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Phenotypic evolution and adaptive strategies in marine phytoplankton (Coccolithophores)

LUKA ŠUPRAHA



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Abstract

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Coccolithophores are biogeochemically important marine algae that interact with the carbon cycle through photosynthesis (CO₂ sink), calcification (CO₂ source) and burial of carbon into oceanic sediments. The group is considered susceptible to the ongoing climate perturbations, in particular to ocean acidification, temperature increase and nutrient limitation. The aim of this thesis was to investigate the adaptation of coccolithophores to environmental change, with the focus on temperature stress and nutrient limitation. The research was conducted in frame of three approaches: experiments testing the physiological response of coccolithophore species *Helicosphaera carteri* and *Coccolithus pelagicus* to phosphorus limitation, field studies on coccolithophore life-cycles with a method comparison and an investigation of the phenotypic evolution of the coccolithophore genus *Helicosphaera* over the past 15 Ma. Experimental results show that the physiology and morphology of large coccolithophores are sensitive to phosphorus limitation, and that the adaptation to low-nutrient conditions can lead to a decrease in calcification rates. Field studies have contributed to our understanding of coccolithophore life cycles, revealing complex ecological patterns within the Mediterranean community which are seemingly regulated by seasonal, temperature-driven environment changes. In addition, the high-throughput sequencing (HTS) molecular method was shown to provide overall good representation of coccolithophore community composition. Finally, the study on *Helicosphaera* evolution showed that adaptation to decreasing CO₂ in higher latitudes involved cell and coccolith size decrease, whereas the adaptation in tropical ecosystems also included a physiological decrease in calcification rates in response to nutrient limitation. This thesis advanced our understanding of coccolithophore adaptive strategies and will improve our predictions on the fate of the group under ongoing climate change.

Keywords: Coccolithophores, Life-Cycle, Phytoplankton, Nutrient limitation, Temperature, Microscopy, High-throughput sequencing, Taxonomy

Luka Šupraha, Department of Earth Sciences, Palaeobiology, Villav. 16, Uppsala University, SE-75236 Uppsala, Sweden.

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To my family

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I **Šupraha, L.**, Gerecht, A., Probert, I., Henderiks, J. (2015). Eco-physiological adaptation shapes the response of calcifying algae to nutrient limitation. + Supplementary information. *Scientific Reports*, 5
- II Gerecht, A., **Šupraha, L.**, Edvardsen, B., Probert, I., Henderiks, J. (2014). High temperature decreases the PIC/POC ratio and increases phosphorus requirements in *Coccolithus pelagicus* (Haptophyta). *Biogeosciences*, 11: 3531-3545
- III Gerecht, A., **Šupraha, L.**, Edvardsen, B., Langer, G., Henderiks, J. (2015). Phosphorus availability modifies carbon production in *Coccolithus pelagicus* (Haptophyta). *Journal of Experimental Marine Biology and Ecology*, 472: 24-31
- IV **Šupraha, L.**, Ljubešić, Z., Mihanović, H., Henderiks, J. (2014). Observations on the life cycle and ecology of *Acanthoica quattropsina* Lohmann from a Mediterranean estuary. *Journal of Nannoplankton Research*, 34(SI): 49-56
- V **Šupraha, L.**, Ljubešić, Z., Mihanović, H., Henderiks, J. (2016). Coccolithophore life-cycle dynamics in a coastal Mediterranean ecosystem: seasonality and species-specific patterns. + Supplementary information. *Journal of Plankton Research*.
- VI Stadniczeňko, S. G.*, **Šupraha, L.***, Egge, E. S., Edvardsen, B. (2016). Haptophyte diversity and vertical distribution explored by 18S and 28S ribosomal RNA gene metabarcoding and scanning electron microscopy. *Manuscript submitted to Journal of Eukaryotic Microbiology*. *equal contribution
- VII **Šupraha, L.**, Henderiks, J. (2016) Nutrient availability modified the evolution of calcifying algae during the past 15 million years. *Manuscript for submission to Nature Publishing Group*.

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Statement of Authorship

Paper 1. L.Š. conducted the experiments, collected and analyzed the data, interpreted results and wrote the manuscript with input from all authors.

Papers 2. and 3. L.Š. contributed to data collection and analysis, interpretation of results and writing of the manuscript.

Paper 4. L.Š. participated in the field work, collected and analyzed the data, interpreted results and wrote the manuscript with input from all authors.

Paper 5. L.Š. participated in the field work, collected and analyzed the data, interpreted results and wrote the manuscript with input from all authors.

Paper 6. L.Š. participated in the field work, contributed to data collection and analysis, interpretation of results and writing of the manuscript.

Paper 7. L.Š. collected and analyzed the data, interpreted results and wrote the manuscript together with the co-author.

Additionally, the following papers were prepared during the course of the PhD but were not included in this thesis:

- I Jordan, R. W., Abe, K., Cruz, J., Eriksen, R., Guerreiro, C., Hagino, K., Heldal, M., Hernández-Becerril, D. U., Malinverno, E., Nishida, S., Patil, S. M., **Šupraha, L.**, Thomsen, H. A., Triantaphyllou, M. V., Young, J. R. (2016) Observations on the morphological diversity and distribution of two siliceous nanoplankton genera, *Hyalolithus* and *Petasaria*. *Micropaleontology*, 61(6): 439-455
- II Korlević, M., **Šupraha, L.**, Ljubešić, Z., Henderiks, J., Ciglencečki, I., Dautović, J., Orlić, S. (2016). Bacterial diversity across a highly stratified ecosystem: A salt-wedge Mediterranean estuary. *Systematic and Applied Microbiology*, 39 (6): 398-408
- III Bosak, S., **Šupraha, L.**, Nanjappa, D., Kooistra, W., Sarno, D. (2015). Morphology and phylogeny of four species from the genus *Bacteriastrum* (Bacillariophyta). *Phycologia*, 54(2): 130-148
- IV **Šupraha, L.**, Bosak, S., Ljubešić, Z., Mihanović, H., Olujić, G. et al. (2014). Cryptophyte bloom in a Mediterranean estuary: High abundance of *Plagioselmis cf. prolunga* in the Krka River estuary (eastern Adriatic Sea). *Scientia Marina*, 78(3): 329-338

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Introduction

Coccolithophores and the Earth system

Coccolithophores (Haptophyta) are a diverse and ubiquitous group of predominantly marine microalgae that cover their cells with tiny calcite plates called coccoliths (Fig. 1). Coccoliths are produced intracellularly through a unique calcification mechanism (Westbroek et al. 1984, Taylor et al. 2007) that incorporates carbon in calcite and releases CO₂ into the environment (Sikes et al. 1980, Westbroek et al. 1993, Paasche 2001, Flynn et al. 2016). Due to this peculiar life-history, coccolithophores interact with the biogeochemical carbon cycle through a balance of photosynthesis (CO₂ sink), calcification (CO₂ source) and long-term deposition of sinking coccoliths to oceanic sediments (Fig. 2).

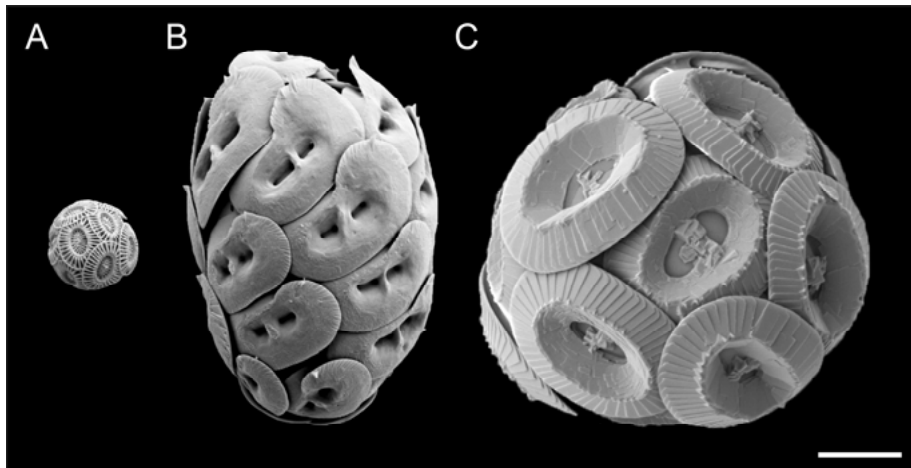


Figure 1. SEM images of common coccolithophore species. A) *Emiliana huxleyi*; B) *Helicosphaera carteri*; C) *Coccolithus pelagicus*. Scale bar = 5µm.

Given their high contribution to the global primary production (up to ~20% of biomass; Rousseaux and Gregg 2013) and the capability to form large blooms (Holligan et al. 1993, Harlay et al. 2010) coccolithophores are considered one of the key components of the biogeochemical carbon cycle (e.g. Iglesias-Rodríguez et al. 2002). The fossil record of the group (Bown et al. 2004) confirms the molecular clock data (de Vargas and Probert 2004, de Vargas et al. 2007), placing the origin of calcification within the Haptophyte lineage in the

late Triassic, ~220 million years ago (Ma). Since its origination, the group has played an important role in chemical buffering of the global oceans and formation of oceanic carbonate sediments, giving rise to the modern Earth system (Ridgwell and Zeebe 2005).

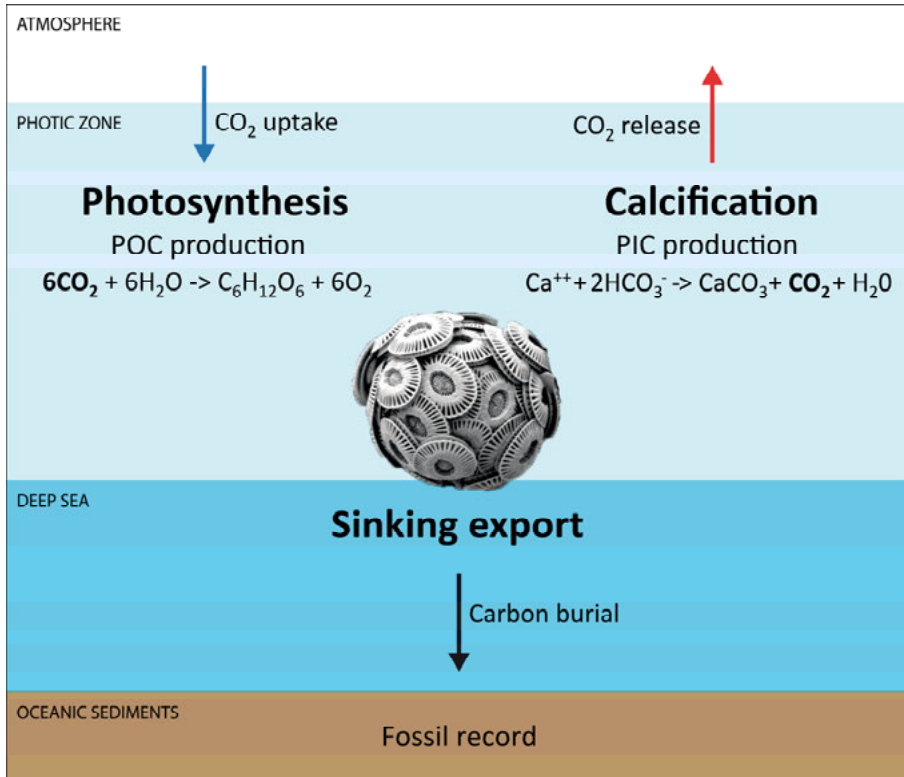


Figure 2. Schematic illustration of the role of coccolithophores in the biogeochemical carbon cycle.

