Universitat Autònoma de Barcelona

Coccolithophore calcification, life-cycle dynamics and diversity response to a warming and acidifying Mediterranean Sea

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“I saw the sky and sea and sand and the flickering flames of the bonfire through my tears.
All at once, it rushed into my head with tremendous speed, and made me feel dizzy.
It was beautiful.”

Banana Yoshimoto

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This thesis is dedicated to all of you,

Barbara

CONTENTS

SUMMARY

This thesis was conducted under the "European Mediterranean Sea Acidification in a changing climate" (MedSeA) project, funded by the European Commission under Framework Program 7 (http://medsea-project.eu; grant number 265103). The MedSeA project stimulated research on the combined effect of ocean warming and acidification on Mediterranean biogeochemistry and ecosystems.

The present thesis focuses on coccolithophores, a group of calcifying phytoplankton tightly connected to the global climate through the carbon cycle. Both calcified life stages (heterococcolithophores and holococcolithophores) of these unicellular algae are commonly found in the Mediterranean Sea. Here, the overall community presents a high degree of species diversity, apparently higher than in the adjacent Red Sea and Atlantic Ocean. On the other hand, the Mediterranean Sea is an area subject to strong environmental seasonal oscillations and anthropogenic pressures. The Mediterranean Sea is considered a "hot spot" for climate change, being among the oceanic regions under faster warming and acidification (The MerMex Group, 2011). These processes are expected to cause not only a rise in temperatures and shifts in the carbonate system, but also to enhance water column stratification. It is anticipated that such environmental changes will influence the coccolithophore populations, in ways that are not yet understood. This thesis contributes to the understanding of coccolithophore responses to a changing Mediterranean Sea based on i) water samples collected along a W – E transect during the MedSeA Ocean Research Cruise (https://medseaoceancruise.wordpress.com), which captured their regional population and diversity (Chapters 2, 3, 4); and ii) water samples collected during the MedSeA Crete mesocosm experiment (https://medseacrete2013.wordpress.com), which tested the combined effects of ocean warming and acidification on the oligotrophic Eastern Mediterranean pelagic ecosystem under nutrient limitation (Chapter 5). These two sets of observations allowed the examination of several aspects of the coccolithophore population such as i) the average coccolith mass of *Emiliania huxleyi*, its distribution and the main morphological / environmental controls (Chapter 2); ii) the heterococcolithophore and holococcolithophore absolute abundances, their relative distributions, diversity patterns, and hypothetical triggers of life phase transformations (Chapter 3 and 4); and iii) the variability in absolute abundance of the total coccolithophore population, total heterococcolithophores and holococcolithophores, and of the predominant species (*E.*

huxleyi, *Rhabdosphaera* spp.) in the coccolithophore community under warming and acidification in an oligotrophic setting (Chapter 5).

Overall, the results presented in this thesis suggest that coccolithophores inhabiting the Mediterranean Sea will be influenced in various ways by projected environmental perturbations: *E. huxleyi* average coccolith mass will change, following future shifts in the proportion of calcification varieties and likely cause changes in the carbonate export production in the Mediterranean Sea; the haploid phase could be favoured over the diploid phase in many coccolithophore species, ultimately increasing the proportion of holo- over heterococcolithophores and holococcolithophore diversity; in the Eastern Mediterranean Sea, warming and nutrient limitation, rather than acidification, tend to reduce the total coccolithophore population, although species specific and strain specific growth optima may modulate this response.

CHAPTER 1

1.1 Introduction

The overall aim of this dissertation is to improve our understanding of the rapid environmental change impacts on the marine ecosystems. In particular, it is focussing on a common and abundant marine calcifying phytoplankton at the base of the food web, coccolithophores, in the Mediterranean Sea. This oceanic region was chosen because of the strong anthropogenic and climate change pressures acting on it, including warming (Sakalli, 2017; Vargas-Yáñez et al., 2008) and acidification (Goyet et al., 2016).

1.1.1 Coccolithophores

Coccolithophores are unicellular calcifying algae, belonging to the clade Haptophyta (class Prymnesiophyceae). Their first representatives appeared in the upper Triassic (~225 million years ago; Bown et al., 2004) and they are highly differentiated in today's oceans, reaching a number of ~280 morphologically defined species (Young et al., 2003). *Emiliania huxleyi*, the most abundant and widespread species, can be found in almost all oceans, from equatorial to sub-polar latitudes (Brown, 1995; McIntyre and Bé, 1967; Winter and Siesser, 1994). Several studies indicate that its adaptability to a wide range of environmental conditions derives from a combination of genetic variability and phenotypic plasticity (Blanco-Ameijeiras et al., 2016; von Dassow et al., 2015; Iglesias-Rodriguez et al., 2002; Iglesias-Rodríguez et al., 2006; Read et al., 2013). Coccolithophore cells are usually enclosed in a coccosphere, an exoskeleton composed by platelets of calcium carbonate called coccoliths. Coccoliths are divided into two main categories: the heterococcoliths, made of variably shaped and sized calcite crystals, and the holococcoliths, made of identically shaped rhombohedral crystallites (< 0.1 μm). It is likely that most coccolithophores have this dimorphic life cycle (Fig. 1.1), producing heterococcoliths during their diploid stage, and holococcoliths during their haploid stage, although this has been directly observed only for a few species (Houdan et al., 2004; Nöel et al., 2004; Parke and Adams, 1960).

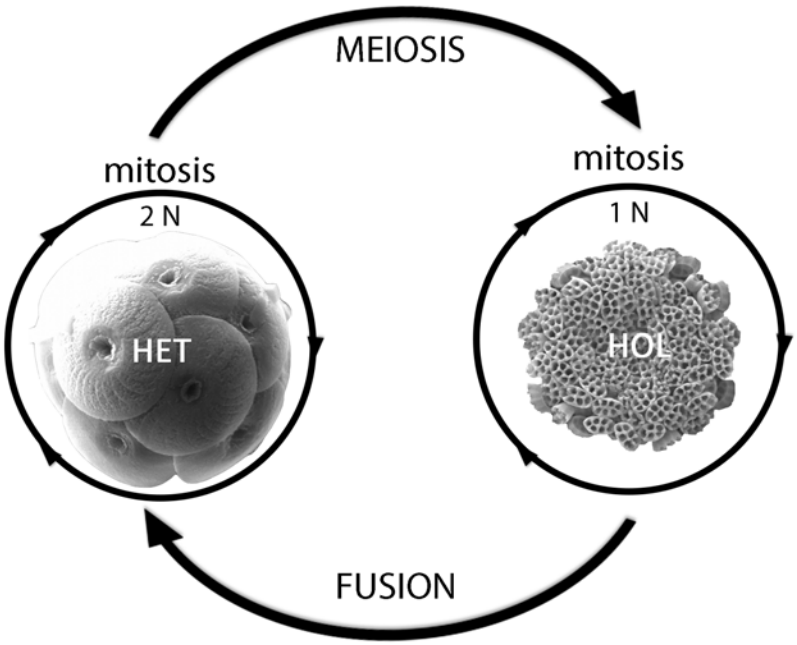

Fig. 1.1 Coccolithophore dimorphic life cycle; photos of *Calcidiscus leptoporus* subsp. *quadriperforatus* HET and HOL.

The two coccolithophore life phases seem to have different physiologies (von Dassow et al., 2015; Frada et al., 2008; Houdan et al., 2006) and modes of calcification, with heterococcoliths produced intracellulary, and holococcoliths produced extracellulary (Rokitta et al., 2016; Rowson et al., 1986; Young and Henriksen, 2003). Some species might not calcify at all while in their haploid stage, like *E. huxleyi* (Frada et al., 2008; Fresnel, 1994), and the triggers of such transitions are still under debate (Cros and Estrada, 2013; Frada et al., 2012; Houdan et al., 2004, 2006; Nöel et al., 2004; Šupraha et al., 2016; Taylor et al., 2017; D'Amario et al., 2017, under review). Figure 1.2 presents a synthesis of the abiotic−biotic factors that might trigger life phase change in coccolithophores, the living conditions and the supposed ecological advantages of each phase. Diploid and haploid cells of *Coccolithus braarudii* and *Calcidiscus leptoporus* showed different sensibilities to inorganic and organic nutrient concentrations, and those of *Coccolithus braarudii* also to turbulence (Houdan et al., 2006).

