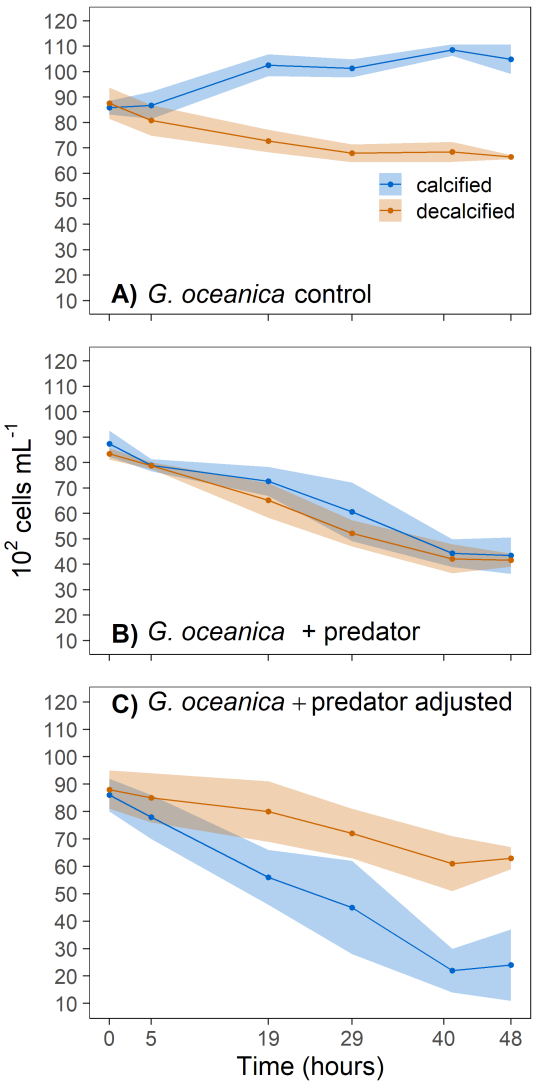

Figure 3 | Mean concentrations ± 1SD (ribbons) of calcified and decalcified *G. oceanica* within the mixed treatment. Decalcified cells produced new coccoliths in the dark and the re-calcified cells were progressively enumerated as calcified cells (compare Fig. 2). This is why the concentrations of calcified cells increased in the controls (A). B) shows the decrease of the cell concentrations in the presence of *A. tonsa* and C) displays the values of B corrected with the controls (see main text for detail).

both types could be clearly distinguished and appeared in two distinct populations with different right-angle light scatter (SSC) values when measured with the flow cytometer (Fig. 2). However, the decalcified cells were able to produce new coccoliths even under dark conditions, which caused an increase in their SSC. Consequently, the two distinct populations overlapped increasingly over the course of the experiment and the decalcified cells could no longer be separated from the calcified cells. Decalcified cells that produced new coccoliths shifted more closely towards (and eventually into) the flow cytometry gate of the calcified cells and were then quantified as such (Fig. 2). Due to this problem, the concentrations of the decalcified cells decreased even without predators, whereas the number of calcified cells increased (Fig. 3 A). When we do not correct for this shift due to "re-calcification" of previously decalcified cells, then both populations (calcified and decalcified) showed a similar decline when *A. tonsa* was present (Figure 3 B). To account for the changes in concentration due to re-calcification, we corrected the cell concentrations in the cultures where *A. tonsa* was present by reference to the concentration change in the controls of the respective treatment (Fig. 3 C). This was done by subtracting the mean treatment concentrations at a given time point from the respective mean treatment concentration of the control at the start. The resulting difference was then subtracted from the respective mean

concentration from the replicates that contained *A. tonsa* at the given time point. The ribbons display the error derived from the standard deviations of the means from the start and the respective time point through error propagation. The decrease of the adjusted cell concentrations shows that the cells counted as calcified decreased stronger compared to the cells counted as decalcified. Thus, this corrected dataset suggests that *A. tonsa* preferably consumed calcified and re-calcified cells compared to decalcified *G. oceanica*, which fits the findings from the pure culture treatments.

Discussion

The coccosphere of *G. oceanica* did not lower the ingestion rate of *A. tonsa*. In contrast, calcified *G. oceanica* were ingested faster than decalcified cells when both types were offered separately (Table 1). Moreover, our data suggests that the copepods even preferred calcified over decalcified *G. oceanica* when both cell types were offered in a mixture. When accounting for changes in the populations in the flow cytometer data evaluation due to recalcification (see results), decalcified *G. oceanica* and cells that had partly rebuilt their coccosphere were preferentially consumed (Fig. 3).