Biogeochemically important physiological traits of modern coccolithophores, above all the production rates of particulate inorganic carbon (PIC) and particulate organic carbon (POC) are highly sensitive to different types of immediate environmental forcing such as temperature stress (Rosas-Navarro et al. 2016), CO_2 concentrations (Riebesell et al. 2000, Zondervan et al. 2002, Borchard et al. 2011, Fiorini et al. 2011) nutrient limitation (Paasche and Brubak 1994, Oviedo et al. 2014) and other (Zondervan 2007). In addition, long-term evolutionary adaptation can change carbon production rates of coccolithophores (e.g. Bolton et al. 2016, Schlüter et al. 2016). Due to this sensitivity to environmental changes, the increasing anthropogenic CO_2 emissions (Le Quéré et al. 2015) and the ongoing climate warming (Roemmich et al. 2015) will likely have a profound effect on the coccolithophore-related biogeochemical balance (Rost and Riebesell 2004). Two processes are considered to be particularly important in this context: the direct effects of increasing CO_2

concentrations and the consequential ocean acidification (Orr et al. 2005, Doney et al. 2009, Meyer and Riebesell 2015), and the rise in ocean temperatures which could lead to increased stratification of the upper photic zone and nutrient limitation (Sarmiento et al. 2004, Behrenfeld et al. 2006). Understanding the adaptive responses of coccolithophores to such perturbations is essential for predicting the fate of the global carbon cycle under changing climate.

Aims of this thesis

The aim of this thesis was to investigate the adaptive responses of coccolithophores to a wide range of environmental parameters, with the focus on temperature and nutrient availability. A multidisciplinary approach was used, spanning a range of research topics linking the cellular physiology and ecology with the evolution of coccolithophores. The research was conducted in frame of three approaches presented in this thesis:

- I Experimental study of the physiological response of globally important coccolithophore species *Helicosphaera carteri* and *Coccolithus pelagicus* to phosphorus limitation. (**Papers I-III**)
- II Field investigations in the Mediterranean Sea and the Oslo Fjord of the environmental drivers regulating coccolithophore life-cycle dynamics, along with a taxonomic study of *Acanthoica quattrosipina* life-cycle and the comparison of methods used in coccolithophore research. (**Papers IV-VI**)
- III Fossil time-series of the macroevolutionary patterns within the *Helicosphaera* genus, an important representative of modern coccolithophore communities over the past 15 Ma, with the focus on coccolith volume and thickness as indicators of cellular calcification in the geological past. (**Paper VII**)

Phosphorus limitation experiments

Response of coccolithophores to phosphorus limitation

The availability of inorganic nutrients, notably phosphorus and nitrogen exhibits a major control on phytoplankton primary production (Klausmeier et al. 2004). Phosphorus in particular is one of the key elements in biological systems, a structural element in nucleic acids, membrane phospholipids and the energy carrier ATP (Westheimer 1987). Due to its overall low concentrations in the modern oceans, high uptake rates of primary producers and bacteria as well as dependence on the external supply (Tyrrell 1999) it is considered a limiting nutrient in many ecosystems such as oceanic gyres (Wu 2000) and the Mediterranean sea (Thingstad 2005). The main processes regulating phosphorus supply to marine surface waters, such as river inflow, atmospheric transport and upwelling are highly sensitive to ongoing climate warming (Roemmich et al. 2015).

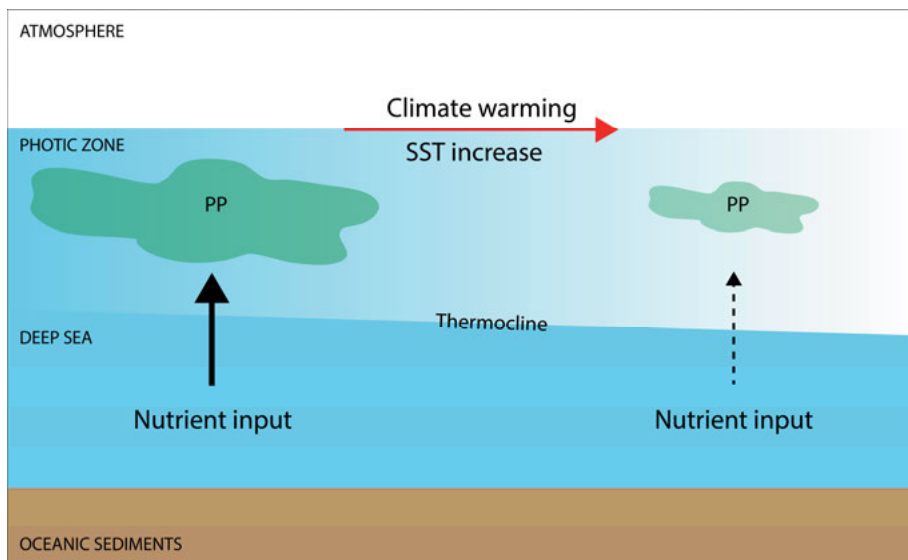


Figure 3. Schematic illustration of putative changes in nutrient input to the photic zone due to climate warming. The increase in global sea surface temperature (SST) will likely lead to increased stratification of the photic zone, decreasing the nutrient input from deep sea. This environmental change will ultimately affect the primary production (PP) of phytoplankton in the photic zone.

Most importantly, ocean warming is predicted to increase the stratification of the upper layers of the ocean and decrease the upwelling of nutrients, including phosphorus to the photic zone (Fig. 3), thus expanding the phosphorus-limited zones (Sarmiento et al. 2004, Falkowski and Oliver 2007). Coccolithophores are no exception to the dependence on phosphorus availability, despite their generally recognized affinity for oligotrophic ecosystems (Margalef 1977) and blooming of some prominent taxa (e.g. *Emiliania huxleyi*) under low-phosphorus conditions (Zondervan 2007). Since the calcification is considered highly resource-consuming (Monteiro et al. 2016), phosphorus limitation may impose energy-deficiency and affect carbon production rates.

Determining the precise physiological response patterns to phosphorus limitation has been a major topic in coccolithophore research during the past ~20 years. This question has commonly been addressed through laboratory experiments using *E. huxleyi* as the model organism. Early studies using both batch culture (Fig. 4A) and chemostat setups suggested that phosphorus limitation can alter the PIC/POC ratio of *E. huxleyi*, i.e. that cells tend to produce multiple layers of coccoliths and tend to slightly increase calcification rates under growth rate-affecting phosphorus limitation (Paasche and Brubak 1994, Paasche 1998).

The same study (Paasche 1998) also suggested that phosphorus limitation might affect the coccolith morphology and increase the prevalence of malformed coccoliths. These studies have also demonstrated that calcification was independent of cell division rates, and that it continued even when cells stopped dividing due to nutrient limitation, as well as in the dark when photosynthesis was stopped. The subsequent batch culture experiments on *Calcidiscus leptoporus* and *E. huxleyi* (Langer et al. 2012, 2013) contradicted these observations on increased inorganic carbon production and malformations. Here, the authors argued that carbon production does not increase under phosphorus limitation, attributing the observed increase in production rates to the methodological error inherent to the variable growth rate during the batch culture experiments. Furthermore, *C. leptoporus* did not show a significant increase in percentage of malformed coccoliths under phosphorus limitation. The experimental study by Riegman et al. (2000) investigated the adaptation of *E. huxleyi* to phosphorus limiting conditions. This study demonstrated that the phosphorus uptake system in *E. huxleyi* shows highest affinity for phosphate observed in any phytoplankton species, enabling it to outcompete other phytoplankton in phosphorus-limited conditions. Borchard et al. (2011), however, showed that phosphorus limitation in combination with elevated pCO₂ and increased temperature stress can decrease the production of particulate organic carbon (POC). Finally, in a comprehensive phosphorus limitation experiment on six Mediterranean strains of *E. huxleyi* using the batch culture method, (Oviedo et al. 2014) observed a range of strain-specific responses, suggesting that the response to phosphorus limitation even within *E. huxleyi*

is highly variable and strain-specific. In this study, *E. huxleyi* morphology was insensitive to phosphorus limitation.

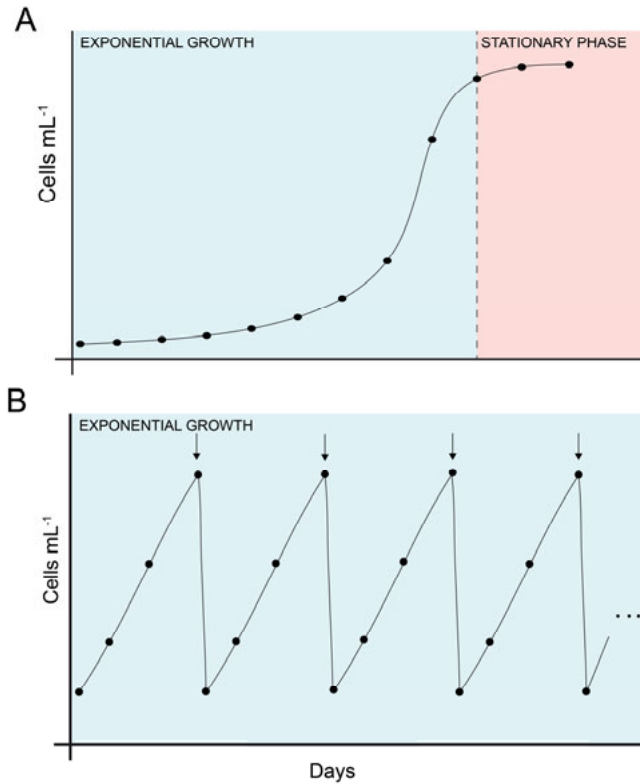


Figure 4. Schematic representation of the two experimental setups used in our experiments: A) In a batch culture setup, cells are inoculated at low density and allowed to grow exponentially. Once all of the phosphate is consumed, cells enter the stationary phase (zero growth) when they are harvested. Control experiments are run in parallel and cells are harvested at the same concentration (cells mL⁻¹) as phosphorus-limited batch; B) In a semi-continuous setup, cells are inoculated at a higher concentration, allowed to grow exponentially and diluted (arrows) using fresh medium before reaching phosphorus limitation and stationary phase. The process is repeated until cells have run through the desired number of generations.

To summarize, experimental data published to date was mostly restricted to *E. huxleyi*, a species shown to be exceptionally adapted to low phosphorus availability (Riegman et al. 2000). This adaptation could underpin the apparent low sensitivity of this species to phosphorus stress in experiments, both in terms of carbon production rates and morphology. On the other hand, a combination of environmental stressors could lead to stronger phosphorus limitation effects, as shown by Borchard et al. (2011). The comparison of *E. huxleyi* strains from oligotrophic and ultra-oligotrophic regions of the Mediterranean sea (Oviedo et al. 2014) did not detect any regional response pattern which

would suggest the existence of eco-physiological adaptation, which could be related to the lack of evolutionary pressure by limiting phosphorus availability in this species. The experiments to date have also demonstrated the importance of experimental set-up, which should be appropriately used when addressing the response of coccolithophores to phosphorus limitation. While the batch culture approach (Fig. 4A) is suitable for investigating malformations and cellular quotas under nutrient limitation, semi-continuous approach (Fig. 4B) is more appropriate for observing changes in elemental production rates within phosphorus limited cultures.

Finally, The observed strain-specific (Oviedo et al. 2014), as well as species specific patterns demonstrated in many studies to date (most of them focused on sea water carbonate chemistry perturbations (Langer et al. 2006, Ridgwell et al. 2009), indicate that more species (preferably of different size, motility, life-phase etc.) should be included in the experimental work in order to obtain a better picture of possible morphological and physiological sensitivity of coccolithophores to phosphorus limitation (Fig. 5).