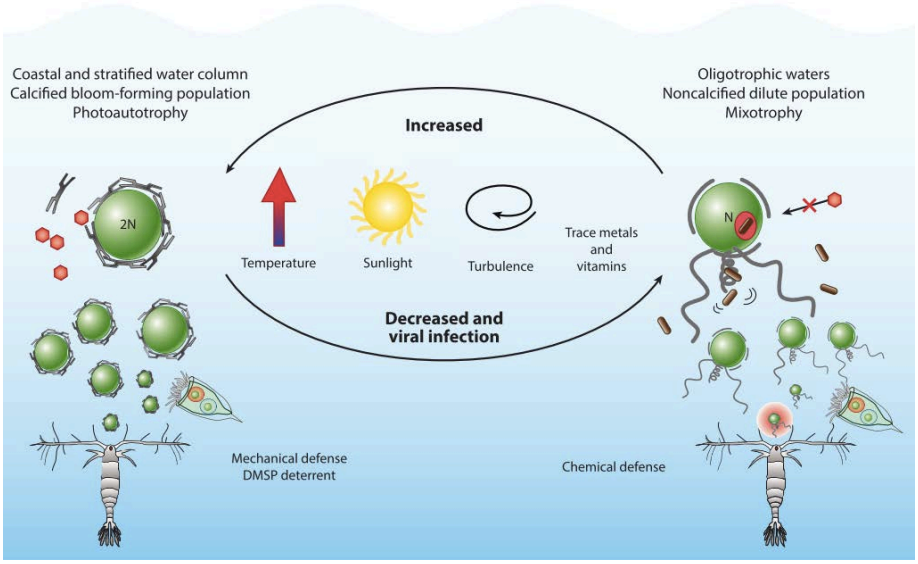

Fig. 1.2 Potential triggers, habitats and ecological advantages of haploid and diploid coccolithophore life phases. The red hexagons represents viruses which can attack only diploid cells, while the brown "pills" represent bacteria which can be ingested by the haploid cells; at the bottom, copepods and ciliates (from Taylor et al., 2017; DMSP = dimethylsulfoniopropionate).

Life phase transitions seem triggered in *Calyptrosphaera sphaeroidea* by temperature and varying concentrations of trace metals and vitamins (Nöel et al., 2004); in *Coccolithus braarudii* by turbulence (Houdan et al., 2006) and in *E. huxleyi* by viral infections (Frada et al., 2008, 2012). The differences between haploid and diploid cells seem not only to expand the ecological niche of the whole coccolithophore population, but also to increase their adaptability to changing environmental conditions.

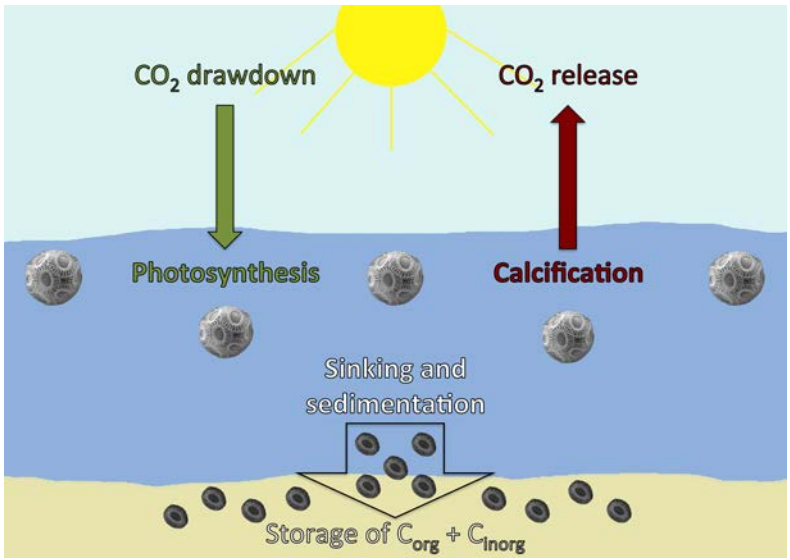

Fig.1.3 Interactions of coccolithophores with the carbon cycle (C_{org}= organic carbon, C_{inorg}= inorganic carbon).

Coccolithophores are not only abundant primary producers at the base of the marine food web, but also important climate regulators (Collins et al., 2013; The Royal Society, 2005). In fact, they are deeply connected with the global climate through the release of dimethyl sulfide (DMS), the processes of photosynthesis and that of calcification (Malin and Steinke, 2004; Rost and Riebesell, 2004). DMS is thought to enhance light backscattering and water acidity in the atmosphere (Charlson et al., 1987); on the other hand, carbon dioxide (CO_2) is a greenhouse gas that can dissolve in marine water, causing at the same time atmospheric warming and ocean acidification (The Royal Society, 2005). Coccolithophores contribute to both the drawdown and the release of CO_2 into the atmosphere (Fig. 1.3), through the organic carbon pump and the calcium carbonate pump (Rost and Riebesell, 2004); they are responsible for more than half of global carbonate production in the photic zone (Balch et al., 2007), contributing to 40 − 60% of $CaCO_3$ deposits in tropical oceans and even more at higher latitudes (Broecker and Clark, 2009).

Due to the close interrelation between coccolithophores and the global climate, the net response of this phytoplankton group to environmental changes has become a highly debated topic of interdisciplinary interest. Ocean acidification is expected to influence both photosynthesis and calcification in coccolithophores (Doney et al., 2009), but the magnitude and quality of responses appears to be species-specific (Langer et al., 2006; Meyer and Riebesell, 2015). Up to present, most research efforts focused on the species *E. huxleyi*, because of its cosmopolitan distribution, abundance and easy maintenance in culture experiments (Paasche, 2002), but also within this species, results have been contradictory concerning both POC (particulate organic carbon) and PIC (particulate inorganic carbon) production (Meyer and Riebesell, 2015). The calcification degree of *Emiliania huxleyi* morphotypes could be distributed in the ocean following carbonate chemistry gradients (heavier calcification in low pCO_2, high pH waters, high carbonate ion concentration and high alkalinity), but a few notable exceptions have already been noticed (Beaufort et al., 2008, 2011; Poulton et al., 2011; Smith et al., 2012), suggesting that the calcification degree might actually differentiate ecophenotypes rather than represent the direct effect of the carbonate system on the coccolithophore calcification process (Cubillos et al., 2007; Poulton et al., 2011; Young et al., 2014a).

Coccolithophore abundance depends on an array of environmental parameters (e.g. temperature, nutrient concentrations, turbulence, carbonate chemistry); the combination of these parameters might regulate their response to ocean acidification (Beaufort et al., 2008; Berger et al., 2014; Horigome et al., 2014; Smith et al., 2012).

For example, coccolithophore response to acidification under nutrient repletion/limitation and under eutrophic conditions seem to vary (Bach et al., 2016; Oviedo et al., 2016;

Rouco et al., 2013; Sala et al., 2016; Sciandra et al., 2003). Moreover, the proportion of different species and morphotypes can fluctuate during the seasonal cycle (Beaufort and Heussner, 2001) or in the years (Beaufort et al., 2011; Meier et al., 2014). The ensemble of current knowledge highlights the genetic complexity (Brand, 1982; Iglesias-Rodriguez et al., 2002; Iglesias-Rodríguez et al., 2006; Read et al., 2013; Schroeder et al., 2005) and potential adaptability of the 'super-species' (*sensu* De Vargas et al., 2004) *E. huxleyi* to acidified conditions (Lohbeck et al., 2014; Schlüter et al., 2016).

Another aspect, which needs attention for the modelling of future responses, are coccolithophore community structure, diversity and morphometrics. The size of coccolithophore cells varies greatly among species; thus, variations in the absolute and relative abundance of species can influence the total coccolithophore contribution to the carbon cycle (Young and Ziveri, 2000; Ziveri et al., 2007). Coccolithophore species are adapted to different ecological niches (e.g. Cros, 2001; Oviedo et al., 2014), and can be expected to respond differently to environmental perturbations: the total diversity in one basin might change as species are selected based on their affinity for the new conditions. Therefore, it has been envisaged that future studies will enhance their predictive power by testing several environmental variables in parallel and registering the response of several species at a time (Hattich et al., 2017; Kroeker et al., 2017; Riebesell and Gattuso, 2015).