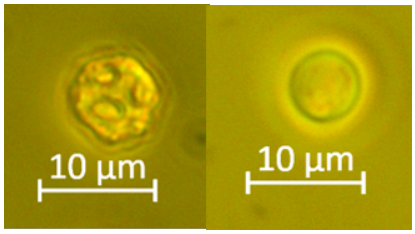

Figure 4 | Microscopic images of calcified and decalcified *G. oceanica*.

Many copepod species including *A. tonsa* are capable of grazing on food items in the size range of coccolithophores such as *G. oceanica*. However, it has been shown that the capture efficiency of food particles in this size range is low (Berggreen et al. 1988; Støttrup and Jensen 1990; Huskin et al. 2000) and in natural communities copepods often discriminate against such small prey and select larger food particles if sufficiently present (Sommer et al. 2000, 2005; Katechakis et al. 2004; Sommer and Sommer 2006). The clearance and ingestion rates of *A. tonsa* in our experiment were low compared to previous studies by Kiørboe et al. (1985), Thor and Wendt (2010) and Katechakis et al. (2004), which may, however, also be due to the different food source offered in these studies compared to ours.

We hypothesize that the apparent preference of *A. tonsa* for calcified cells was probably due to the size difference between calcified and decalcified cells. Calcified *G. oceanica* are about 7 µm in diameter. Decalcified cells are smaller (about 5.4 µm in diameter, Fig.

4) and represent the lower end of the prey size spectrum of *A. tonsa* (diameters of 4-5 µm, Berggreen et al. 1988; Kiørboe 2011a). *A. tonsa* feeds on non-motile prey particles by generating a feeding current of which the individual prey particles are detected and ingested individually (Kiørboe 2011b). It is believed that the copepod detects prey within this scanning current by chemosensory perception of the solvents leaking out of the viscous boundary layer that surrounds the phytoplankton cell (Kiørboe 2011a). The larger the size of the prey cell that is swept through the feeding current, the stronger the chemical signal that can be detected by the copepod, and prey cells of sizes at the lower limit are often not noticed early enough to ensure an effective capture (Kiørboe 2011a). Due to their lower size, the decalcified *G. oceanica* probably more often passed the feeding current undetected compared to calcified cells.

Huskin et al. (2000) examined the predation of *Calanus helgolandicus* on different prey species and found low ingestion rates on the coccolithophores *Emiliania huxleyi* and *Coccolithus pelagicus*. The authors showed that the copepods fed most efficiently on larger prey. Despite low ingestion rates, Huskin et al. (2000) measured a high egg production of *C. helgolandicus* that fed on *C. pelagicus*. The authors pointed out that the ingestion rate of copepods on coccolithophores varies according to the season and referred to Nejstgaard et al. (1997) who found high ingestion rates and egg production of *C. finmarchicus* on *E. huxleyi* during late spring when both species are naturally abundant.

Small coccolithophores may or may not represent an adequate food source for copepods, but so far there is no evidence that copepods discriminate against coccolithophores because of their coccosphere. Sikes and Wilbur (1982) reported equal ingestion rates of *C. finmarchicus* on a calcifying and a non-calcifying cells of *E. huxleyi* and *Chrysotila carterae* and concluded that coccoliths do not protect against predation. In line with their study, our results show that *A. tonsa* did not select actively for decalcified cells or against calcified cells of *G. oceanica*. Altogether this suggests that even in the case of *G. oceanica*, which is a heavily calcified coccolithophore, the coccosphere does not offer protection against the globally relevant copepod *A. tonsa*.

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5 | Synthesis

The results from my experimental work did not support the hypothesis that the coccosphere protects against viruses or grazing. It was not the case that coccolithophore cells without a calcite shell were predominantly infected by the *Emiliania huxleyi* virus 86, while those with shells were spared. Nor was it the case that naked cells were eaten preferentially by *Oxyrrhis marina*, or *Acartia tonsa*. Thus, the experiments did not yield clear evidence that the coccosphere provided an effective protection against the virus, the heterotrophic dinoflagellate, or the calanoid copepod.

Based on the results of the experiments with *E. huxleyi* and the *E. huxleyi virus 86*, I concluded that the coccosphere provides at best limited protection against viral infection. I had initially suspected that the coccosphere provides a barrier that prevents viral particles from infecting the cell. However, recent findings and additional results from a further long-term experiment I conducted raise doubts about a protective function of the coccosphere against viral infection, which will be discussed in the following section.