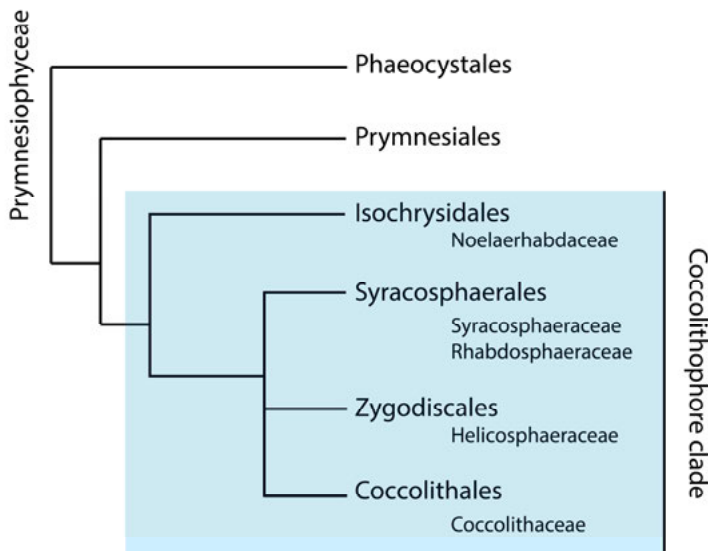


Figure 5. Schematic tree showing the phylogenetic relationships between major coccolithophore families discussed in this thesis and the position of coccolithophore clade within the haptophyte class Prymnesiophyceae. Adopted and modified from Young et al. (2005).

Phosphorus limitation changes carbon production rates in large coccolithophores

The aim of our experiments was to test the response of large and heavily calcified coccolithophore species, *Helicosphaera carteri* and *Coccolithus pelagicus* to phosphorus limitation. Previous experimental studies on this topic have almost exclusively been performed on the model species *Emiliania huxleyi*. Despite being perhaps the single most important coccolithophore species from the biogeochemical perspective, it represents only a tiny fraction of coccolithophore diversity. Therefore, one of the goals of our work was to include new coccolithophore species in the available experimental databases which would account for the taxonomic, morphological and functional diversity within the group. Both investigated species are important contributors to deep-sea carbonate fluxes (Baumann et al. 2004, Ziveri et al. 2007), with recent studies indicating that *C. pelagicus* could be the most important calcifier in the Arctic Ocean (Daniels et al. 2014).

Earlier investigations on coccolithophore physiological responses to different perturbations in the environment revealed that coccolithophores exhibit species- and even strain- specific response patterns, the origin of which has not yet been fully understood (Langer et al. 2006; Ridgwell et al. 2009; Oviedo et al. 2014). Here, we compared strains of *H. carteri* and two subspecies of *C. pelagicus* isolated from contrasting nutrient regimes, aiming to relate their response patterns to local eco-physiological adaptation (Fig. 6).

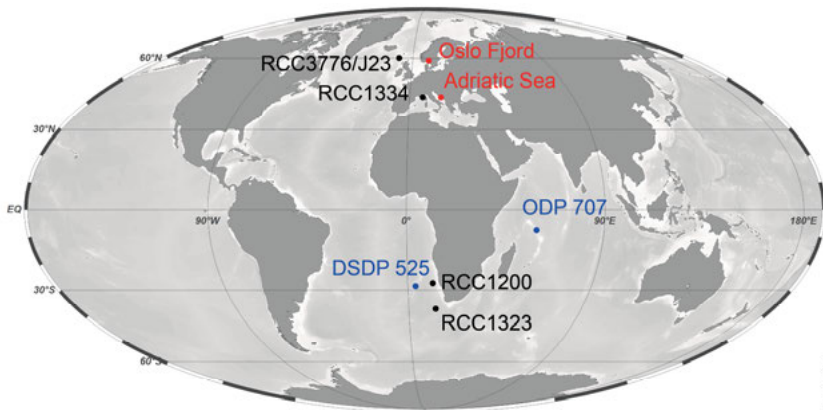


Figure 6. Map showing the geographical locations relevant for this thesis: culture isolation sites (black); locations of field studies (red); deep-sea drilling sites (blue). Map source: Schlitzer, R., Ocean Data View, odv.awi.de, 2015.

One of our goals was to investigate physiological responses to phosphorus limitation by using culture set-ups which are appropriate for answering specific research questions. Previously, Langer et al. (2013) have argued that the batch culture setup might not be appropriate for investigating the changes in production rates, due to variable growth rates during the course of the experiment in phosphorus limited batches (Fig. 4A). Therefore, we complemented our batch culture study on *C. pelagicus* with another study using a semi-continuous culture approach (Fig. 4B). This allowed us to observe the short-term adaptation of carbon production rates to different levels of phosphorus limitation in this species.

The experiments on *H. carteri*, presented in **Paper I**, demonstrated that the adaptation to local nutrient availability can lead to changes in cell physiology as well as strain-specific carbon production rates. Ultimately, such physiological adaptations can define the response patterns of different strains to nutrient limitation. In this experiment, we compared two strains that were isolated in contrasting nutrient regimes (Fig. 6): the oligotrophic and phosphorus limited Mediterranean Sea (RCC1334) and high production upwelling area of South Atlantic (RCC1323). The two strains exhibited different physiologies, as reflected in specific nutrient requirements, cell size, and calcification rates. The results indicate that the Mediterranean strain, under evolutionary pressure from phosphorus limitation, has adapted its physiology by decreasing phosphorus and nitrogen requirements and allocating limiting resources to photosynthesis, which resulted in decreased calcification rates. On the other hand, lower phosphorus requirements allowed the Mediterranean strain to reach 2.6 times higher cell concentrations under the same phosphorus concentrations compared to the Atlantic strain (Fig. 7). Despite distinct physiologies of the two strains, the batch culture setup did not allow for the precise detection of within-strain responses to phosphorus limitation. However, the same cellular PIC content values between the control and phosphorus limited treatments in batch setup may indicate a decrease in production rates when slower average growth in the phosphorus limited cultures is taken into account (Langer et al. 2013). This means that both strains likely exhibited an immediate decrease in PIC production in phosphorus-limited batches.

Our study suggests that *H. carteri* experiences stronger evolutionary pressure from phosphorus limitation than smaller species such as *E. huxleyi*, leading to divergence of different strains in terms of phosphorus physiology. This observation also explains the apparent lack of eco-physiological adaptation in respect to phosphorus in *E. huxleyi* (Oviedo et al. 2014), a species with high affinity for phosphorus (Riegman et al. 2000) and orders of magnitude lower phosphorus requirements compared to *H. carteri*. Finally, we observed a statistically non-significant signal of decreased coccolith volume in the Mediterranean strain with lower calcification rates, which could have implications for the interpretation of fossil coccolith volume data.

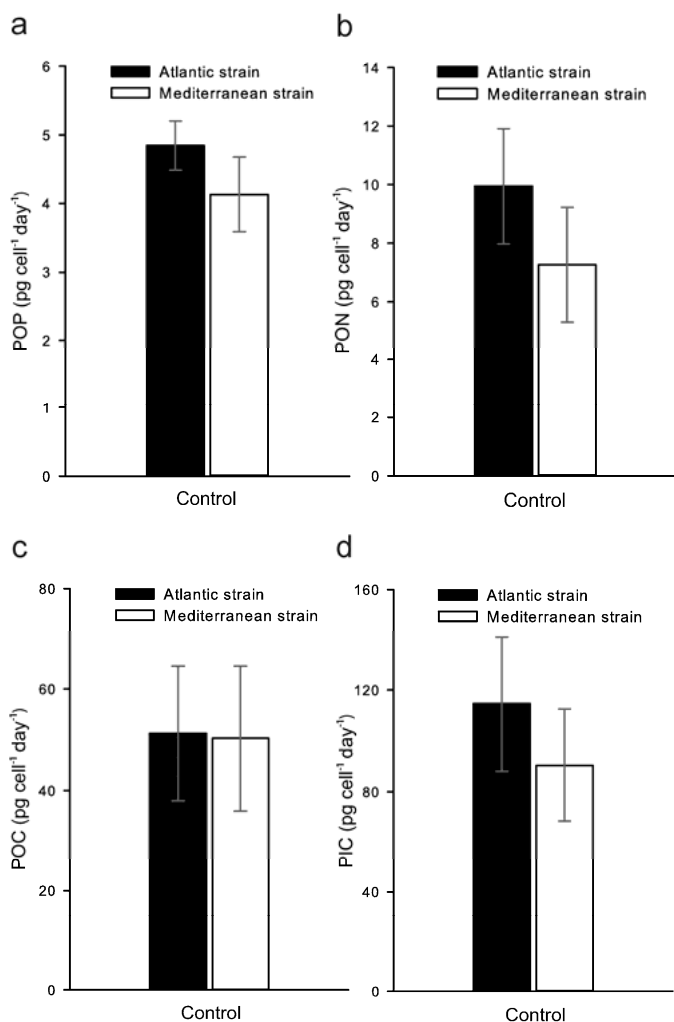


Figure 7. Cellular production rates of a) POP, b) PON, c) POC and d) PIC in the control cultures. Each bar represents pooled measurements from the three replicates. Two samples were taken from each replicate (total N=6). Error bars show standard deviation. Adopted from **Paper I**.

The sensitivity of large and heavily calcified coccolithophores to phosphorus limitation was confirmed in our experiments on *C. pelagicus*. In our batch culture study (**Paper II**), we showed that phosphorus limitation can lead to an increase in coccolith malformations, confirming the early findings by Paasche (1998). At the same time, high temperature stress enhanced the negative effects of phosphorus limitation on coccolith morphology (Fig. 8), showing that multiple stressors can add up to produce stronger effects, as reported by Borchard et al. (2011). Cellular carbon quotas on the other hand did not significantly change under phosphorus limitation in both subspecies of *C. pelagicus* during the batch culture experiments. However, temperature stress (+5°C)

increased the phosphorus requirements in the high latitude subspecies *C. pelagicus* ssp. *pelagicus*. Interestingly, increased phosphorus requirements and higher uptake rate of phosphorus due to temperature stress were used to sustain POC production and growth rate at the expense of calcification, decreasing the PIC/POC production ratio of cells grown at higher temperature. The allocation of resources from calcification to photosynthesis and POC production is in agreement with our observations on *H. carteri*.

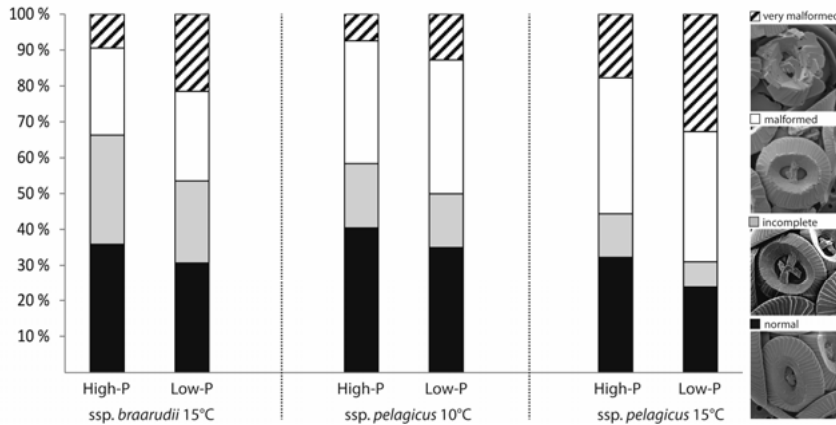


Figure. 8. Morphology of coccoliths observed under scanning electron microscopy of *Coccolithus pelagicus* ssp. *braarudii* at 15°C (n>2782), and ssp. *pelagicus* at 10°C (circles, n>1389) and 15°C (rhombus, n>1324), subdivided into the four categories normal (black bars), incomplete (grey bars), malformed (white bars), and very malformed (striped bars). Adopted from **Paper II**.