1.1.2 The Mediterranean Sea

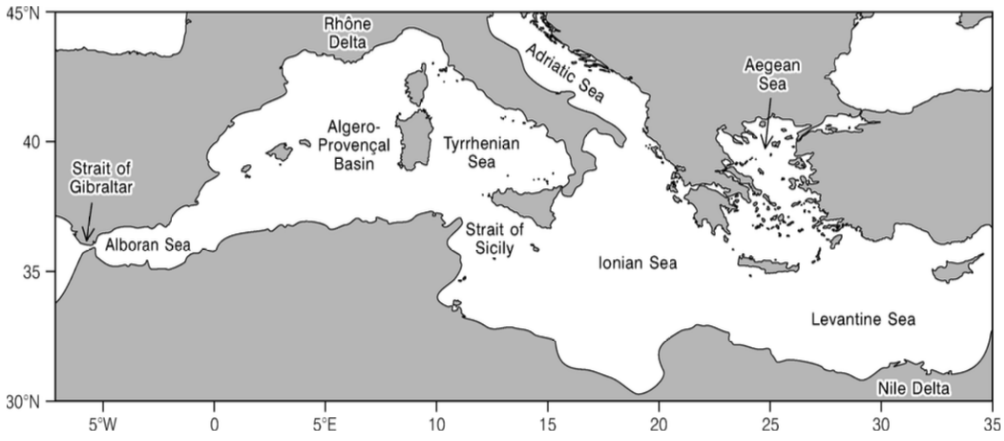

Fig. 1.4 Map of the Mediterranean Sea and main sub-basins (from Rohling et al., 2009).

The Mediterranean Sea is located at the transition between the subtropical and temperate climate zones, and extends longitudinally from ~ 5° W to 35° E. The Strait of Sicily, at 11° E, conventionally divides the basin into the Western and Eastern Mediterranean, although each of these two areas further contains a number of sub-basins (Fig. 1.4). The

Mediterranean Sea is semi-enclosed, communicating only with the Atlantic Ocean, the Black Sea and the Red Sea, and is subject to excess evaporation: the main source of relatively cold and fresh water is the Atlantic Ocean, through the Strait of Gibraltar. Strong temperature and salinity gradients between different parts of the basin trigger a thermohaline anti-estuarine circulation. Once entered into the Mediterranean, the Atlantic water continues to flow eastward, branches in reaching new sub-basins, and gets frequently deviated into mesoscale structures like gyres and eddies (Pinardi et al., 2015; Rohling et al., 2009; Tanhua et al., 2013b).

Environmental gradients are particularly evident between the Western and Eastern Mediterranean. In the western basins, waters tend to be colder and less saline; also, they are generally characterized by higher pCO_2, lower pH and lower alkalinity than the eastern basins (Álvarez et al., 2014; Hassoun et al., 2015a, 2015c; Rivaro et al., 2010). The Mediterranean is overall oligotrophic, with chlorophyll *a* values ≤ 2 – 3 mg/m^3 in the open sea (D'Ortenzio and D'Alcalà, 2009); this feature is particularly evident in the eastern basin, where the nitrogen:phosphorus ratio is extremely low (Krom et al., 1991; Tanhua et al., 2013b). Open-ocean primary production varies also seasonally, reaching maximum values during the winter-spring bloom events in the NW basin, and minimum values during summer in the SE basin (D'Ortenzio and D'Alcalà, 2009; Siokou-Frangou et al., 2010).

The Mediterranean Sea is one of the most prominent hot-spots for climate change (The MerMex Group, 2011). Based on the IPCC CO_2 emission scenarios (IPCC, 2007; Pachauri et al., 2014), it has been projected in some regions an increase of 6°C in sea surface temperatures (Sakalli, 2017) by year 2100 in respect to present values. Moreover, compared to pre-industrial times, a pH reductions comprised between 0.245 - 0.462 in the Western basin, and between 0.242 – 0.457 in the Eastern basin have been projected for year 2100 (Goyet et al., 2016). Although the Mediterranean Sea has high alkalinity and it is characterized by a carbonate saturation state always > 1 (Álvarez et al., 2014; Hassoun et al., 2015a, 2015c; Rivaro et al., 2010), the absorption of atmospheric CO_2 shifts the whole equilibrium, reducing water pH under critical levels for certain marine organisms, especially calcifiers (Doney et al., 2009; Gattuso et al., 2015; Hall-Spencer et al., 2008; Ziveri et al., 2014).

1.1.3 Coccolithophores in a changing Mediterranean Sea

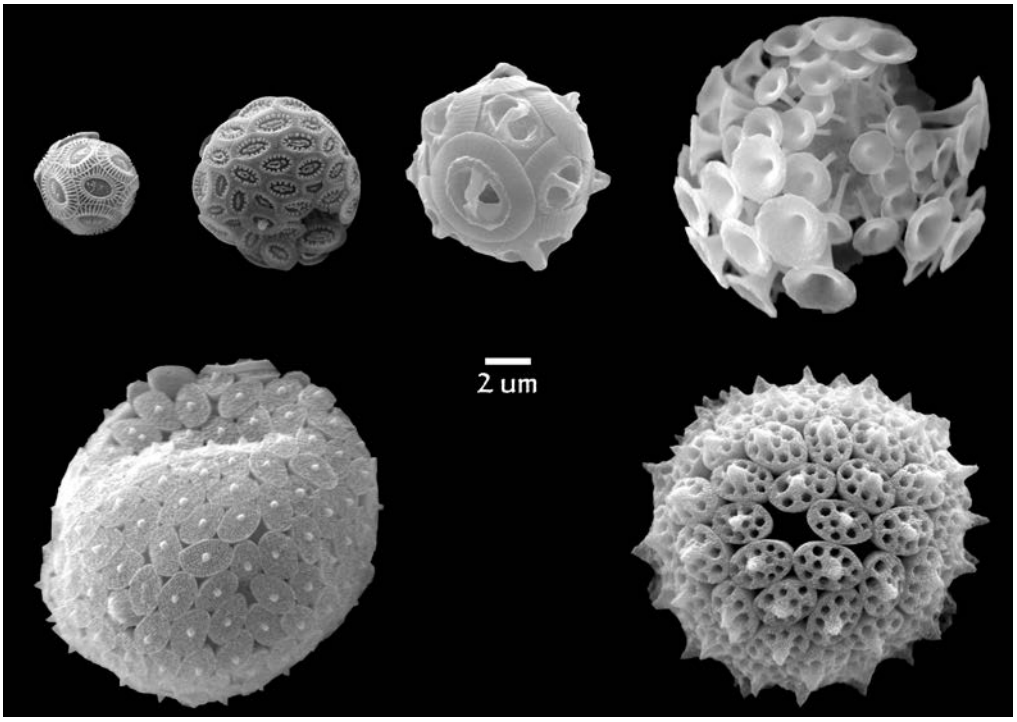

Fig. 1.5 Some coccolithophore species from the Western and Eastern Mediterranean Sea. From the top left to the bottom right: *Emiliania huxleyi*, *Syracosphaera molischii*, *Gephyrocapsa oceanica*, *Discosphaera tubifera*, *Coronosphaera mediterranea* HOL *hellenica* type, *Helicosphaera carteri* HOL perforate.

Coccolithophores are one of the most common groups of phytoplankton in the Mediterranean Sea (e.g. Bárcena et al., 2004; Ignatiades et al., 2009; Malinverno, 2003; Ziveri et al., 2000). Here, they can dominate over siliceous phytoplankton (Oviedo et al., 2015) and their coccoliths can contribute up to ~ 87% of the total $CaCO_3$ in superficial oceanic sediments (Broecker and Clark, 2009). These organisms live within the photic zone and reach high diversity in the Mediterranean Sea (Fig. 1.5; Cros and Fortuño, 2002; Ignatiades et al., 2009; Oviedo et al., 2014). Their distribution is variable, both in spatial (patchiness) and temporal (seasonality) terms (Ignatiades et al., 2009; Kleijne, 1991; Knappertsbusch, 1993; Oviedo et al., 2015); although a high variability has been registered throughout the whole basin, they seem to reach highest concentrations in the eastern basin during winter, and in the western basin during spring-summer (Knappertsbusch, 1993; Oviedo et al., 2015). The two main Mediterranean basins host quite distinct coccolithophore populations, the western basin featuring a higher proportion of eutrophic species than the eastern basin; moreover, coccolithophore species diversity can be different in the western and eastern basins, with apparently opposite trends for the haploid and diploid phases (Ignatiades et al., 2009; Oviedo et al., 2015).