The experiments with *O. marina* showed that the coccosphere did not deter the grazer from ingesting calcified coccolithophore cells. However, the results showed that the calcite affected the food uptake and growth of the phagotrophic protozoan. Therefore, I concluded that calcification serves as indirect protection, promoting enhanced growth of coccolithophores due to the decreasing grazing pressure caused by calcite ingestion. However, the energy investment of coccolithophores in calcification puts them at a competitive disadvantage compared to phytoplankton species that do not invest in armor, but which also benefit from an overall reduced grazing pressure. According to this conclusion, the effect of the coccosphere becomes important only when coccolithophores are largely dominating the phytoplankton community (although determining the level of dominance needed to make calcification an advantage would require dedicated modelling).

All studies that I know of, which have been conducted to this topic found that inorganic cell coverings of marine phytoplankton do not serve as defense against phagotrophic protozoa (Harvey et al., 2015; Strom et al., 2018; Pančić et al., 2019; Mayers et al., 2020), only one of which examined microzooplankton predation in natural phytoplankton assemblages (Mayers et al., 2020). However, it has been argued that a specific trait does not have to perform perfectly to be maintained, but can be retained in a population as long as the benefit of this trait offsets its costs (Finkel and Kotrc, 2010, cited from Pančić et al. 2019). Future studies will help to discover whether relevant species in the very diverse group of protozoans, or mixotrophic protists, possess the capability of selecting against calcified phytoplankton species. Nevertheless, further below I question my conclusion that calcification implies a competitive disadvantage under non-selective predation and present some studies that I think provide an alternative perspective on this topic.

Copepods were shown to actively select for less armored prey and the diatom frustule is thought to serve as defense against copepod grazing (Hamm et al., 2003; Friedrichs et al., 2013; Pančić et al., 2019). Their chewing mouthparts that are edged with silica, and the diatom frustule are often referred to as a perfect example for the coevolution and continuous arms-race of predators and their prey (Hamm and Smetacek, 2007). The results of the third chapter of this thesis show that the calcite shell of *Gephyrocapsa oceanica* does not offer protection against predation by a copepod. The coccolithophore cells appeared to be at the lower end of the food size spec-

tra of adult *Acartia tonsa*. Berggreen et al. (1988) showed that the lower size limit for particle capture is relatively constant throughout the developmental stages of *A. tonsa*. Furthermore, the authors found similar or even higher minimum particle sizes in other pelagic copepod species (Berggreen et al., 1988), which suggests that smaller coccolithophore species do not fall into the main prey spectrum of copepods. Nevertheless, the coccosphere of larger coccolithophore species may offer a direct protection against copepod grazing. The impact of calcite on copepod digestion could be significant (Jansen and Wolf-Gladrow, 2001), but little is known whether the ingested calcite can affect the food uptake and growth of copepods, too, which would again offer indirect protection against copepod grazing, implying the same arguments with respect to the selective advantage of bearing a calcite shell as mentioned above for phagotrophic protozoa.

5.1 Adaptations in the *Emiliania huxleyi-E. huxleyi* virus system

At the time my experiments were conducted the *E. huxleyi virus 86* was characterized to follow a lytic cycle of viral reproduction (Thyrhaug et al., 2003). This means that the virus enters the host cell and is replicated by its metabolic machinery, which then leads to the lysis of the host cell and to the release of viral progeny into the surrounding medium. The simple extrapolation of the model that every infection is followed by lysis predicts the extinction of both, the host and the virus. The virus will eventually extinguish all host cells (Emiliani, 1993a,b) and thus its own basis of existence (Bratbak et al., 1998). However, unicellular organisms exhibit immune mechanisms and are equipped with a variety of biochemical mechanisms to detect and defuse alien nucleic acids, and, if nothing helps, eventually undergo programmed cell death to impede or suppress the infection and proliferation of a virus (Danilova, 2006). This mechanism ultimately offers protection to the clonal relatives of the host cell, since the target of selection in asexually reproducing organisms is the sum total of all cells comprising the clone (Mayr, 2001, cited from Hamm and Smetacek 2007). On the other hand, selection favors viruses that are able to evade the immune responses of the cell (Richard and Tulasne, 2012). Viruses often incorporate pieces of the host's DNA and can obtain entire genes through horizontal gene transfer. The *EhV 86* contains several genes for the biosynthesis of certain lipids that are involved in the programmed cell death machinery, and is thought to manipulate this cellular pathway for its own propagation (Monier et al., 2009; Bidle and Vardi, 2011; Rosenwasser et al., 2014). The stable coexistence of *E. huxleyi* and the *EhV's* thus can be explained as a result of the continuous arms race between the host and the virus, reminiscent to the Red Queen Model (Bidle and Vardi, 2011), which proposes that a species must constantly adapt in order to survive in a world where it is confronted with ever-adapting enemies and competitors (Van Valen, 1973). From the perspective of the pathogen, selection leads to a trade-off in its virulence (Frank, 1996; Ewald, 1998; Van Baalen, 1998). Virulence refers to the degree of damage that a pathogen causes to its host, so the loss in fitness of the host due to the pathogen. The fitness of the pathogen depends on the fitness of its resource, so that evolution streamlines the virulence of a pathogen in accordance with its transmission to other hosts (Ewald, 1998). More virulent pathogens efficiently exploit their hosts for their own reproduction, but also cause much harm. They spread early and kill early, which at some point reduces their transmission rate due to the decimation of hosts. Less virulent pathogens exploit their hosts in a more sustainable way, which increases the probability to encounter new hosts, but