In a third study, the response of *C. pelagicus* to phosphorus limitation was tested using the semi-continuous experimental setup where cells are kept in exponential growth, allowing for a more precise quantification of cellular carbon production rates and acclimation strategies (**Paper III**). Here, we demonstrated that different levels of phosphorus limitation cause different response patterns. The initial reduction in phosphorus availability to 1 μ M decreased the POC production, while growth-limiting, severe phosphorus limitation (0.5 μ M) led to a decrease in PIC production (Fig. 9). The decrease in both PIC production and PIC content per cell means that coccolithophores do not calcify more under nutrient limitation, and argues against the hypothesized function of coccoliths to provide ballast to reach nutrient-rich deeper layers (Young 1994). This response pattern of decreased calcification as a short term response to phosphorus limitation was therefore also documented in this study and it is in agreement with our observations on *H. carteri*.

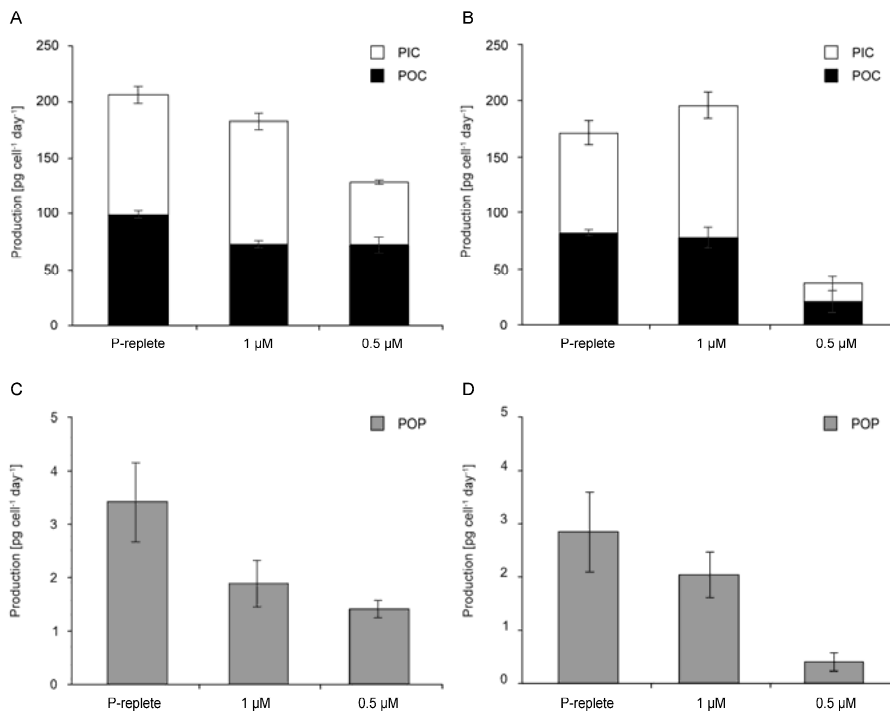


Figure 9. Production of particulate inorganic and organic carbon (PIC, POC: A, B) and phosphorus (POP: C, D) in semi-continuous cultures of *Coccotholus pelagicus* subsp. *braarudii* at three initial phosphate concentrations (P-replete, 1, 0.5 μ M), calculated using the average growth rate over all dilution cycles (A, C) and the growth rate of the last dilution cycle only (B, D). Average \pm SD, n=3. Adopted from **Paper III**.

Large coccolithophores are proven to be highly sensitive to phosphorus limitation, both in terms of carbon production and in terms of coccolith morphology. Under nutrient limitation, they seem to prioritize photosynthesis and POC production over calcification, an expensive and arguably not vital process. In combination with elevated temperature stress, the effects of phosphorus limitation are even stronger, which means that future climate scenarios with increased ocean temperatures (Roemmich et al. 2015) and enhanced nutrient limitation (Sarmiento et al. 2004) can have a strong negative effect on these marine calcifiers. Our findings are also highly relevant as they shed new light on coccolith size changes and changes in coccolith calcification that are observed in the fossil record (Henderiks and Pagani 2008, Suchéras-Marx et al. 2010, Herrmann et al. 2012, Bolton et al. 2016). Future interpretations of the fossil record should therefore take into account primary production levels and nutrient availability.

Field studies on extant coccolithophores

Coccolithophore life-cycles

The majority of ~280 coccolithophore morphospecies exhibit a haplo-diploid life cycle (Young et al. 2005). In this life-cycle, four haploid cells are formed after the meiotic division of a diploid cell and the formation of a zygote by fusion of two haploids starts a new diploid generation (Houdan et al. 2004). The coccolithophore life cycle is heteromorphic, meaning that the two life-phases exhibit distinct morphology, in most taxa in the form of a heterococcolith-bearing diploid phase and a holococcolith-bearing haploid phase (Fig. 10). The two types of coccoliths have different ultrastructure and are produced by distinct calcification mechanisms. The heterococcoliths are intracellularly produced calcite platelets made of alternating crystalline units (Westbroek et al. 1984, Young et al. 1999, Taylor et al. 2007). On the other hand, holococcoliths are platelets composed of tiny calcite crystalloliths, assembled extracellularly in a controlled environment (Klaveness 1973, Rowson et al. 1986).

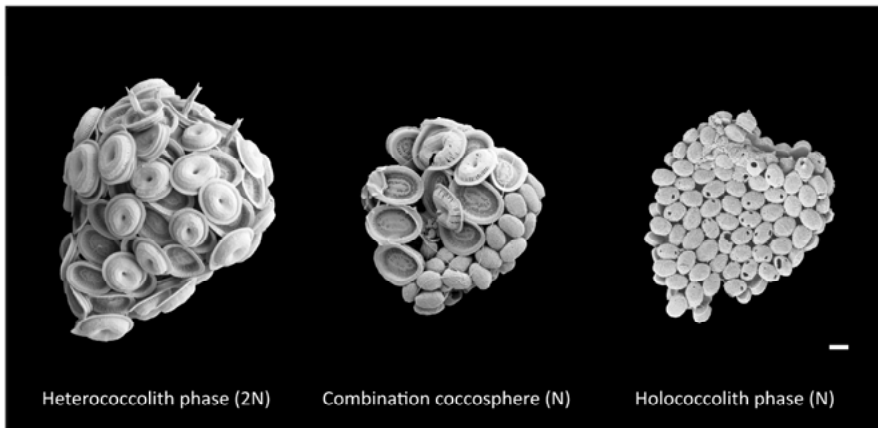


Figure 10. Life-phases of *Syracosphaera pulchra*, a common coccolithophore species. The combination coccosphere represents the first generation of haploid cells after the meiotic division of heterococcolith-bearing diploid cell. Scale bar=1 μ m. Adopted from **Paper V**.

The exceptions to heterococcolith-holococcolith life-cycle include the absence of calcite plates in haploid phases of the Noelaerhabdaceae (Klaveness

and Paasche 1971), Pleurochrysidaceae (Gayral and Fresnel 1983) and Hymenomonadaceae families (Fresnel 1994), as well as the production of nannoliths in the haploid phase of Ceratolithaceae (Sprengel and Young 2000) and Alisphaeraceae (Cros and Fortuño 2002).

The heterococcolith-holococcolith life-cycle was first described by Parke and Adams (1960) who revealed that the heterococcolithophore species *Coccolithus pelagicus* and holococcolithophore species *Crystallolithus hyalinus* represent two life-phases of the same species, based on culture observations. This heteromorphic nature of the life-cycle later proved to be problematic for the taxonomy and nomenclature of coccolithophores, as a large number of described species likely represent a phase in the life-cycle of other described species. Observations of combination coccospheres (Fig. 10) are the most common means of resolving this taxonomic problem. These cells are covered with both heterococcoliths and holococcoliths of the same species, and represent either the first generation after the meiotic division, or the cell formed by syngamy of the two haploids (Cros et al. 2000, Geisen et al. 2002). Although combination coccospheres are generally rare in field samples, a number of publications in the past 30 years have helped in reconstructing the relationships between the most common heterococcolith and holococcolith taxa and establishing the modern coccolithophore taxonomy (Samtleben and Schröder 1992, Alcober and Jordan 1997, Cortés 2000, Cros et al. 2000, Cortés and Bollmann 2002, Geisen et al. 2002, Triantaphyllou and Dimiza 2003, Triantaphyllou 2004, 2010, Malinverno et al. 2008, Couapel et al. 2009, Frada et al. 2009, Daniels et al. 2014b, Karatsolis B. et al. 2014, Triantaphyllou et al. 2015). Still, many of the life-cycle associations remain unresolved and more taxonomic investigations are needed. The highly diverse coccolithophore families such as Syracosphaeraceae and Rhabdosphaeraceae are particularly under-investigated, as many heterococcolithophore species from these families are still not associated with their holococcolithophore counterparts. Future studies of coccolithophore diversity in areas known for high abundances of these taxa such as the Mediterranean Sea (Kleijne 1991, Cros et al. 2000, Triantaphyllou et al. 2015) will likely result in more taxonomically resolved life-cycles.

The discovery of the coccolithophore life-cycle and subsequent advances in resolving coccolithophore taxonomy have opened new questions and objectives in coccolithophore research. The most important questions involve the physiology and ecology of the two life phases, and the implications of the life-cycle on the biogeochemistry, adaptation and evolution of the group. Early studies on the physiology of the two life-phases have shown that they are not only morphologically, but also physiologically distinct. Houdan et al. (2006) showed that the two life phases of *C. pelagicus* and *C. leptoporus* have specific ecological preference. In their growth experiments, the holococcolith phases of the two species performed better under nutrient depleted conditions, whereas the heterococcolith phases had optimal growth rates in the nutrient-

rich medium. In addition, the holococcolith phase of *C. pelagicus* (which is flagellated as opposed to the heterococcolith phase) exhibited mixotrophic nutrition by feeding on bacteria provided in the medium. In the same study, the heterococcolith phases showed higher resilience to water movement, suggesting that they might be more successful in environments of higher turbidity.

Findings from the above-mentioned experiments were supported by field data, which revealed that coccolithophore life-phases show distinct seasonal occurrence, corresponding to seasonal patterns in nutrient availability and turbidity of the water column. Cros & Estrada (2013) found that the holococcolith phase prevails during periods of pronounced stratification and oligotrophic conditions, while the heterococcolith phase prefers mixed and nutrient rich water column in the western Mediterranean Sea. Similar patterns in seasonal occurrence of life-phases were observed in the coccolithophore assemblages of the Aegean Sea (Triantaphyllou et al. 2002, Dimiza et al. 2008) and in different areas of the North Atlantic (Haidar and Thierstein 2001, Renaud 2001, Silva et al. 2013) and detected in coccolith fluxes as sampled by sediment traps in the Eastern Mediterranean Sea (Malinverno et al. 2009). Clear ecological preferences, confirmed experimentally and in field studies, indicate that the coccolithophore life-cycle represents an adaptation to changing environments, especially in the ecosystems with pronounced seasonality.

The ecological findings are in accordance with general observations on lower resource requirements of the haploid phases in phytoplankton (Lewis 1985) and the hypothesis that haplo-diploid life-cycle represents an adaptation to seasonal environments (Richerd et al. 1993). However, most of the available life-phase patterns to date were observed on the community level, and have not yet accounted for the high diversity of coccolithophores and possible species- or even strain-specific behaviors which are quite common in coccolithophores (Langer et al. 2006; Oviedo et al. 2014).