Within the coccolithophore population, heterococcolithophores and holococcolithophores show different preferential distributions in the Mediterranean Sea. Holococcolithophores have an ubiquitous tendency to concentrate in the upper layers of the water column, in contrast with the heterococcolithophores, which instead can reach the lower photic zone (Cros, 2001; Oviedo et al., 2015; D'Amario et al., 2017, under review). The reasons for these patterns are still under debate and likely depend of the distinct physiology of coccolithophore diploid and haploid life phases. An inverse relationship between holococcolithophore abundance and nutrient concentration has been noticed repeatedly in field studies done in the Mediterranean Sea, suggesting that holococcolithophores might be especially adapted to oligotrophic conditions (Cros and Estrada, 2013; Kleijne, 1991; Oviedo et al., 2015; D'Amario et al., 2017, under review). Additional environmental parameters might have a role on their distribution and in triggering the life cycle (section 1.1; Taylor et al., 2017); among them, field studies in the Mediterranean Sea have highlighted the potential impacts of temperature and carbonate system (Oviedo et al., 2015; Šupraha et al., 2016).

A large number of abiotic and biotic factors seem to affect phytoplankton populations in the oligotrophic Mediterranean Sea. The net response of coccolithophores to environmental perturbations in this basin may be assessed by coupling field observations on their abundance, diversity, calcified life stages and calcification patterns, with mesocosm and culture experiments on local strains.

1.2 Objectives

The main overall objective of this thesis was to identify the environmental controls operating on coccolithophores in the Mediterranean Sea, and to assess their potential response to climate change. In this study we mainly consider some key environmental parameters such as seawater carbonate chemistry, temperature and inorganic nutrient concentrations (nitrate, nitrite, phosphate). These environmental parameters are changing rapidly under climate change. The scientific approach was based mainly on the study of samples collected in the natural setting of the Mediterranean Sea, and partly on a mesocosm experiment. The coccolithophore community was analysed through a combination of quantitative and qualitative techniques: species / morphotypes identification and counting through light (LM) and scanning electron microscopy (SEM), image analyses for *E. huxleyi* coccolith morphometrics, and automated measurements of *E. huxleyi* coccolith mass with the Système de Reconnaissance Automatique de Coccolithes (SYRACO; Beaufort and Dollfus, 2004; Dollfus and Beaufort, 1999).

The thesis includes four main chapters. Chapters 2, 3 and 4 are based on water samples collected during the 2011 Meteor M84/3 and the 2013 MedSeA oceanographic cruises, which encompassed the Western and the Eastern Mediterranean; the corresponding west-east transect was characterized by strong environmental gradients. Chapter 2 focuses on *Emiliania huxleyi* coccolith mass and the drivers of its variability; in the discussion were included morphological and environmental parameters. Chapter 3 concentrates on the relative proportion of heterococcolithophores and holococcolithophores, expressions of two coccolithophore life phases. The differential distribution of these two categories of coccolithophores was analysed in detail. Chapter 4 takes in exam the total coccolithophore, heterococcolithophore and holococcolithophore diversities, in particular their different patterns along the longitudinal transect and their potential environmental controls. In the first three chapters, the observed coccolithophore patterns and environmental correlations were discussed in view of ongoing climate changes.

Finally, Chapter 5 was based on water samples collected during a mesocosm experiment performed on phytoplankton populations from the SE Mediterranean (Cretan Sea); the experiment tested the effects of warming and acidification, both separately and in combination. The evolution of the coccolithophore population under perturbed conditions is discussed based on the absolute and relative abundance of the dominant species.

CHAPTER 2

Environmental control on the *Emiliania huxleyi* coccolith mass and calcification degree in the Mediterranean Sea

Abstract

The Mediterranean Sea is undergoing warming and ocean acidification, two processes which are expected to impact coccolithophore distribution and perhaps their net calcification. The responses of *Emiliania huxleyi*, the most abundant and widely distributed coccolithophore species, appear to be strain-specific. We analyzed marine water samples collected along a W-E Mediterranean transect during April 2011 (Meteor cruise M84/3) and May 2013 (MedSeA cruise). Based on morphometric measurements (SYRACO) we obtained the average mass, length and calcification degree of *E. huxleyi* coccoliths; also, four main calcification varieties were identified by scanning electron microscope (SEM) observations. The results suggest that *E. huxleyi* coccolith mass in the Mediterranean Sea is not only significantly correlated to coccolith length and calcification degree, but also to the relative abundance of the calcification varieties: in particular, the small undercalcified A1 and the large overcalcified A3b. The distribution of the calcification varieties seem to follow the main environmental gradients, in particular the carbonate system parameters, and will likely be influenced by climate change.

2.1 Introduction

Coccolithophores are the most prominent calcifying phytoplankton group on Earth and their remains constitute 20 to 40 % of total open ocean carbonate sedimentation, from equatorial to sub-polar regions (Broecker and Clark, 2009; Honjo et al., 1982; Winter and Siesser, 1994). They interact with atmospheric CO_2 through photosynthesis and calcification: these two biological processes are tied to the organic carbon pump and the carbonate counter pump, respectively, influencing the 'rain ratio' (Paasche, 1964), or particulate inorganic carbon (PIC): particulate organic carbon (POC) ratio. The accumulation of human-induced atmospheric CO_2 is altering the global climate and driving rapid changes in the carbonate chemistry of surface seawaters. For example, it has been shown that since the industrial revolution, the global ocean surface pH has decreased by 0.1 units and a supplementary decrease of 0.06 to 0.32 units is expected by the end of the 21st century (Orr et al., 2005; Pachauri et al., 2014). It has been predicted that these changes will cause a decline in marine carbonate production (Kroeker et al., 2013; Portner

et al., 2014). However, it is still unknown how large such a potential reduction in calcification will be in the future and what will be the effects on the marine community structure and marine biogeochemistry.

The family Noelaerhabdaceae is the most abundant in present-day oceans, and there is evidence that they may be impacted by ocean acidification (Jin and Gao, 2016; Meyer and Riebesell, 2015; Milner et al., 2016; Müller et al., 2017). On a global scale, coccolith mass within this family has been found to be correlated to carbonate system parameters: seawater carbonate ion (CO_3^{2-}) concentration, calcite saturation state, pH. These parameters apparently control the distribution of differently calcified taxa, primarily species and morphotypes of the genera *Gephyrocapsa* and *Emiliania* (Beaufort et al., 2011; Cubillos et al., 2007; Rickaby et al., 2016). Most laboratory experimental results on *Emiliania huxleyi*, as reviewed by Meyer and Riebesell (2015), showed a tendency for decreasing particulate inorganic carbon (PIC) production and PIC:POC ratio in high pCO_2 conditions. Nonetheless, variable and sometimes contradictory responses have been found in respect to ocean acidification for *E. huxleyi* (e.g. Beaufort et al., 2011; Hoppe et al., 2011; Langer et al., 2011), probably due to the large number of genotypes included in this species (Bendif et al., 2014; von Dassow et al., 2015; Hagino et al., 2011; Read et al., 2013). A complication is that, in laboratory and field studies, two different concepts of "calcification" are used: calcification rate and calcification degree. During cultures, PIC production and PIC:POC ratio are obtained, while in filtered marine water samples and sediments it is necessary to refer to coccolith calcite mass and coccolith size. Recent work by Rosas-Navarro et al. (2016) however, indicates that high coccolith mass might be a good indicator of high PIC production.

Emiliania huxleyi was classified into four genotypically-controlled morphotypes, A, B, C and corona, by Young and Westbroek (1991). The combination of microscopic observations and molecular studies has uncovered the existence of additional morphotypes and highlighted the genetic complexity of this species (Bendif et al., 2014; van Bleijswijk et al., 1991; Cook et al., 2011; Hagino et al., 2005, 2011; Medlin et al., 1996; Paasche, 2002; Schroeder et al., 2005), which shows an array of distinct biogeographical and seasonal distributions (Hagino et al., 2011; McIntyre and Bé, 1967; Poulton et al., 2011). Up to present, five morphotypes of *E. huxleyi* (A, B, B/C, C and R) have been proven to remain consistent when reproducing. Additionally, all known morphological varieties and their intergradational forms can be informally divided between two main groups: A and B (Young et al., 2003, 2014b; Young and Westbroek, 1991). It has been suggested that coccolith mass variability within this species might depend mostly on morphotype distribution, combined with the specific responses of the

predominant strains in the area of interest (Boeckel and Baumann, 2008; Cubillos et al., 2007; Henderiks et al., 2012; Poulton et al., 2011; Saruwatari et al., 2016; Smith et al., 2012; Young et al., 2014a).