also the risk of being outcompeted by more virulent pathogens. Thus, selection favors a trade-off that enhances the virulence of pathogen up to the point where it is outweighed by the cost of transmission due to host death (Frank, 1996; Ewald, 1998; Van Baalen, 1998). Different strains of *E. huxleyi* vary in their susceptibility to a certain *EhV* (Allen et al., 2007; Kegel et al., 2013) and there is a high level of competition between different *EhV* strains, such as more virulent mutants quickly outcompete less harmful ones (Nissimov et al., 2016).

However, the results in Publication 1 show a suspicious connection between the magnitude of the decline of the host cell concentrations due to viral infection and the peak abundance of the host cells, which seem to correlate negatively. Moreover, the results from a long-term experiment show that the population of *E. huxleyi* always recovered although the virus was still present in high concentrations (Fig. 5.1 A). On the basis of the Red Queen Hypothesis, one could explain this with the evolution of resistant host cells, which then were again infected by viruses that caught up and adapted to overcome the host immunity, so that the cell concentration declined again. However, the second increase in the host concentrations happened at about the same time in all replicates and the general development of the host and virus concentrations were similar (Fig. 5.1 B, C). Thyrhaug et al. (2003) made similar findings and proposed a dynamic feedback mechanism between phytoplankton hosts and their viruses. Recently, it has finally been shown that the *E. huxleyi-EhV* system follows density dependent virulent dynamics and that the *EhV* is actually a temperate virus, similar to a herpes virus, which is expressed under certain circumstances and is probably able to stay dormant in the host until the stress level of the latter increases (Knowles et al., 2020). In the dilute marine environment, selection apparently favors viruses whose virulence changes in accordance with the density of the host, and thus aligns with the amount of host cells present. Many phytoplankton viruses probably capable of adapting their virulence according to the host population (Thyrhaug et al., 2003).

5.2 The role of the coccosphere in blocking viral particles

Knowles et al. (2020) proposed that viral infection may be controlled by the host's physiology since stressed cells show a weaker immune response. *E. huxleyi* cells that are treated with acid and base will experience more stress than those that are not treated and the difference in viral susceptibility between decalcified and calcified cells could had been due to the treatment instead of a protective effect of the coccosphere. The cells of the non-calcifying *E. huxleyi* strain that were treated with acid and base showed a decreased susceptibility to the virus, which could had been due to a larger damage of the receptors that facilitate the entry of the virus due to the treatment.