Another important question in coccolithophore life-cycle research is the regulation of life-cycle transitions. Evident seasonality and ecological preferences of the two life-phases suggest that there could be one or more environmental parameters or biological factors that trigger the life-phase transition process in coccolithophores. Experimental studies and culture observations suggest that different types of nutrient- (Inouye and Chihara 1979, Gayral and Fresnel 1983, Noël et al. 2004) and temperature- (Leadbeater 1970, Noël et al. 2004) stress can trigger life-phase transitions. In addition, the aging of cultures was proposed by Parke & Adams (1960) as a possible trigger. Houdan et al. (2004) observed spontaneous life-phase transitions at distinct times of the year, indicating a possible role of either a biological clock or seasonal chemical variations in stock water used for medium preparation. Finally, studies on *E. huxleyi* have provided strong evidence that the haploid phase is resistant to viral attacks and could represent a means of avoiding viral infections

in late stages of algal blooms (Frada et al. 2008, 2012). In this sense, the emergence of haploid cells in late stages of *Syracosphaera bannockii* bloom detected by Daniels et al. (2014) could have been triggered by viral infections.

In conclusion, the coccolithophore life-cycle represents a challenging research topic since it involves complex taxonomical problems that need to be solved in order to obtain information on coccolithophore ecology from field studies. Therefore, more data of high taxonomic resolution coupled with detailed surveys of environmental parameters on the life-phase level is needed to obtain better picture of ecological preferences and adaptational benefits of the coccolithophore life phases on the species and community level. This data will certainly shed more light on the possible biogeochemical implications of changes in life-phase structure of coccolithophore assemblages, and help understand the role of the life-cycle in the evolutionary history of the group.

Description of the *Acanthoica quattropsina* life-cycle

Resolving taxonomical relationships between heterococcolithophore and holococcolithophore taxa is a prerequisite for conducting ecological studies on life-cycle dynamics. In **Paper IV**, we provided a detailed taxonomical description of the life cycle of a common coccolithophore species *Acanthoica quattropsina* from the highly diverse Rhabdosphaeraceae family. The species was first described in its heterococcolith phase by Lohmann (1903), and combination coccospheres suggesting a life-cycle association with a previously undescribed member of holococcolithophore genus *Sphaerocalyptra* were observed by Cros & Fortuño (2002) and Cros et al. (2000). During our field survey of coccolithophore communities of the Krka River estuary located on the Eastern Adriatic coast (Mediterranean Sea), we collected exceptional material abundant with all life-phases of *A. quattropsina*, which allowed for a detailed description of the life-cycle (Fig. 11).

Our observations show that *A. quattropsina* exhibits a typical haplo-diploid life-cycle with the alternation of the two life-phases and the formation of combination coccospheres after the meiotic division of the diploid cell. A high degree of morphological variability was documented, both within the heterococcolith and the holococcolith phase. In the heterococcolith phase, the variability was most pronounced in the morphology of the central conical area of rhabdololiths and in the number and morphology of apical spines. This might indicate that there is more than one distinct species within the *A. quattropsina* complex, as was suggested by Kleijne (1992). In the holococcolith phase, we observed a strong variability in the morphology of the conical central area. Interestingly, the morphological variability followed a distinct pattern which could represent different phases in the development of the holococcolith. This hypothesis is supported by observations on holococcolith production (Klaveness 1973, Rowson et al. 1986, Noël et al. 2004) which takes place on

the surface of the cell, unlike the production of heterococcoliths which are exported on the cell surface only after they are completely constructed intracellularly (Westbroek et al. 1984, Taylor et al. 2007).

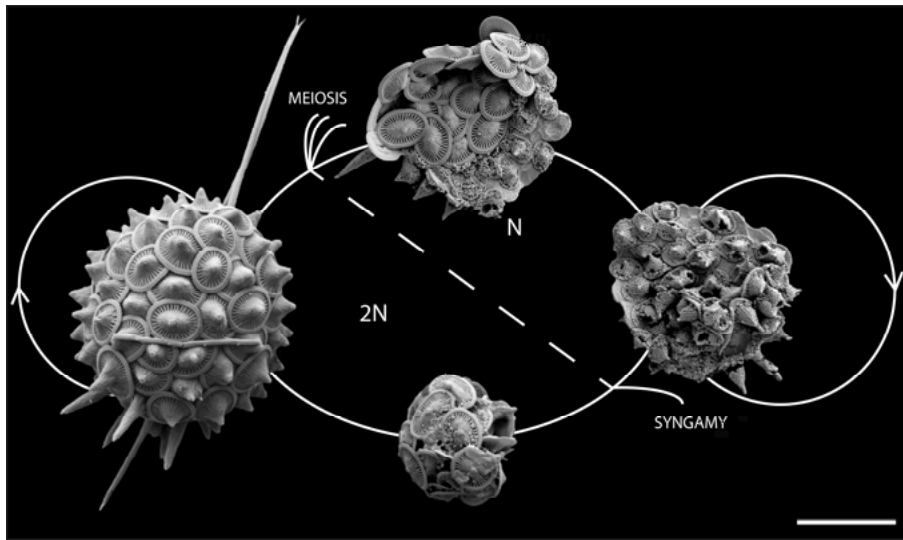


Figure 11. Life cycle of *Acanthoica quattropsina*, as interpreted from our results. The species exhibits a haplo-diploid life cycle, with mitotic divisions taking place within both life phases. Scale bar=5 μ m. Adopted and modified from **Paper IV**.

Moreover, our study suggests that other heterococcolithophore members of the Rhabdosphaeraceae family probably have life-cycle associations with members of the holococcolithophore genus *Sphaerocalyptra*. Another association showing this pattern is *Algirosphaera robusta* - *Sphaerocalyptra quadridentata* (Triantaphyllou and Dimiza 2003). Several combination coccospheres supporting this relationship have been observed by others (e.g. Cros & Fortuño 2002, Fig. 110 A-D; Triantaphyllou et al. 2015, PLATE 5:5-6). Still, other holococcolithophore taxa were also found to form combination coccospheres with Rhabdosphaeraceae, as demonstrated by the possible *Cyrtosphaera aculeata* - *Holococcolithophora heimdaliae* combination coccosphere. Given that the Rhabdosphaeraceae family includes at least 18 species (Kleijne 1992, Jordan et al. 2004), most of the life-cycle associations remain to be determined.

This study also presented the vertical distribution patterns of the *A. quattropsina* life-phases suggesting an affinity of the species for brackish waters and an exceptional tolerance to low salinity and even fresh-water conditions, in agreement with observations from the Baltic Sea (Meier et al. 2014). The detailed description of the life-cycle provided here will enable future field studies to link the life-phases with the precise environmental conditions under which they occur, advancing the knowledge on coccolithophore life-cycle dynamics.

Environmental regulation of coccolithophore life-cycle dynamics

The aim of our field study conducted along the Eastern Adriatic coast in winter and summer 2013 was to investigate the dynamics of coccolithophore life-cycles along strong spatial and seasonal environmental gradients (**Paper V**). The investigated area included oligotrophic coastal stations as well as the highly stratified and nutrient-rich Krka River estuary. The inflow of fresh water in the Krka system created strong vertical and longitudinal gradients of salinity, temperature and nutrient concentrations, which allowed for a multivariate analysis of coccolithophore life phase abundances and environmental parameters. The analysis was restricted to 14 coccolithophore species with taxonomically verified and well established life-cycles. We observed a distinct seasonality of life phases, in accordance with previous studies (Cros and Estrada 2013, Dimiza et al. 2015). The heterococcolith phases of the investigated species dominated winter communities, whereas the holococcolithophore phases thrived in the summer conditions. Nutritional preferences of the two life-phases observed in experiments (Houdan et al. 2006) are clearly in agreement with our field data, as life phases indeed seem to be adapted to distinct nutrient levels. In this context, the smaller cell size of a holococcolith phase, combined with smaller genetic material and probably lower calcite content of holococcoliths likely represent an adaptation to limited resources.

The hypothesis suggesting that a holococcolith phase overcomes nutrient stress with mixotrophic nutrition, as was shown for the flagellated holococcolith phase of *C. pelagicus* (Houdan et al. 2006) is likely not applicable to the Mediterranean communities. Here, most of the heterococcolithophores are also flagellated and likely mixotrophic, which could represent an adaptation of both life phases to an environment which is oligotrophic in both seasons.

While the adaptational benefits of the two life-phases are evident and can explain their occurrence and dominance patterns in our data, the parameters regulating life-phase transitions are less clear. Our sampling frequency, with two samplings (winter and summer) could not determine the precise triggers of life-phase changes. However, our observations can provide guidelines for future investigations of coccolithophore life-phase transitions, both in field and culture studies. The nutrient availability itself could not explain the occurrence of life-phases in our study, despite some experimental observations suggesting that it is the main trigger of transitions (Inouye and Chihara 1979, Gayral and Fresnel 1983, Noël et al. 2004). Instead, the multivariate analysis of life-phase prevalence on the community level showed that the holococcolith phase was becoming more dominant along a gradient of increasing temperature (Fig. 12). This finding was particularly interesting since it suggests that the holococcolithophore dominance has an optimum distribution curve, with highest prevalence in ~14-21°C range. Similar distribution in respect to temperature gradients can be inferred from other studies in the Mediterranean

which analyzed distribution of coccolithophore life phases (Dimiza et al. 2008, Cros and Estrada 2013, Oviedo et al. 2015). The temperature signal observed in our study could mean that a certain temperature threshold (e.g. $\sim 14.5^{\circ}\text{C}$ in the Eastern Mediterranean) directly triggers the life-phase transition, but it could also indicate that life-phase transitions are triggered by other environmental parameters regulated with temperature. These could include insolation, day-length or water column structure regulating mixing and nutrient levels in the photic zone.

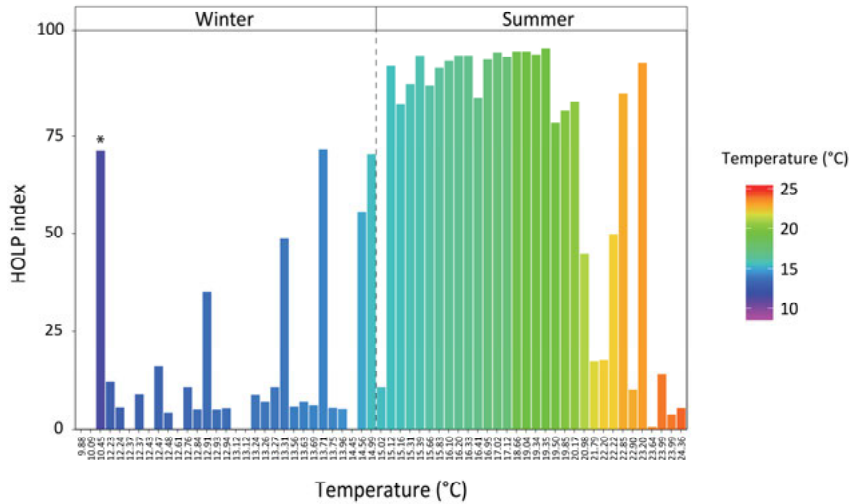


Figure 12. Bar chart showing the prevalence of holococcolithophores (HOLP index) from both sampling seasons ranked by temperature. Sample labels are listed above the plot, marking the season, station name and the sample depth. (*) estuarine sample collected in winter at the E3 station (4 m depth). Due to the sharp halocline at 4 m depth, the assigned temperature is likely inaccurate and the species composition of the sample is similar to the warmer samples from deeper depths. Adopted and modified from **Paper V**.

A number of species in our study exhibited distinct ecological preferences which were not following the common seasonal abundance pattern. The estuarine samples during winter were dominated by the holococcolith phases of *A. quattrosphina*, *Syracosphaera pulchra* and *S. mediterranea*, while the heterococcolith phase of *S. halldalii* bloomed in the estuary during summer. These findings clearly demonstrate that coccolithophore life-cycle dynamics cannot be explained solely by seasonal phenomena, but they are likely regulated by a combination of seasonal changes and species-specific ecological preferences. In addition, observations on mixed life-phase blooms in this study could indicate that biological factors such as viral infections during late-bloom conditions could also help trigger the life-phase transitions, as was suggested by several other studies (Frada et al. 2008, 2012, Daniels et al. 2014b).