The region of focus for this paper is the Mediterranean Sea; a small-scale ocean with steep W−E physicochemical gradients and a fast overturning circulation (80 to 100 years; Kershaw, 2013). It is located at the transition between the subtropical high-pressure zone and the European temperate zone, and therefore is particularly sensitive to climate change (Boucher, 1975; Rohling et al., 2015; Tanhua et al., 2013b). Mediterranean waters are supersaturated with respect to aragonite and calcite (Gemayel et al., 2015; Schneider et al., 2007), and are characterized by relatively high alkalinity: an average of 2588 µmol kg^{-1} during the MedSeA cruise, increasing eastward (Hassoun et al., 2015c). Due to the relatively short residence time, the entire water column of the Mediterranean Sea has already been invaded by anthropogenic CO_2 (Rivaro et al., 2010) and acidification can be detected in the long-term time series in the North Western Mediterranean Sea (REFS) with a decrease in pH of 0.09 units (from 0.05 to 0.14 since the preindustrial era; Touratier and Goyet, 2011). These changes, accompanied by a general rapid increase in temperature and salinity, are likely to reduce nutrient supply in the upper layers of the water column (Hoegh-Guldberg et al., 2014). The most abundant morphotype of *E. huxleyi* in the Mediterranean Sea is type A, (Knappertsbusch, 1993; Oviedo et al., 2015). The existence of different degrees of calcification in *E. huxleyi* type A has been documented for several oceanic basins (e.g. Beaufort et al., 2011; Cubillos et al., 2007; Henderiks et al., 2012; Smith et al., 2012; Young, 1994; Young et al., 2014a; Ziveri et al., 1995), including the Mediterranean Sea (Triantaphyllou et al., 2010).

In this study, we differentiate four main varieties of the dominant *E. huxleyi* Type A morphotype, characterized by different calcification degrees. We sought to test the hypothesis that measured differences in their coccolith mass were mainly driven by variations in the degree of calcification, rather than variations in coccolith size (length).

2.1.1 Oceanographic setting during the M84/3 and MedSeA cruises

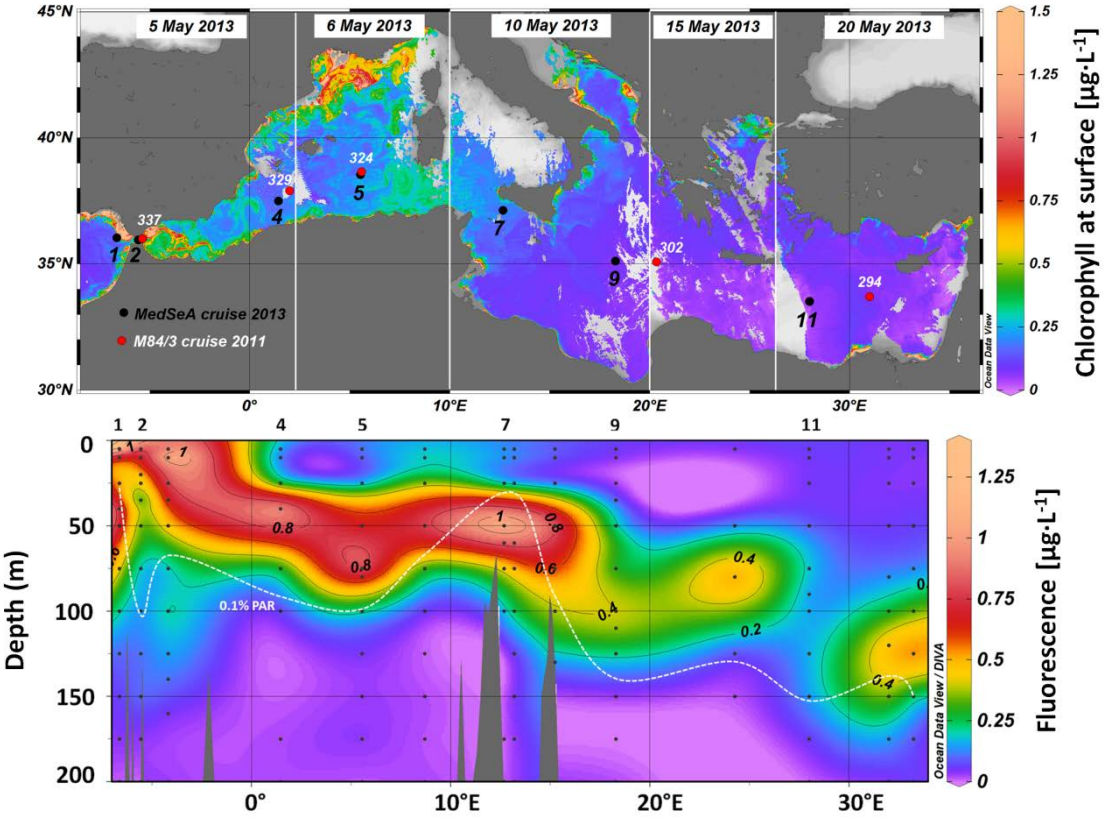

Fig. 2.1 Above: superficial Chl *a* (MODIS Aqua L2) during the MedSeA cruise and location of sampled stations from both cruises. Below: fluorescence for the first 200 m during the MedSea cruise and correspondent limit of the 0.1% PAR; note the general deepening of this limit in the eastern Mediterranean. Only the numbered stations have been analyzed for coccolithophores, the remnant points have been used only to reconstruct the fluorescence profile.

The oceanographic data presented and discussed in this paper were retrieved along a west – east transect extending from the Atlantic Ocean (- 6.64° E, 36.03° N) to the Levantine Basin of the Mediterranean Sea (31.00° E, 33.70° N; Fig. 2.1), during the Meteor M84/3 (6 − 28 April 2011) and the MedSeA (1 − 31 May 2013) oceanographic cruises.

The Mediterranean Sea is a semi-enclosed basin functioning as a miniature ocean. Water exchanges with the Atlantic Ocean, through the Gibraltar Strait, are forced by the excess of evaporation over precipitation and runoff in the basin. The Atlantic surface waters which enter the Mediterranean Sea flow eastward along the northern coast of the Maghreb. This flow is subsequently divided into different branches feeding the western Mediterranean Sea through the Tyrrhenian cyclonic circulation and reaching the Lion Gyre to the north, and the eastern Mediterranean Sea through the Sicily Strait. Biogeochemical and physical parameters varied longitudinally and with depth during the two cruises, following similar trends (Fig. S1, S2, S3, S4; Oviedo et al., 2015). We describe here their variability and

distribution in the first 200 m of the water column of the MedSeA stations analyzed; for a detailed description regarding the M84/3 cruise, please refer to the works of Hainbucher et al. (2013), Oviedo et al. (2015) and Tanhua et al. (2013a). During the MedSeA cruise, temperature and salinity both increased eastward (17 − 21° C; 35 − 39 PSU); along the water column though, temperature decreased, while salinity increased with depth. Dissolved oxygen (O_2) concentration was highest in the southern Alguero-Balearic Basin at 50 m depth (~ 249 µmol kg^{-1}), and lowest in the Gibraltar Strait at 175 m depth (~ 165 µmol kg^{-1}). Total alkalinity increased eastward and with depth, usually reflecting salinity gradients (Hassoun et al., 2015b): within the first 200 m of the analyzed MedSeA stations, it varied from a minimum of 2348 µmol kg^{-1} in the SW Mediterranean, to a maximum of 2653 µmol kg^{-1} in the SE Mediterranean. The average carbonate ion (CO_3^{2-}) and dissolved inorganic carbon (DIC) concentrations also increased eastward; however, while [CO_3^{2-}] was always higher close to the surface, DIC increased with depth. [CO_3^{2-}] oscillated between 150 µmol kg^{-1} in the Western Basin (at 200 m), and 256 µmol kg^{-1} in Eastern Basin (at 10 m). DIC varied between 2048 µmol kg^{-1} in the SW basin (at 5 m), and 2334 µmol kg^{-1} in the SE basin (at 200 m). Average pH values in the first 200 were overall higher in the SE (8.12) than in the SW Mediterranean (8.08). However, the lowest and highest pH values were both included in the Atlantic station (7.99 at 200 m; 8.19 at 5 m), highlighting the existence of strong vertical gradients. The partial pressure of carbon dioxide (pCO_2) was higher on average in the SW (388 µatm) than in the SE Mediterranean (359 µatm), while the most extreme values were found within the Atlantic station (279 µatm at 5 m depth, 461 µatm at 200 m). Similar observations on the carbonate system were made previously in the Mediterranean Sea (Copin-Montégut, 1993; Millero et al., 1979; Schneider et al., 2007, 2010). Dissolved nutrients were scarce, especially phosphate in the SE basin, like in previous studies (Crombet et al., 2011; Ribera d'Alcalà, 2003; Tanhua et al., 2013b). Surface Chlorophyll *a* (Chl *a*) followed the same pattern as nutrients, decreasing eastward (Fig. 2.1, S2; Oviedo et al., 2015; Rahav et al., 2013). The Deep Chlorophyll Maximum (DCM) is a common and persistent feature of the eastern Mediterranean Sea, except during winter mixing (Crise et al., 1999; Ediger and Yilmaz, 1996; Oguz et al., 2013; Raimbault et al., 1993; Siokou-Frangou et al., 2010). The DCM can be identified from fluorescence and from the 0.1% PAR (photosynthetically active radiation) profiles of the MedSeA cruise transect (Fig. 2.1). The eastward deepening of the fluorescence maximum is a common feature in the Mediterranean and it was observed during both the MedSeA and the M84/3 cruises: it reached, and occasionally exceeded, 100 m depth in the Levantine Basin. The physicochemical gradients were similar for the MedSeA 2013 cruise and the Meteor cruise M84/3, allowing