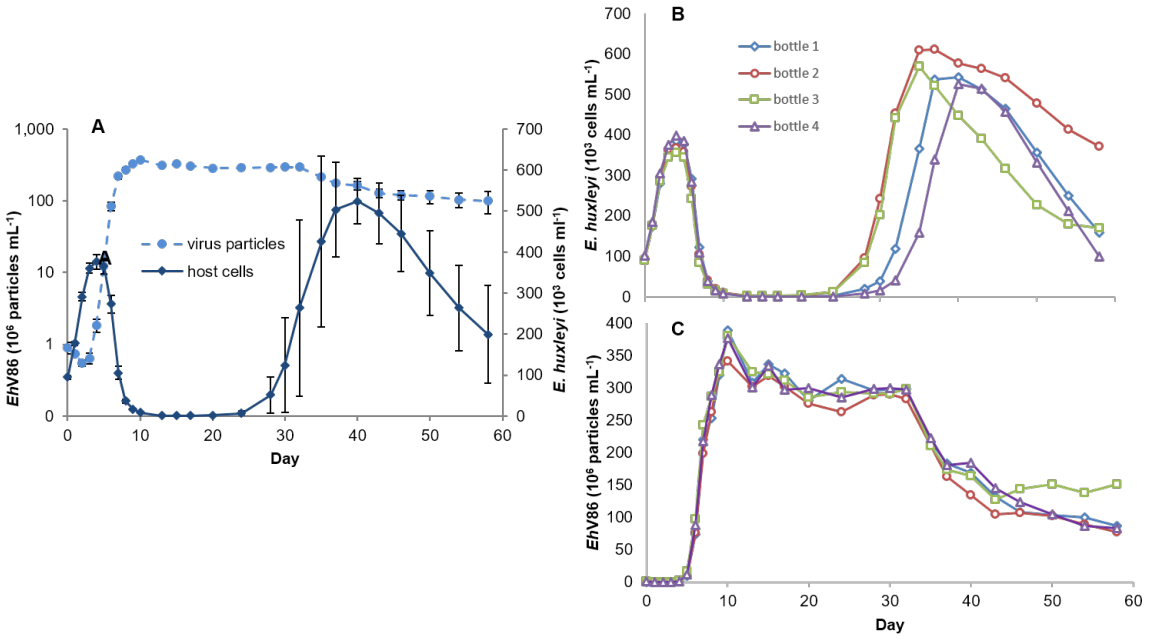

Figure 5.1: Long term experiment of virus infected *E. huxleyi* cultures. (A) Development of the concentrations of the host cells and viral particles over time (Mean ± SD, n=4, host cells on normal axis, viral particles on log-axis). Development of the host cells (B) and viral particles (C) within the individual replicate bottles.

Furthermore, it has been shown that the infection rate depends on the number of viral particles that are initially added to the *E. huxleyi* cultures. The more viral particles are added, the later crashes the host population and the earlier it recovers (Thyrhaug et al., 2003, compare Experiments 1 and 5 in Publication 1). These findings suggest that there is more than just the physiological stress of the host that determines the density dependent dynamics in the host-virus system.

In view of the findings of Thyrhaug et al. (2003), who discovered the feedback mechanism also in non-calcifying phytoplankton species the protective role of the coccosphere within these dynamics can be questioned. We hypothesized that the coccosphere keeps off viral particles from attaching and penetrating the cell membrane, and that infection can occur only due to gaps between the coccoliths. However, the results from Walker et al. (2018) and Kottmeier et al. (2020) indicate that the coccosphere is well maintained throughout the cell cycle and gaps between coccoliths do not really occur. Non-dividing calcified cells in the dark got infected (Publication 1) and the non-calcifying strain of *E. huxleyi* investigated in Publication 1 was generally less susceptible to viral infection than the calcified strain.

E. huxleyi is osmotrophic, so able to take up different organic compounds from the surrounding medium (Godrijan et al., 2020), and has been shown to be capable of phagotrophy, ingesting bacteria (Rokitta et al., 2011; Avrahami and Frada, 2020). Thus, the cell membrane of *E. huxleyi* is probably well equipped with receptors for detecting organic compounds that sustain growth. *EhV* particles may just hijack an uptake mechanism that is meant for useful organic substances and may be taken up actively by the cell instead of having to penetrate the coccosphere. It has

been shown that *EhV* particles enter the cell via lipid rafts, which are special microdomains in the membrane of the host cells that are formed by certain lipids and contain receptors the infective particles bind to (Rose et al., 2014). Such a mechanism could match with density regulated host-virus dynamics since the expression of certain receptors on the cell surface of the host will probably change with nutrient availability and thus growth and abundance of the host cells.

5.3 Virus infection and the *E. huxleyi* life cycle

In contrast to diploid cells, haploid cells of *E. huxleyi* do not suffer from viral infection (Frada et al., 2008). It has been stated that viral infection of the diploid life phase promotes sexual cycling and the transition to haploid cells, either by inducing meiosis, or by removing the competitive diploid cells, leaving over haploids (Frada et al., 2008). During the infection experiments, I was never able to observe (via microscopy and flow cytometry) haploid cells of *E. huxleyi*. However, I used to transfer a small volume of three replicates of a continuous *E. huxleyi* culture infected with the *EhV 86* into fresh medium every 6-8 weeks, keeping the cultures running on the side in a light cabinet. Diploid calcified cells sink to the bottom of the culture flasks and form a green carpet while the supernatant medium becomes transparent. I used to gently shake the cultures once a day to resuspend the cells. Now after about 2 years doing this, the cultures remain turbid and less cells sink to the bottom. Microscopic observations show that a large fraction of the cells inside do not possess coccoliths and are able to swim, what only haploid cells can do (Fig. 5.2). However, Frada et al. (2017) observed that viral infection does not necessarily induce meiosis, but also favors resistant diploid cells or the emergence of aneuploid cells. Moreover, there is evidence that the virus is present in haploid cells, too, but does not lead on to lysis in this case (Mordecai et al., 2017).