Our finding on the possible role of temperature in the direct or indirect regulation of Mediterranean coccolithophore life phases has important implications from a biogeochemical perspective. As opposed to mostly robust and heavily calcified heterococcoliths, fragile holococcoliths commonly dissolve before reaching marine sediments. This means that their contribution to carbon burial and ballasting of organic carbon transport is minor. It is therefore essential to further constrain the environmental controls on life phase transitions, as well as better understand the carbon production patterns within holococcolithophores and their response to global changes. The dataset presented herein is an important step in this direction. We predict that ongoing warming of the global ocean, coupled with stratification of the upper layers and decreased input of nutrients (Sarmiento et al. 2004) may favor the prevalence of holococcolith phases, with major implications for the biogeochemical performance of the group.

Using molecular methods in coccolithophore research

For many years, scanning electron microscopy (SEM) has been used as a standard tool for investigating modern coccolithophore communities (Okada and McIntyre 1977, Kleijne 1991, Dimiza et al. 2008, 2015). Since the taxonomy of modern coccolithophores is well established and mostly based on SEM observations (Cros and Fortuño 2002, Young et al. 2003), the SEM-based methods can provide detailed and accurate qualitative and quantitative information (Bollmann et al. 2002). Still, certain downsides of the SEM-based methods have also been identified. Firstly, the methods require taxonomic expertise to precisely identify coccolithophores. Secondly, some important taxa with non-calcifying haploid life-phases (e.g. *E. huxleyi*) are not accounted for in SEM surveys. Lastly, quantitative analysis is commonly based on counts performed on many fields of view and can be time-consuming. Therefore, the method is not optimal for routine monitoring of coccolithophore communities or surveys with large number of samples.

Understanding of haptophyte diversity and ecological importance in global oceans was markedly advanced by the usage of molecular methods (Edwardsen et al. 2016), which are able to identify fragile species which could not be detected using the standard microscopy-based approach. Two molecular methods have been used extensively for haptophyte research: Sanger sequencing of clone libraries from environmental samples or environmental sequencing method (e.g. Moon & van der Staay et al. 2000, Moon-van der Staay et al. 2001) and high-throughput sequencing (HTS) (e.g. Liu et al. 2009, Bittner et al. 2013, Egge et al. 2013, 2015a, 2015b). Both methods are based on a similar principle of amplifying marker genes from the environmental samples and subsequently obtaining sequences or reads, with much higher yield using HTS compared to Sanger sequencing clone libraries. However, the

final taxonomical performance of molecular methods is constrained by the availability of reference sequences obtained either from monoclonal culture material or pipetted single cells, or taxonomically verified environmental sequences. In addition, the quantitative analysis using molecular methods can be problematic due to various biases, e.g. group- or species-specific rRNA content or rDNA copy number (Egge et al. 2013).

The application of the environmental sequencing approach in investigating coccolithophore communities was tested by Young et al. (2014). The study found overall good taxonomical correspondence of 28S rDNA molecular markers with the SEM analysis of the same samples, but found no strong correlation of numbers of reads and SEM counts. While this result suggest that the molecular methods could be used for coccolithophore research, more method testing is needed. In particular, the methods yielding more sequences than clone libraries such as the HTS methods could prove to be more appropriate for quantitative investigations of coccolithophore communities. In addition, higher yield of reads in HTS methods combined with larger volume of seawater analyzed in HTS methods compared to SEM methods might provide better information on taxonomic diversity. Finally, two most common markers, 18S rDNA and 28S rDNA should be compared to find the optimal approach in analyzing coccolithophore communities using the HTS methods.

SEM vs. HTS method

The comparison of the scanning electron microscopy (SEM) method and the high-throughput sequencing (HTS) method for investigating extant coccolithophore communities was conducted using samples collected in the Outer Oslofjord (Norway) in summer 2013 (**Paper VI**). Our results demonstrate that the HTS methods show promising potential for investigating coccolithophore communities, despite certain downsides that were observed in our study. Previous studies have shown that the HTS methods are optimal for haptophyte research (Edvardsen et al. 2016), especially for identifying non-calcifying taxa which are not detectable under light or electron microscope during the routine counts (Egge et al. 2015). For these taxa, the alternative methods are time consuming and commonly restricted to qualitative analysis using transmission electron microscopy. On the other hand, even the most fragile coccolithophores are routinely preserved in samples prepared for SEM analysis, which allows for detailed and precise quantitative and qualitative investigations (Bollmann et al. 2002, Young et al. 2003). Still, the downsides of the SEM method, such as time-consuming analysis that can omit rare species and the need for taxonomic expertise were all recognized during the method comparison.

Paper VI shows that the 18S rDNA marker gives overall better representation of the relative abundance of coccolithophores at the family level (Fig.

13), and that the number of reads obtained using this marker corresponds to number of morphospecies detected under the SEM. The 28S rDNA approach seemed to overestimate the coccolithophore diversity compared to the SEM, and did not provide satisfactory relative abundance estimates. These observations are in agreement with a previous study comparing SEM and a molecular (clone library *i.e.* environmental sequencing) approach that used the 28S rDNA marker (Young et al. 2014). On the other hand, 18S rDNA underestimated species diversity within the genera rich in morphospecies such as Syracosphaeraceae and Rhabdosphaeraceae. The usage of HTS methods for estimating cell abundance is already known to be problematic, mostly due to different rDNA copy number or interspecific RNA contents within haptophytes, as well as variations of the RNA content along different physiological traits such as size and growth rate (Egge et al. 2013). The divergence in diversity estimates observed by the two markers is commonly attributed to the overall faster evolutionary rates in parts of the 28S RNA gene, which has been suggested to be more useful for differentiating closely related taxa (Liu et al. 2009).

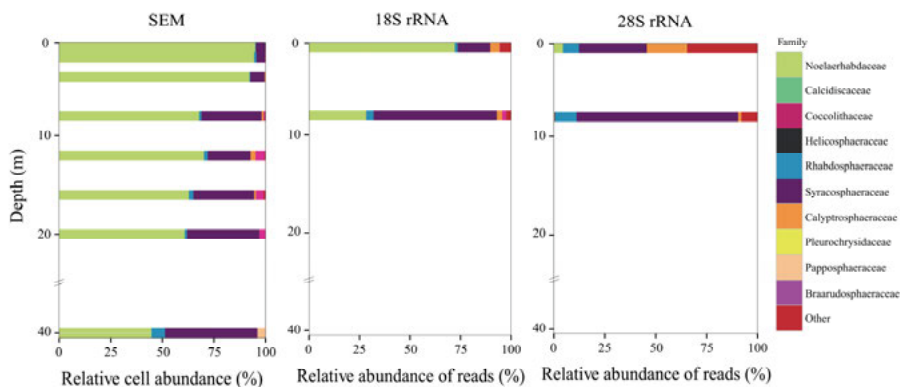


Figure 13. Relative abundance of coccolithophore families inferred from SEM counts at 8 depths and number of reads obtained using 18S and 28S rDNA markers at 1 and 8 m depths. Adopted from **Paper VI**.

Another important remark about the HTS method is that only a small portion of detected operational taxonomic units (OTUs) could be identified to the species level. A number of species were only detected using the HTS method, and identified with high certainty, demonstrating the power of HTS methods in cases when sequences of taxonomically verified species are present in the reference sequence databases. This result also shows that the taxonomical performance of the method is highly constrained by the quality of the reference database used for taxonomic assignment, an issue which was recognized in a

previous molecular study on coccolithophores (Young et al. 2014). The availability of taxonomically verified coccolithophore sequences in the reference database mostly depends on the number of taxonomically analyzed culture isolates. Coccolithophore reference sequences derived from cultures are still highly biased towards the most common species such as *E. huxleyi* or coastal species, which is largely related to the difficulties in culturing most of the coccolithophore species. Therefore, new approaches should be developed which could collect genetic data from taxonomically verified single-cells picked from water samples without establishment of cultures. The structural robustness of coccoliths, which are key to morphological identification, is clearly advantageous feature which should be used for combining single-cell molecular analysis and SEM-based taxonomy. The enhancement of reference databases from single-cell isolates in combination with HTS methods could provide an optimal approach for studying coccolithophore communities in future, although certain features of coccolithophore communities such as the life-cycles are to date not detectable by the molecular methods, but rely on SEM methods (**Paper V**).

Fossil time-series of *Helicosphaera* evolution

Evolution of coccolithophores over the past 15 Ma

The exceptional fossil record of coccolithophores can be traced back to the late Triassic (~220 Ma), allowing for detailed investigations of the evolution of the group (Bown et al. 2004). The early evolution of coccolithophores was marked by a steady increase in diversity (Bown et al. 2004, Bown 2005, Fig. 14) that peaked during the warm and high-CO₂ environment of the late Cretaceous (Fletcher et al. 2008). The major extinction event at the K/Pg boundary (~66 Ma) resulted in the disappearance of 93% of coccolithophore species from the fossil record (Bown et al. 2004). Subsequent radiation of surviving taxa led to a recovery of coccolithophore diversity during the Paleogene and a diversity maximum in the late Paleocene-early Eocene. The group then decreased in prominence during the Oligocene (~29-26 Ma ago) when it also reached a minimum of Cenozoic diversity. A short recovery in diversity was observed in the Miocene with a new decline in the Pliocene and Pleistocene (Bown et al. 2004).

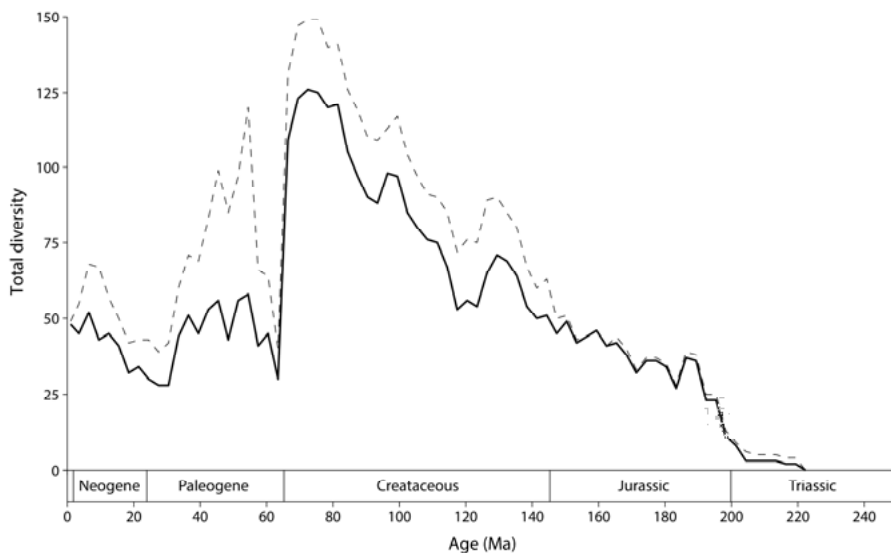


Figure 14. Diversity of calcareous nannoplankton (dashed line) and coccolithophores (solid line) during the past ~250 Ma. Adopted and modified from Bown (2005)

Along with an overall decrease in diversity, the Cenozoic fossil record also shows a decrease in coccolith size (Hannisdal et al. 2012, Herrmann and Thierstein 2012) and decreased coccolithophore carbonate fluxes over the past 17 Ma (Suchéras-Marx and Henderiks 2014). The macroevolution of coccolithophores during the Cenozoic also shows a clear correspondence to global changes in atmospheric CO₂ (Pagani et al. 2005, 2011, Zhang et al. 2013), suggesting that CO₂ concentrations could have been the main driver of the macroevolution of the group (Henderiks and Pagani 2008, Hannisdal et al. 2012). This hypothesis argues that the emergence of smaller species and extinction or decrease in prominence of larger taxa over the Cenozoic are probably related to the evolutionary pressure of decreasing CO₂ and a shift to smaller-sized cells which are less sensitive to CO₂ limitation (Henderiks and Rickaby 2007, Henderiks and Pagani 2008). Furthermore, a decrease of CO₂ concentrations below a physiological threshold of ~500ppmv in the late Miocene (~7 Ma) likely led to the emergence of carbon concentrating mechanisms in coccolithophores (Bolton and Stoll 2013) as well as in other phytoplankton groups (Raven et al. 2008). This evolutionary adaptation is recorded in modern taxa (and the fossil record) in the form of ‘vital effects’ *i.e.* an offset in isotopic fractionation between the biogenic carbonate compared to non-biogenically precipitated carbonate (Ziveri et al. 2003). Recent analyses of the isotopic fractionation in fossil coccoliths as well as their thickness have related a Miocene decline in CO₂ with negative trends in cellular calcification rates within the globally important Noelaerhabdaceae family (Bolton et al. 2016). This decrease in calcification was arguably related to allocation of limiting carbon to photosynthesis.