a conjunct analysis of the associated biological datasets. The surface waters (0 − 200 m) of both cruises, according to the Temperature − Salinity − Density (T − S − D) plot, can be grouped under three regional domains (Fig. 2.2). Region 1 represents the Atlantic Water (AW) entering the Gibraltar Strait and expanding into the Alboran Sea (36 − 38.5 PSU; 13 − 18°C); region 2 includes the Modified Atlantic Water (MAW) expanding from the southern Alguero-Balearic Basin to the Sicily Strait (37 − 39 PSU; 13 − 18°C); and region 3 consists of saltier and warmer MAW, located between the Ionian Sea and the Levantine Basin (38.5 − 39 PSU; 15.5 − 20.5°C).

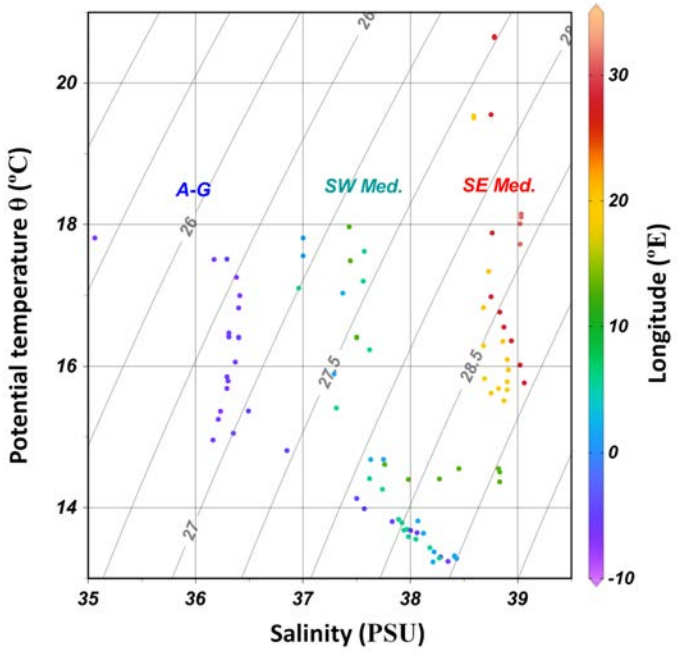

Fig. 2.2 Main water masses within the first 200 m during the MedSeA and M84/3 cruises. A-G = Atlantic Water from the eastern Atlantic Ocean and the Gibraltar Strait; SW Med. = Modified Atlantic Water from the South Western Mediterranean; SE Med. = Modified Atlantic Water from the South Eastern Mediterranean.

2.2 Material and methods

2.2.1 Environmental parameters

During the MedSeA cruise, temperature and salinity, along with fluorescence and dissolved oxygen concentration, have been measured at various depths by a CTD system (Sea-Bird Electronics 991 plus) attached to a rosette of 12 x 24 liters Niskin bottles (Ziveri and Grelaud, 2015).

Total alkalinity and dissolved inorganic carbon for this cruise have been reported in (Hassoun et al., 2015c). These two parameters of the carbonate system were combined in CO2Sys (Lewis and Wallace, 1998) to calculate carbonate ion concentration, pH and pCO_2; following Álvarez et al. (2014), we chose the equilibrium constants of Mehrbach et al. (1973) refitted by Dickson and Millero (1987).

Phosphate (PO_4^{3-}), nitrate (NO_3^-) and nitrite (NO_2^-) concentrations were obtained by OGS (Italian National Institute of Oceanography and Experimental Geophysics). Water samples were filtered over glass fiber filters (Whatman GF/F; 0.7 µm) onboard, stored at - 20 °C, and analyzed on land using a Bran+Luebbe3 AutoAnalyzer, following Grasshoff et al. (1999). Nitrite and nitrate concentrations had a detection limit of 0.02 µM, while phosphate concentration a detection limit of 0.01 µM; inter-comparison exercises (QUASIMEME) confirmed the validity of nutrient measurements.

Methods of collection and analysis of environmental parameters during the M84/3 cruise have been presented in Oviedo et al. (2015), Rahav et al. (2013) and Tanhua et al. (2013a, 2013b). Environmental CTD data for this cruise are available at CLIVAR and at the Carbon Hydrographic Data Office (CCHDO), UCSD Scripps Institution of Oceanography; http://cchdo.ucsd.edu/cruise/06MT20110405. Correspondent bottle data can be found at the Carbon Dioxide Information Analysis Center (CDIAC), (http://cdiac.ornl.gov/oceans/Coastal/Meteor_Med_Sea.html). Additional documentation on the M84/3 cruise can be downloaded from PANGAEA (http://www.pangaea.de/PHP/CruiseReports.php?b=Meteor_1986).

2.2.2 Phytoplankton samples

A total of 55 water samples from the two cruises were analyzed combining morphometric measurements (mass, length, width) and / or detailed morphology recognition. Along the transect, all the samples were collected between 5 and 150 m depth (Table 2.1), following the eastward deepening of the photic zone (see chlorophyll profile in Fig. 2.1).

Station	Date	Coordinates		Depth (m)										
	dd/m/yy	°E	°N	5	10	25	40	50	75	80	100	110	125	150
1	2/5/13	-6.64	36.03	a,b		a	a,b							
2	3/5/13	-5.56	35.95	a,b	a,b	a		a,b	a					
4	7/5/13	1.45	37.49	a,b		a	a,b				a			
5	8/5/13	5.55	38.52	a		a,b		a	a	a,b			a	
7	11/5/13	12.68	37.12	a,b		a		a			a,b			
9	12/5/13	18.29	35.11	a		a,b		a				a	a	a,b
11	15/5/13	28.00	33.50	a		a,b		a	a		a,b		a,b	a
294	10/4/11	31.00	33.70	a,b		a,b		a,b			a,b			
302	13/4/11	20.35	35.07			a,b		a,b			a,b			
324	21/4/11	5.60	38.65	a,b		a,b		a,b			a,b			
329	23/4/11	2.00	37.90	a,b		a,b		a,b			a,b			
337	25/4/11	-5.36	36.00	a,b		a,b		a,b			a,b			

Table 2.1 List of samples analyzed using SYRACO (a) and/or SEM (b).

Water samples of both cruises were extracted from Niskin bottles and filtered (2.5 − 5 liters per sample) onto cellulose acetate-nitrate filters (Millipore, Ø 47 mm, 0.45 µm). A hydraulic vacuum pump system (Eyela, A-1000S) was used at low pressure, to obtain an even distribution of particles on the filter. Each filter was then rinsed with distilled water buffered with ammonia (63 ml NH_3 + 500 ml of distilled water) in order to remove salt residues, and oven-dried at 40 °C for about eight hours.