Coccolithoviruses belong to the NCLDV's (Nucleocytoplasmic Large DNA Viruses), a monophyletic group of viruses that derive from a common ancestor and probably diversified even before eukaryotes evolved (Guglielmini et al., 2019). NCLDV's exhibit larger genomes than many bacteria, which arguably blurs the line between non-living viruses and living cells (Yutin et al., 2014). They had formerly been suggested as the origin of the nucleus in eukaryotes (Bell, 2001), and even to constitute a forth domain of life (Colson et al., 2012). The *Coccolithoviruses* have been discovered as pathogens that follow simple lytic infection dynamics, but the *E. huxleyi – EhV* system is being intensively studied and turns out to be very complex. I came to this topic with the relatively simple hypothesis that cells of *E. huxleyi* whose coccosphere has been edged away suffer more from viral infection while calcified cells do less. I did not find evidence that the purpose of the coccosphere is the defense against *Coccolithoviruses*. Instead, the results of this work, together with the growing body of literature, suggest that the *EhV's* are deeply ingrained with their hosts and that the host-virus system of Coccolithophores and *Coccolithoviruses* (and potentially other phytoplankton and viruses) is far more complex than appreciated in the wider oceanographic community.

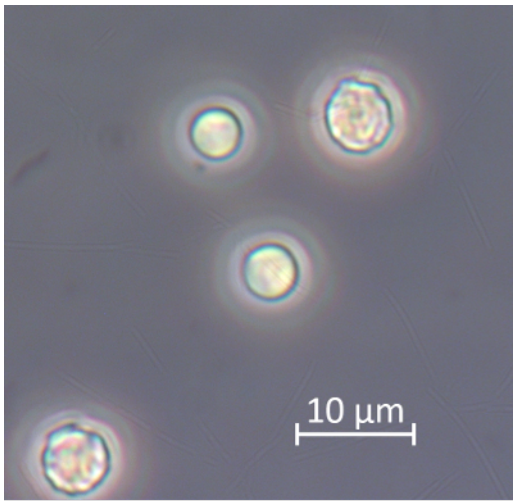

Figure 5.2: Non-calcified cells that are able to swim inside an infected *E. huxleyi* culture that has been transferred into fresh medium regularly.

5.4 Can the coccosphere serve as protection against non-selective microzooplankton grazing?

The results in Publication 2 show that calcification in coccolithophores impedes the digestion and food uptake capacity as well as the growth of a phagotrophic predator, but does not deter grazing. Thus, it provides indirect protection, but also favors competitive phytoplankton species that do not invest energy in "armor", but in growth instead, and do likewise benefit from the energy investment of the coccolithophores. One would assume that evolution favors grazers that are capable to detect calcite and choose non-calcified prey instead. Indeed, grazers who could differentiate between calcified and non-calcified prey would likely have a major advantage over predators that cannot distinguish and thus stodge useless inorganic food. However, you can only choose when you have a choice, and one could just as well speculate that this kind of selection does not occur, simply because of the dilute conditions in the marine environment. Protozoan predators live in a viscous and dilute environment and have to browse large volumes of seawater to find enough food to subsist (105–107 times their own body volume per day) (Kiørboe, 2011). Thus, protozoa may only have a limited choice for food even during phytoplankton blooms, and even less throughout the remaining season. Under such conditions, selection favors the ability to detect potential prey items on the basis of their taste/smell. Consequently, protozoa are well-equipped with chemoreceptors to search for an adequate nutrition along chemical gradients (Wootton et al., 2007; Martel, 2009; Breckels et al., 2011), in order to increase the probability of finding enough food at all, or all the elements needed to make a living. When one of these creatures that follows the signal of necessary nutrients encounters a coccolithophore, it may just take what it gets to be able to survive and to possibly even reproduce in order to maintain a population.

In the experiments with *O. marina*, I aimed to set up an initial prey density appropriate to ensure feeding saturation of the predators to ensure maximum feeding rates, which is quite the opposite situation to the one described above. Holling (1959) proposed that the intake rate of

a predator is dependent on the prey abundance and increases with increasing prey density, but saturates with higher prey densities as the intake rate is limited by the predators' capability to handle the prey. Since it has been shown that *O. marina* exhibits such a functional response of its feeding rate according to the prey density as proposed by Holling (Roberts et al., 2011), I hypothesized that *O. marina* would be able to actively select against calcified cells when enough food is provided and the grazers have a real choice of what prey they take up next. However, as already mentioned, one can question whether such food saturating conditions are characteristic for the pelagic ocean, or prevail often enough to promote the evolution of grazers that actively avoid ingesting calcified cells.