Patterns in coccolithophore diversity, supported by biogeochemical data and changes in coccolith size and calcification rates during the Cenozoic indeed suggest that atmospheric CO₂ and its aqueous concentrations in the surface ocean are a major driver of the phenotypic evolution of coccolithophores. However, observations from culture experiments showing that nutrient (Paasche and Brubak 1994, Paasche 1998, Oviedo et al. 2014) and temperature stress (Rosas-Navarro et al. 2016) as well as a number of other parameters (Zondervan 2007) affect carbon production, should be considered when interpreting phenotypic evolution of coccolithophores. This is especially relevant for larger and heavily-calcified coccolithophore taxa, which are more likely to be affected by any resource limitation. In addition, experiments indicate that a combination of stressors can have stronger effects on carbonate production in coccolithophores (Borchard et al. 2011). Clearly, any environmental forcing on coccolithophore evolution is complex and could involve multiple stressors.

Ongoing climate change is manifested by a number of environmental perturbations, from ocean acidification (Doney et al. 2009) to ocean temperature increase (Roemmich et al. 2015) and nutrient limitation (Sarmiento et al. 2004). In this context, Finkel et al. (2010) suggested that climate forcing with

all of its different elements is driving phytoplankton evolution rather than CO₂ concentration by itself, as evident from the Cenozoic macroevolutionary patterns of non-calcifying phytoplankton groups such as diatoms and dinoflagellates. Climate-regulated upwelling of nutrients in the photic zone was also proposed as an important evolutionary driver (Falkowski and Oliver 2007). Therefore, interpreting signals from the fossil record requires a good understanding of the physiology and ecology of living coccolithophores and their responses to environmental perturbations. On the other hand, a better understanding of the evolutionary drivers and responses will improve our predictions of the behavior of coccolithophores in a changing ocean system.

Nutrient limitation and the phenotypic evolution of *Helicosphaera* spp.

Our observations from the culture experiments as well as field investigations were used to interpret the evolutionary trends in coccolith volume and thickness (indicators of calcite production) within the *Helicosphaera* genus over the past 15 million years (**Paper VII**). Fossil material was sampled from oceanic sediments collected at two sites: South Atlantic DSDP Site 525 and western Indian Ocean ODP Site 707. The evolutionary patterns in coccolith volume and thickness were clearly distinct between the two sites, suggesting that local environmental conditions modified the phenotypic evolution of the genus (Fig. 15). Most of the studies to date agree that atmospheric CO₂ levels have exerted the major control on the evolutionary patterns of coccolithophores during the Cenozoic (Pagani et al. 2005, Henderiks and Rickaby 2007, Bolton et al. 2016). In this study, we found strong evidence to support that local productivity levels have also played an important role in modifying the calcification response to globally decreasing CO₂. This observation is in agreement with our experimental results, which show that large coccolithophores can modify their PIC production as an adaptation to nutrient limitation (**Papers I-III**).

Two main evolutionary patterns can be inferred from our data. At the eutrophic Site 525, located in the upwelling area of the south-eastern Atlantic, main evolutionary pressure was probably related to progressively limiting CO₂ concentrations. Here, the decrease in average coccolith volume was more prominent compared to the tropical site in the Western Indian Ocean. The coccolith volume pattern showed a gradual decrease, which was mostly related to the emergence of smaller morphotypes, as inferred from the lack of strong signal in thickness decrease. Similar adaptation patterns based on the occurrence of smaller taxa under the pressure of decreasing CO₂ was proposed for the Paleogene radiation within the *Reticulofenestra* genus (Henderiks and

Pagani 2008). This pattern is evident from the occurrence of smaller size classes after a ~500 ppmv threshold in limiting CO₂ concentrations ~7 Ma (Bolton and Stoll 2013). Smaller cells are less sensitive to resource limitation and produce smaller coccoliths, which seems to be a common and successful adaptation strategy (Henderiks 2008).

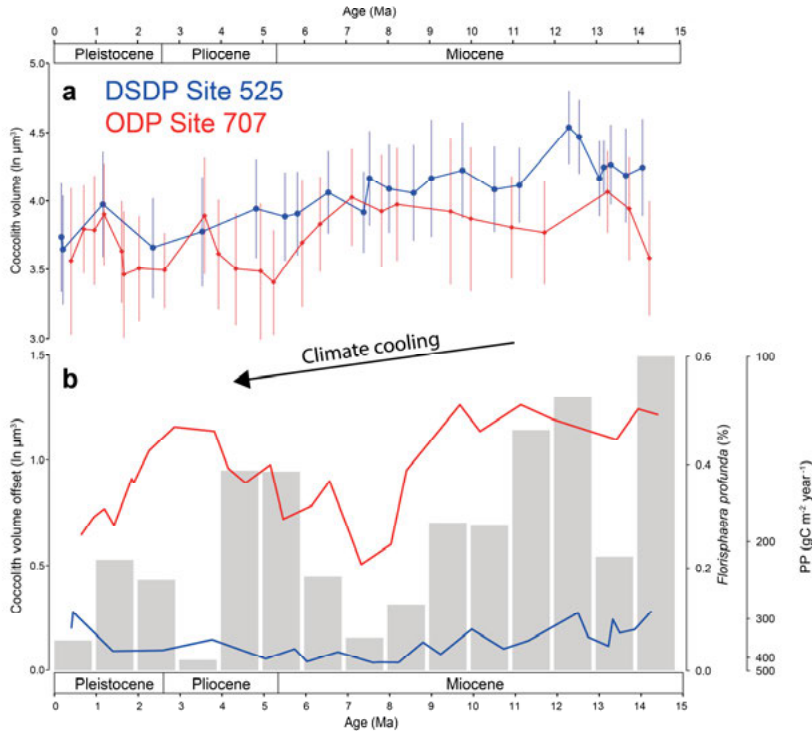


Figure 15. a) Time series of coccolith volume measurements within the *Helicosphaera* genus at Site 525 (blue) and Site 707 (red) over the past 15 Ma. For each data point, N=50. Error bars show standard deviation. b) Relative abundance of *Florisphaera profunda*, an indicator of the depth of the photic zone and primary productivity values calculated from *F. profunda* relative abundance as described in ref. 57, superimposed over a bar chart showing the average difference in coccolith volume between sites (Site 525 > Site 707) calculated over 1 million-year intervals. Adopted and modified from **Paper VII**.

Our results, combined with the biostratigraphic data on the occurrence of species since the middle Miocene show that the transition to smaller cells can happen both within a species as well as by occurrence of new, smaller species. The latter trend is evident across the coccolithophore lineage during the past 15 Ma (Herrmann and Thierstein 2012, Suchéras-Marx and Henderiks 2014). In *Helicosphaera*, the time period with below-threshold levels of CO₂ was marked by the emergence of several smaller taxa (e.g. *H. hyalina*, *H. pavimentum*, *H. wallichii*) and the extinction of heavily-calcified *H. granulata*. In

fact, *H. carteri* is the only survivor of the Miocene climate transition in this genus, suggesting that adaptive strategies other than size decrease could have taken place during this period.

Insights into alternative adaptive strategies can be inferred from the volume and thickness patterns at the tropical Site 707. Here, we observed intervals of strong decrease in coccolith volume and coccolith thickness, which was not observed at Site 525. The decrease in coccolith thickness was used as a calcification proxy in a recent study on the evolution of Noelaerhabdaceae (Bolton et al. 2016). Therefore, decrease in volume coupled with a decrease in thickness likely represents a signal in physiological adaptation which allocated limiting resources from calcification to photosynthesis, thus reducing the calcification rates. The fact that these intervals occurred even before the late Miocene, below-threshold values of CO₂ suggests that other environmental parameters could lead to such physiological adaptive response. We argue that the intervals in decreased calcification are likely caused by nutrient limitation, following the adaptive strategy observed in experiments presented in **Papers I-III** of this thesis. The nutrient limitation at this site was confirmed by a number of independent primary production proxies and data on total fluxes (burial rates) of calcifying nannoplankton at Site 707. This physiological response was observed in all major *Helicosphaera* species and can arguably also be noticed in Noelaerhabdaceae trends from a nearby tropical site (Bolton et al. 2016).

Our findings suggest that the phenotypic evolution of coccolithophores was regulated by the interaction of many environmental parameters, from global CO₂ and ocean carbonate chemistry to regional nutrient availability. Coccolithophore calcification seems to be a highly plastic process and can be modified by different types of environmental forcing (Zondervan 2007). Therefore, this range of possible evolutionary drivers further complicates the prediction of response patterns of coccolithophore communities to the ongoing climate change. The complexity is further increased by species- and strain-specific response patterns, as well as the presence of life-phases with physiologically and biogeochemically distinct functions. This thesis aimed to address part of this complexity in order to better understand coccolithophore adaptation and its relevance for biogeochemical processes.

Conclusions and future directions

This thesis presents a multidisciplinary approach to investigating climatic adaptation in coccolithophores. The main aim of the thesis, linking cellular physiology and community ecology of living coccolithophores with the long-term evolutionary trends has succeeded in providing new perspectives on coccolithophore adaptation and evolution. At the same time, the work presented in this thesis refined the taxonomy of coccolithophores and helped introduce new molecular methods in coccolithophore research. In this chapter, the summary of main findings presented in this thesis will be followed by the discussion on possible new research directions that have been established by this work.

Our experimental results have revealed the sensitivity of large coccolithophore species to phosphorus limitation, with the coccolith morphology and calcification rates being the most affected traits. Phosphorus limitation leads to allocation of the limiting resource to the biologically most important process – photosynthesis, at the expense of calcification and regulation of coccolith morphology. In addition, temperature stress, when coupled with phosphorus limitation, further enhanced the negative effects, a finding which is highly relevant for future climate scenarios.

Field studies in the Adriatic Sea have provided new insights into coccolithophore life-cycle dynamics and their relationship with the environmental forcing. Our results combined with previous studies suggest that temperature gradients seem like the most probable regulator of community-level coccolithophore life-cycle dynamics in the Mediterranean Sea. However, a high degree of species-specific variations in ecological preferences points out that the life-cycle dynamics are far more complex.

Taxonomic investigations have provided detailed description of the life-cycle of a common coccolithophore species *Acanthoica quattrosipina*. The exceptional material analyzed for this study has also revealed a high degree of morphological variability which could be related to holococcolith production mechanism, a first such observation in a field material.

Finally, our culture experiments enabled us to relate the trends in phenotypic evolution of the large coccolithophore genus *Helicosphaera* with the long term patterns in nutrient availability and primary productivity over the past 15 Ma. In particular, our results show that nutrient limitation had strong negative effects on calcification rates and consequently on carbon burial. They

also point to the complexity of climate changes, which bring a number of different environmental perturbations, each with its peculiar effect on coccolithophore physiology and evolution.