A portion of filter was mounted on a glass slide, soaked in immersion oil and secured applying a coverslip and tape on the borders of the slide. The sample slides were then analyzed by a Leica DM6000B cross polarized light microscope fitted to a SPOT Insight Camera, at x 1000 magnification. The luminosity level of the microscope was set prior to microscope analysis, following the recommendations of Horigome et al. (2014). A minimum of 40 pictures were taken for each sample and the digital images were processed with SYRACO (SYstème de Reconnaissance Automatique de COccolithes), a software system developed for the automatic recognition of coccoliths (Beaufort and Dollfus, 2004; Dollfus and Beaufort, 1999). The results for *E. huxleyi* were isolated, with usually a minimum of 300 coccoliths per sample being measured (100 in a few low abundance samples), reaching a total of 23255 coccoliths. As the birefringence of calcite particles, when viewed in cross-polarized light, increases with their thickness (up to 1.5 µm), the brightness of *E. huxleyi* coccoliths was measured on a grey level scale and transformed into mass for each coccolith (Beaufort, 2005). Coccolith length was also measured with SYRACO and converted from pixels (px) to micrometers (µm); the error of the measurement (± 0.15 µm) derives from the image resolution: 1 px ≈ 0.15 µm. Finally, the average coccolith mass (M_s) and length were calculated for each sample.

The average *E. huxleyi* coccolith mass was compared with the relative abundance of different calcification varieties of *E. huxleyi* type A, this being largely the predominant morphotype along the transect (2.. 3). *Emiliania huxleyi* type A coccoliths (a mean of 377 per sample, for a total of 13192) were identified by SEM observation and assigned to one of the following categories: low−calcified (A1), medium−calcified (A2) and high−calcified (A3a, A3b). The main distinguishing feature between A1, A2 and A3b was the ratio between slit length (SL) and tube width (TW) on the distal shield of the coccolith (Fig. 2.4): in A1, SL > TW; in A2, SL ≈ TW; in A3b, SL < TW. The main feature characterizing A3a coccoliths was instead the nearly, or completely closed, central area; in this case, the ratio between SL and TW was very variable and not taken into account.

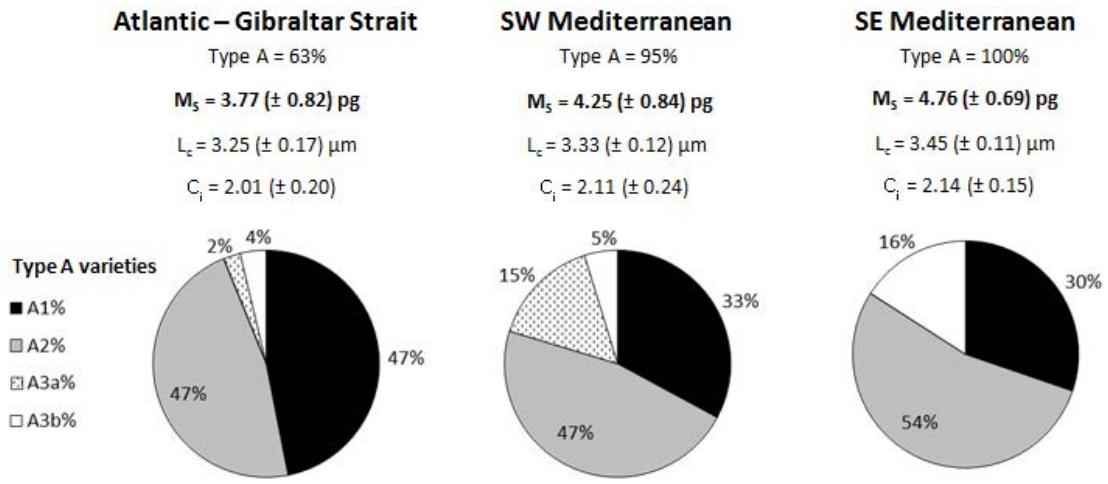

Fig. 2.3 Percentage of type A coccospheres in respect to the total *E. huxleyi* (Type A), average SYRACO coccolith mass (M_S), corrected length (L_C) and calcification index (C_i); the relative abundances of the type A calcification varieties are shown in the pie charts. Data from the M84/3 and MedSeA cruises have been combined for each province described in Fig. 2.2.

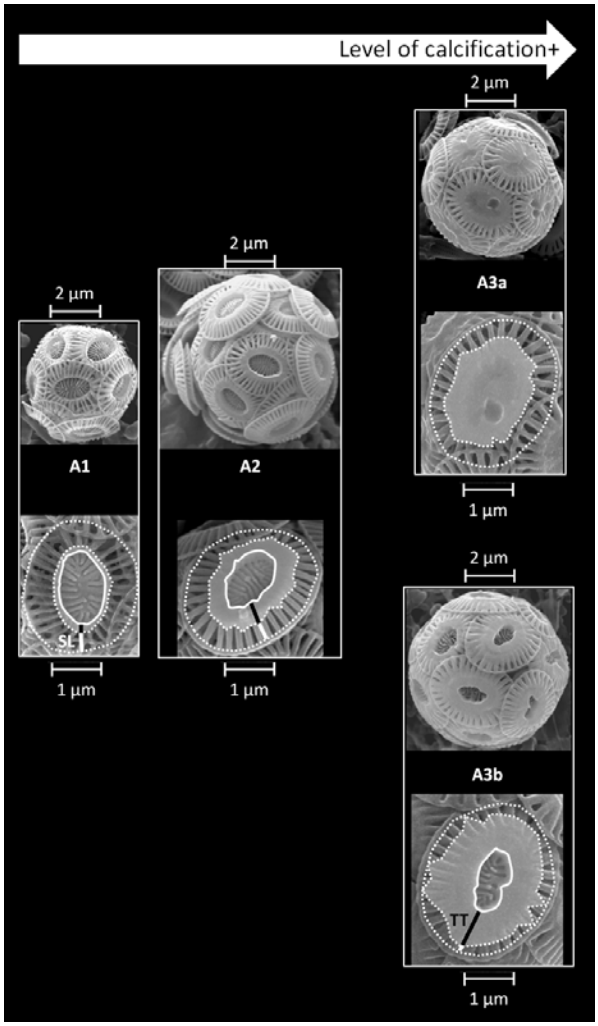

Fig. 2.4 *Emiliania huxleyi* Type A calcification varieties in detail. Open central areas have been highlighted with a continuous white line; SL = slit length, marked by a straight white line; TW = tube width, marked by a straight black line. The scale of 2 µm refers to the coccospheres, the scale of 1 µm to the coccoliths.

Each coccolith was assigned to a single variety and the contribution of each variety to the total *E. huxleyi* type A coccoliths was calculated in terms of relative abundance. The sum of A1, A2, A3a and A3b percentages in each sample is therefore equal to 100%. Notably, we counted free coccoliths, already detached from the coccosphere. Our observations suggest that the norm is for single coccospheres to be entirely formed of coccoliths of one variety; however, on a few occasions we did observe a mix of calcification varieties on a single coccosphere (Fig. S5).

We expected the distal shield to be systematically underestimated by SYRACO, due to the extreme brightness of the calcitic coccolith tube in comparison to the peripheral and thinner area of the shield, when the coccolith is observed in cross-polarized light (Beaufort et al., 2008). To quantify this underestimation, we selected six samples from those already analyzed with SYRACO, so as to include a wide range of coccolith sizes. Fifty micrographs of flat-lying *E. huxleyi* type A coccoliths were captured per sample, using a Zeiss EVO MA 10 scanning electron microscope (SEM) at 30 000 X magnification. Those images were then analyzed with the open source software Fiji (Schindelin et al., 2012), a distribution of ImageJ (Schneider et al., 2012). The Coccobiom2 macro (http://ina.tmsoc.org/nannos/coccobiom/Usernotes.html; Young et al., 2014), developed specifically for coccoliths, was chosen to facilitate the measurements. Through this method, we obtained accurate measurements of the central area (C.A. length) plus tube length, and of the total distal shield length for each coccolith (Fig. S6).

Coccolith mass depends on both coccolith length and its degree of calcification. To express coccolith calcification degree independently from length, we used a calcification index (C_i), calculated following Eq. (1):

$$C_i = \frac{M_s}{M_n} \quad (1)$$

Where M_s is the coccolith mass measured with SYRACO, and M_n is the predicted "normalized mass" for a coccolith of a certain length. M_n was calculated based on Young & Ziveri (2000), using their estimated shape dependent constant (k_s) for *E. huxleyi* coccoliths with medium degree of calcification (= 0.02). The coccolith volume was obtained as in Eq. (2):

$$V = k_s \times length \quad (2)$$

The volume was finally used to calculate the normalized mass, as in Eq. (3):

$$M_n = \mathrm{V} \times \rho_c \quad (3)$$

in which ρ_C is the density of calcite (= 2.7 pg μm^{-3}).