5.5 Different perspectives on predator-prey dynamics

Holling's model is identical to the Monod model, which describes the growth rate of microorganisms in an aqueous medium as a function of the absolute nutrient concentration. Both models assume that the consumption or growth rate of a consumer is not affected by its own population density, which is often not correct because individuals usually influence or interfere with each other (Jost, 2000; Arditi and Ginzburg, 2012, see Lobry and Harmand 2006). The functional response forms a cornerstone of the most common predator-prey model (Lotka-Volterra) used to explain natural predator-prey population dynamics (Rosenzweig and MacArthur, 1963). In essence, the model predicts periodic oscillations of the prey and the predator abundances around an equilibrium. Arditi and Ginzburg (1989) criticized the assumption that the functional response of a predator depends on the prey density alone and proposed that the per capita predation rate rather depends on the ratio of prey density to predator density. The authors argued that the prey equilibrium abundance in the common predator-prey model is determined by the upper trophic level alone, thus perfectly top-down controlled, and entirely unaffected by the characteristics of the prey, such as its growth rate or maximum sustainable population size in a given environment (Arditi and Ginzburg, 2012). Such a top-down controlled way of thinking was also the reasoning behind my conclusion, that calcification implies a competitive disadvantage against non-selective grazers. However, it may be questionable, as it implies that the phytoplankton biomass is strongly controlled by competition and predation. Murray (1982, 1999) criticized this way of thinking and argued that the size of populations is limited by the amount of resources available instead of being regulated by density-dependent processes such as predation or competition. This view seems in line with the one of Arditi and Ginzburg (1989, 2012), which, in contrast to the common predator-prey model mentioned above, allows joint exponential growth of interacting populations when resources are unlimited (Arditi and Ginzburg, 2012). White (2001, 2005, 2007) concluded that most trophic systems are bottom-up regulated. The author argued that predators are inefficient at controlling their prey because the prey have evolved ways to become inaccessible (White, 2005). This view is conform with the properties of ratio-dependent predator-prey models in contrast to the models that are based on prey density alone (Tyutyunov and Titova, 2020).

What are the implications of these theoretical models for the potential role of the coccosphere in predator-prey dynamics? Are phytoplankton species that do not invest in armor better off in competition under non-selective grazing? Competition commences when some essential element needed for the buildup of biomass becomes scarce. It should be less important during

the winter and pre-bloom bloom phase and becomes increasingly important when the stratification of the water column increases and nutrients become scarce. Just in that part of the year during which the phytoplankton assemblages in the temperate ocean are successively dominated by diatoms, coccolithophores and dinoflagellates. The three phytoplankton groups that manage to grow out of the microbial food web and whose species are armored with opal, calcite, or cellulose plates, respectively, thus invest resources and energy in armor.

It has been argued that predation is equally important in shaping marine ecosystems as bottom-up factors like resource acquisition, growth and competition are, and that the evolution of the organisms in the pelagic environment is shaped from both directions (Verity and Smetacek, 1996; Verity et al., 2002). The seasonal succession of phytoplankton groups in the temperate ocean is driven by the availability of nutrients and light, but also strongly influenced by predation (Sverdrup, 1953; Margalef, 1978; Smetacek and Passow, 1990; Barber and Hiscock, 2006; Behrenfeld, 2010). The mixed layer that comprises most of the living plankton increases with decreasing sea surface temperatures during winter, whereby it is enriched with remineralized nutrients from deeper water layers. The phytoplankton as well as its main predators, the microzooplankton get diluted during mixing, but the depth integrated phytoplankton biomass already begins to increase during winter (Behrenfeld, 2010). It has been argued that small cells, which have a high nutrient uptake capacity, also profit from the nutrient flush and the increasingly favorable conditions towards spring, which promote a "rising tide that lifts all phytoplankton" (Barber and Hiscock, 2006). Protozoan predators quickly catch up with their prey and consume most of the biomass production of the small and fast-growing phytoplankton, which leads to an increasing throughput of matter through the microbial food web. The impact of grazing proportionally increases with increasing phytoplankton division rates. As a result most of the primary production that is formed over the course of the season is lost to grazing (Lochte et al., 1993; Behrenfeld, 2014).