Future studies on the topic of coccolithophore adaptation and fate of coccolithophores under ongoing climate change should address some of the topics opened in this thesis. For example, nutrient limitation should also be investigated on gene expression level to reveal possible trade-offs and resource allocation patterns in more detail. This approach will also require the identification of genes involved in the calcification process that likely define the biogeochemical performance of coccolithophores. While certain steps in this direction have already been made using *E. huxleyi* cultures (Lohbeck et al. 2014, Rokitta et al. 2014, 2016), large coccolithophores should clearly be included in such investigations, as they show highest sensitivity to phosphorus limitation and resource limitation in general.

Moreover, our field data on coccolithophore life-cycles suggests that a future warmer and more oligotrophic ocean will likely favor holococcolithophore communities. Such change would have strong effects on the biogeochemical processes. Therefore, future experimental studies should also address the carbon production patterns of the holococcolith phase and better constrain their role in the carbon cycle.

Detailed field studies on the coccolithophore life-cycle dynamics are still limited by the unresolved taxonomic relationships. Therefore, more taxonomic studies are needed, preferably in areas known for high diversity of holococcolith phase such as the Mediterranean Sea. Coupling taxonomic investigations with detailed analysis of environmental data and higher sampling frequency would advance our understanding of life-cycle regulation.

Molecular methods tested in this thesis were constrained by the lack of taxonomically verified sequences in reference libraries. Developing methods for single-cell genomics coupled with morphology-based taxonomical identification of sequenced individual coccolithophore cells could prove to be the appropriate way to fill gaps in reference libraries. This would allow for more precise taxonomic analysis using the molecular methods.

Finally, we have shown a clear relationship between coccolith size parameters (volume and thickness) of *Helicosphaera* spp. and different evolutionary strategies. Future studies on coccolith volume should include more sites with contrasting production levels which could further constrain this relationship. Also, experimental studies could help establish stronger and more precise relationships between calcification rates and size parameters in large coccolithophores, which could then be used to interpret production levels in different paleo-environments. Finally, morphometric analysis of coccolith thickness across the modern assemblages of large coccolithophore species from contrasting environments may reveal patterns related to nutrient availability.

Svensk sammanfattning

Introduktion

Marina fytoplankton är en viktig del inom flera av jordens system. De utgör grunden inom den marina näringskedjan och har även en betydande roll i kretsens globala kretslopp. De fytoplankton som är kalkproducerande, coccolitoformerna (inom divisionen Haptophyta, även kända som fästalger), är en stor och artrik grupp mikroalger och karaktäriseras av att dess celler är täckta av små kalcitplattor, kända som coccoliter. Coccoliter produceras intracellulärt genom en unik kalcifieringsmekanism, där kalcit binds med kol och på samma gång frigör koldioxid. Detta gör att coccoliterna i hög grad är kopplade till den biogeokemiska kolcykeln och även kan påverka denna genom att förändra balansen mellan fotosyntes (en koldioxidsänka), kalcifiering (en koldioxidkälla) och långvarig avsättning, där coccoliter sjunker ner till havssedimenten. Sedan coccolitoformernas uppkomst under den senare delen av trias för ungefär 220 miljoner år sedan har de haft en stor betydelse för hur jordens kretslopp kommit att utvecklas och hur de ser ut idag.

Den cellulära produktionshastigheten av partikulärt inorganiskt kol (PIC) liksom av partikulärt organiskt kol (POC) påverkas mycket lätt negativt av olika typer av miljöfaktorer, exempelvis för hög eller låg temperatur, koldioxidhalt och brist på näringsämnen. På grund av denna känslighet inför miljörelaterade förändringar kommer de ökande antropogena utsläppen av koldioxid och den pågående uppvärmningen av jorden sannolikt ha en påtaglig effekt på coccolitoformerna, och därigenom även på den biogeokemiska balansen. I detta sammanhang finns det två processer som är av särskilt intresse: de direkta effekterna av ökande koldioxidutsläpp och den havsförurning som följer, samt de stigande havstemperaturerna som riskerar leda till en ökad stratifiering av den övre fotiska zonen och näringsbrist. Att ta reda på hur coccolitoformerna reagerar på denna typ av förändringar är av yttersta vikt för att förstå kolcykeln, och hur den i framtiden kommer förändras på grund av det förändrade klimatet.

Målet med denna avhandling var att undersöka hur coccolitoformerna anpassats till en rad miljörelaterade förändringar, med särskilt fokus på temperatur och näringstillgång. Forskningen genomfördes inom ramen för tre olika delområden: a) experimentella studier av hur coccolitoformer svarar fysiologiskt på fosforbrist (**Artikel I-III**), b) fältundersökningar i Medelhavet och Oslofjorden (**Artikel IV-VI**), samt c) en tidsserie av evolutionen hos det

coccolitofora släktet *Helicosphaera* under de senaste 15 miljoner åren (**Artikel VIII**).

Experiment med begränsning av fosfor

Målet med våra experiment var att testa hur de stora och kraftigt förkalkade coccolitoforarterna *Helicosphaera carteri* och *Coccolithus pelagicus* reagerade vid fosforbrist. Tidigare experimentiella studier i ämnet har nästan uteslutande genomförts på modellarten *Emiliania huxleyi*, trots att denna endast utgör en bråkdel av coccolitoforernas totala mångfald. Vi jämförde stammar av *H. carteri* och två underarter av *C. pelagicus*, varav hälften isolerats från en näringsrik miljö, hälften från en näringsfattig, med syftet att utforska om deras fysiologiska gensvar motsvarar en ekofysiologisk anpassning.

I experimentet som utfördes med *H. carteri* (**Artikel I**) jämförde vi två stammar från olika näringsförhållanden: den ena från det oligotrofiska och fosforfattiga Medelhavet, den andra från ett område i Sydatlanten med hög primärproduktion. De två stammarna uppvisade olika krav på näringstillgång, och skilde även i cellstorlek och kalcifieringshastighet. Resultaten indikerade att stammen från Medelhavet, där den begränsade tillgången på fosfor utgjort ett evolutionärt tryck, har anpassat sin fysiologi genom att minska sina fosfor- och kvävebehov och istället lägga sina om än begränsade resurser på fotosyntes, något som resulterat i sänkt kalcifieringshastighet.

De stora och kraftigt kalcifierade coccolitoforernas känslighet för begränsad fosfortillgång bekräftades genom våra experiment på *C. pelagicus* (**Artikel II**). Här visade vi att en begränsad tillgång på fosfor kan leda till att coccoliterna blir missbildade. Tillsammans med den stress som en högre temperatur innebär ökade de negativa effekterna av fosforbegränsningen ytterligare, vilket visar att när flera stressfaktorer kombineras så blir konsekvenserna också mer omfattande.

I vår andra studie på *C. pelagicus* (**Artikel III**) visade vi att om tillgången på fosfor kraftigt begränsas, leder detta till en långsammare kalcifieringshastighet. Stora coccolitoforer har visats vara mycket känsliga för just tillgången på fosfor, både när det gäller kolproduktion liksom för morfologin hos dess coccoliter. Vid låg näringstillgång tycks de prioritera fotosyntes och produktionen av POC över kalcifiering, en reaktion som sker i än högre utsträckning om coccoliterna samtidigt stressas av en högre temperatur. Detta innebär att framtidens varmare klimat med förhöjda havsvattentemperaturer och minskad tillgång på näringsämnen kan ha kraftigt negativa effekter på dessa marina kalkbildande organismer.

Fältstudier

Syftet med våra fältstudier i Adriatiska havet var att lösa det taxonomiska släktskapet mellan coccolitoforenas olika morfotyper, vilka representerar olika stadier i deras livscykel. Först efter att detta problem kunnat lösas blev det möjligt att gå vidare till nästa steg, där dynamiken i deras livscyklar studerades ur ett ekologiskt perspektiv.

I **Artikel IV** presenterar vi en detaljerad taxonomisk beskrivning av livscykeln hos en vanlig coccolitoforart, *Acanthoica quattropsina*, vilken tillhör den mycket artrika familjen Rhabdosphaeraceae. Utifrån våra observationer visade vi att *A. quattropsina* har en typisk haploid-diploid livscykel, där dess två livsstadier alterneras med bildandet av kombinerade coccosfärer efter att den diploida cellen genomgått meios. Vi noterade även att det fanns en stor morfologisk variation i båda livsstadier.

Vår fältstudie som presenteras i **Artikel V** syftade till att undersöka dynamiken i coccolitoforenas livscykel i relation till förändringar i säsong, liksom förändringar i deras ekologiska levnadsmiljö. Vi kunde se hur deras livsfaser skiftade beroende på säsong, där den diploida fasen dominerade coccolitsamhället under vinterhalvåret, medan den haploida var som vanligast under sommaren. Vi observerade dock även arter som hade särskilda preferenser gällande levnadsmiljön, och till skillnad mot våra tidigare observationer, skiftade dessas livsfaser inte beroende på säsong. Enligt vår dataanalys styrs coccolitsamhällets livsfaser sannolikt av säsongrelaterade skillnader i temperatur i kombination med de ekologiska preferenser som är specifika för en viss art.

Den tredje fältstudien utfördes i Oslofjorden (**Artikel VI**), med syftet att testa användbarheten av molekylära undersökningsmetoder inom coccolitoforforskningen. Metoden som användes, ”high-throughput sequencing” (HTS), jämfördes med det vanligt förekommande tillvägagångssättet där coccolitoforer undersöks med hjälp av ett svepelektronmikroskop. Resultaten från HTS visade sig på det stora hela ge en god uppfattning av coccolitoforsamhällets sammansättning. Medan användningen av markörgenen 18S rDNA gav en bättre uppskattning gällande relativ förekomst, gav markörgenen 28S rDNA en bättre taxonomisk beskrivning av coccolitoforsamhället.

Fossilbaserad tidsserie

Våra observationer från odlingsexperiment (**Artikel I**) liksom från fältundersökningar (**Artikel V**) användes för att med hjälp av volymen och tjockleken (en indikator för kalcitproduktion) hos coccoliter från släktet *Helicosphaera* tolka de evolutionära förändringarna som ägt rum under de senaste 15 miljoner åren. Proverna ur vilka fossilen förberetts hade hämtats från Sydatlanten samt västra delen av Indiska oceanen. De evolutionära mönstret visade sig

skilja signifikant mellan de två platserna, vilket tyder på att lokala förhållanden i miljön har påverkat släktets fenotypiska evolution. Vi fann även starka bevis för att också lokala produktivitetsnivåer har haft en stor inverkan på hur responsen blivit vid en global minskning av koldioxidhalten. Detta stämmer väl överens med resultaten från våra experimentella studier, där vi visade hur stora coccolitoforer kan modifiera sin produktion av PIC som en anpassning till rådande näringsförhållanden (**Artikel I-III**).

Vidare visar våra data hur *Helicosphaera* spp. huvudsakligen haft två större evolutionära mönster. I området beläget i Sydatlanten, där minskande koldioxidnivåer utgjorde det främsta evolutionära trycket, minskade coccoliterna i volym som en konsekvens till uppkomsten av mindre morfotyper. Förutom att små celler inte är lika känsliga för näringsbrist, producerar de även mindre coccoliter, och denna anpassning förefaller vara en både vanlig och framgångsrik. Hos coccolites från Indiska oceanen såg vi istället en minskning i tjocklek, vilket är en indikation på att kalcifieringen är på tillbakagång. Denna typ av anpassning berodde sannolikt på att näringstillgången periodvis var kraftigt begränsad, något som också stöddes av våra egna mätningar av den primära produktionen. Utifrån våra resultat verkar det som att coccolitoforer-
nas evolution styrdes av ett samspel mellan flera olika miljörelaterade parametrar, från de globala koldioxidnivåerna och havets karbonathalt till den lokala tillgången på näring.

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