In the open ocean, the growth of the dominant photosynthetic picoplankton is thought to be balanced by grazing (Landry et al., 1997). The primary production is based on regenerated production and directly disappears again in the microbial food web (Dugdale and Goering, 1967; Sarmiento and Gruber, 2006). However, the herbivores obviously do not reduce their food to zero (Banse, 2013). Strom et al. (2000) compared trophodynamic plankton models with observations to examine the reasons that determine the lower limit of phytoplankton biomass in the open ocean. They concluded that spatial heterogeneity, or the top-down control of herbivores through carnivores are no convincing reasons why the phytoplankton never vanishes (Strom et al., 2000). In order to explain the issue of why "consumers avoid exterminating their prey", the authors proposed that protozoa exhibit highly plastic feeding capabilities and just switch to other prey (e.g. bacteria) when the phytoplankton biomass becomes low (Strom et al., 2000). However, Strom et al. (2000) criticized that models assume microherbivores to exhibit no intrinsic mortality besides predation, which was inconsistent with several studies that found high mortality rates under very low food levels. Nevertheless, the authors argued that the phytoplankton biomass is tightly controlled by protozoan grazing. This raises the question whether microherbivores regulate the phytoplankton biomass or if they are themselves just limited? Predators require a certain minimum prey density to achieve a positive net growth rate. An upper limit for the conversion of prey into predator biomass is set by the second law of thermodynamics, with efficiencies of 70–80% for a maximally efficient heterotroph, and around 60% for bacteria

and protozoa in culture, but many if not most consumers cannot reach this high levels (Calow, 1977; Sterner and Elser, 2017). Furthermore, autotrophs exhibit a less strict elemental homeostasis than heterotrophs, which can lead to stoichiometric imbalances between consumers and their resources (Sterner and Elser, 2017), so that a certain prey can potentially fail to satisfy the predator's stoichiometric needs although it is present in some amount. Following the view of White (2005, 2013), phytoplankton species may have evolved to become nutritionally inadequate for their predators, or inaccessible by producing cell walls, shells or toxins, which is widespread among the phytoplankton.

I doubt that these defense mechanisms only help against selective grazers and imply a competitive disadvantage against non-selective predators since they are costly and thus benefit competitive species that do not invest these costs. Therefore, I conclude that the view on this topic is currently incomplete. The view of Murray (1982, 1999) and White (2001, 2005, 2007, 2013) is very different from the one I learned and provides a different way of thinking. Arditi and Ginzburg (1989, 2012) proposed an alternative model that does not exclude that perspective. It differs from the one I know, the standard prey-dependent view. The authors noted that both theories are not the only alternatives but ends of a spectrum (Arditi and Ginzburg, 2012). I believe that these different views can help to understand how evolution in the plankton works. Is it the survival of the fittest, or rather the non-survival of the non-fit (den Boer, 1999)? Are populations regulated or limited? I believe that these questions are strongly related to the question asked in the title of my thesis "Why do coccolithophores calcify?". The fastest growing phytoplankton species may not always be the most successful ones under conditions that allow joint exponential growth of interacting populations, but their numeric gain may quickly disappear in the prevailing grazer community since it attracts and facilitates higher predator densities, thus being channeled again quickly into the microbial food web. Müller (2019) pointed out that the development of the coccosphere was probably not an instant but a stepwise process. It probably started with the ability to precipitate calcium carbonate inside the cell in simple crystals and evolved to the ability to produce intricately built coccoliths. The coccosphere may not serve as armor at all, however, the impact of coccolithophore calcification on the food uptake and growth of their main predators could have paid off right from the beginning. How did the arms race begin? Did cells develop defense systems that deterred their predators, or did they develop traits that caused them to be eaten less than others, thereby increasing their own share of an environment's carrying capacity?

Verity and Smetacek (1996) argued that the "theoretical basis for contemporary research concerning the structure and function of marine pelagic ecosystems is self-limiting". The authors advocated for detailed studies of the dominant taxa from different environments with the goal of identifying the reasons for their success. In contrast, Murray (2001) criticized that biology has many inductive generalizations but is missing universal laws and deductive theory.

While my work could not give a simple answer to the seemingly simple questions why coccolithophores calcify, the results from my work provided new insights into the complexity of host-virus interactions and pelagic food-web controls, which hopefully stimulates discussion among plankton ecologists, as well as providing a motivation for further work on these fundamental and important topics.

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Eidesstattliche Erklärung

Hiermit bestätige ich, dass die vorliegende Arbeit mit dem Titel:

Why do coccolithophores calcify? Does the calcium carbonate shell serve as protection against viral infection and predation?

von mir selbstständig verfasst worden ist und keine weiteren Quellen und Hilfsmittel als die angegebenen verwendet wurden.

Die vorliegende Arbeit ist unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft entstanden und wurde weder im Rahmen eines Präfungsverfahrens an anderer Stelle vorgelegt noch veröffentlicht.

Veröffentlichte oder zur Veröffentlichung eingereichte Manuskripte wurden kenntlich gemacht.

Ich erkläre mich einverstanden, dass diese Arbeit an die Bibliothek des GEOMAR Helmholtz Zentrum für Ozeanforschung Kiel und die Universitätsbibliothek der Christian-Albrechts-Universität zu Kiel weitergeleitet wird.

Kiel, Oktober 2021 Mathias Haunost