2.2.3 Statistics

A series of Spearman's rank correlations and a Canonical Correspondence Analysis (CCA) were performed on the dataset. The Spearman's analyses (Tables A1−A3) tested the correlations between morphological parameters (M_s, corrected length, C_i), the relative abundances of the calcification varieties (A1, A2, A3a, A3b) and the environmental variables; coefficients were regarded significant for $p \leq 0.05$. The CCA is a multivariate analysis, particularly useful to clarify the distribution of species in relation to environmental gradients (Palmer, 1993). In Fig. 2.6, the ordination axes represent linear combinations of environmental parameters, while the vectors radiating for the center illustrate single environmental gradients; the relative abundances of the calcification varieties and the average coccolith mass are supposed to depend from these gradients. The software PAST 3.14 (Hammer et al., 2001) was used for such statistical tests. Regression analyses and correspondent coefficients (R^2) were instead obtained plotting data in Grapher^{TM} 12 (Golden Software, LLC).

2.3 Results

2.3.1 Coccolith length and calcification index

Regression analysis was performed to test the similarity between the length obtained by automated SYRACO light microscopy technique and SEM micrographs ($R^2 = 0.97$; Fig. S6c). The length obtained with SYRACO was systematically larger than the SEM-derived coccolith central area and tube length (Fig. S6a), but smaller than the coccolith length measured with Fiji (an extended version of ImageJ, see Material and Methods section; Fig. S7c); the equation of this last regression was used to correct the SYRACO-derived lengths of all remaining samples. When the corrected lengths (L_c) and M_s were plotted together, the slope of the regression line visibly increased (Fig. S8). The average L_c and C_i were positively correlated (Table A1; $p < 0.01$), implying that in our samples large coccoliths tended to be more heavily calcified than small ones.

2.3.2 Longitudinal distributions

Data from the M84/3 (April 2011) and MedSeA cruises (May 2013) have been combined to obtain the average values for all morphological variables, the relative abundances of the *E. huxleyi* morphotypes, and those of the Type A calcification varieties. The largely dominant *E. huxleyi* morphotype in our samples was Type A. Type B/C, the only other type of *E. huxleyi* observed, was poorly represented: ~ 5% of the total *E. huxleyi* community on average, except for St. 337 (the westernmost station sampled during the

M84/3 cruise), where it reached ~ 73%; moreover, Type B/C coccoliths were almost never retrieved in the SE Mediterranean (0 – 1% of the total *E. huxleyi*).

A1 and A2 were the most abundant calcification varieties along the transect. The average percentage of A1 decreased from the Atlantic-Gibraltar Strait (47% of the total *E. huxleyi* Type A) towards the SE Mediterranean (30%), while A2 increased (from 47% to 54%). The overcalcified forms included A3a and A3b: A3a was present only in the Atlantic-Gibraltar Strait domain (2%) and in the SW Mediterranean (15%), while A3b was found in the whole transect and increased in relative abundance eastwards, 4% to 16%. Coccolith mass (M_s) increased eastward (from 3.77 pg to 4.76 pg), in parallel with L_c (from 3.25 µm to 3.45 µm) and C_i (from 2.01 to 2.14).

2.3.3 Spearman correlations and CCA

Emiliania huxleyi coccolith mass (M_s) was positively correlated with C_i and L_c (Fig. S9, Table A1), and with the relative abundances of A1 and A3b (Fig. 2.5, Table A2). Length (L_c) had similar coefficients in respect to A1 and A3b, but was also significantly correlated with A2 and the relative abundance of *E. huxleyi* Type A; C_i was instead correlated only with A3b. Other significant correlations were found between morphological and environmental parameters (Table A3). Mass (M_s), C_i and L_c were all strongly correlated with salinity and pH. These morphological variables also showed some significant relationships with pCO_2, $[CO_3^{2-}]$, temperature and dissolved oxygen. The Type A% showed the strongest correlations with the carbonate system, salinity and nutrients, followed by temperature (Table A3). The correlation coefficients with respect to the environmental parameters varied among calcification varieties. All of them displayed significant correlations with at least one carbonate system parameter; furthermore, A1 and A3b were strongly linked by inverse relationships with salinity, while variety A3a had distinctive correlations with temperature and nutrients. The CCA (Fig. 2.6) revealed that 83.84% of the variance in the relative abundance of *E. huxleyi* Type A and its four calcification varieties was explained by axis 1 (52.84%) and 2 (31.00%). Axis scores showed that the three major contributors to axis 1 were pCO_2 (- 0.45), pH (0.43) and $[CO_3^{2-}]$ (0.34); the three major contributors to axis 2 were salinity (- 0.38), $[CO_3^{2-}]$ (- 0.38) and temperature (- 0.32).

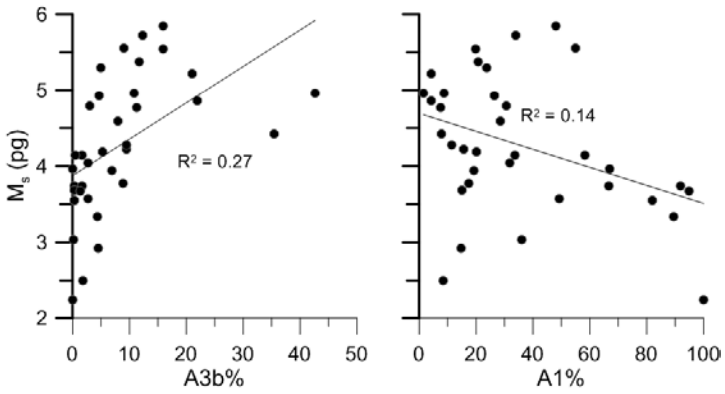

Fig. 2.5 Relationships between coccolith mass (M_s) and the percentages of two calcification varieties (percentages calculated in respect to the absolute abudance of *E. huxleyi* Type A coccoliths). The black line represent the linear regression between the pairs of variables.

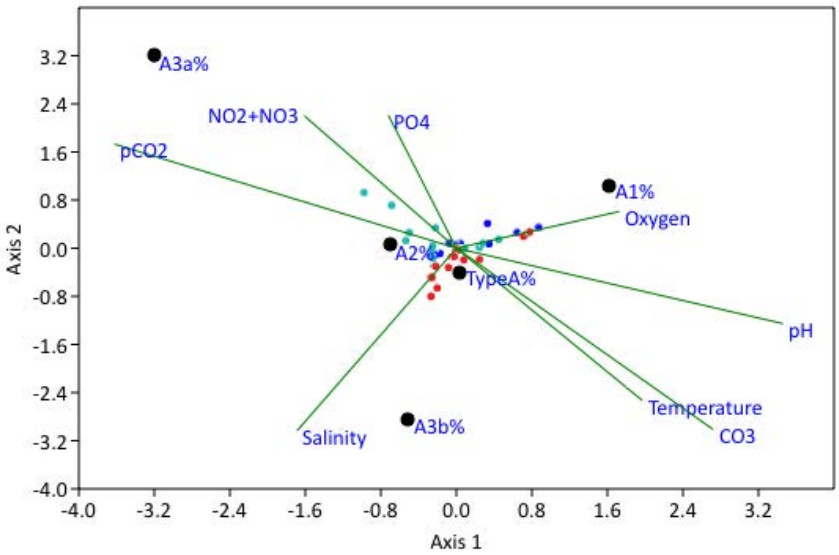

Fig. 2.6 Biplot of the CCA analysis. Vectors radiating from the centre symbolize the environmental gradients; their length is proportional to the strenght of the gradient. The black dots are the centroids of *E. huxleyi* Type A and of its four calcification varieties. The coloured dots represent individual samples, differentiated by marine province (blue = A-G, aquamarine = SW Med., red = SE Med.).

2.4 Discussion

2.4.1 Morphological controls over *E. huxleyi* coccolith mass

Emiliania huxleyi coccolith mass gradually increased eastward, from the Atlantic−Gibraltar domain to the SE Mediterranean. Our results suggest that it was controlled by two main morphological features: distal shield length and the degree of calcification (Fig. S9, Table A1; Bach et al., 2012; Berger et al., 2014; Horigome et al., 2014; Meier et al., 2014). The index used here to represent coccolith calcification degree (C_i) is size−independent, and might be applied as an alternative to the “relative tube width” of Young et al. (2014a) when only SYRACO data are available.

Emiliania huxleyi coccolith mass in the Mediterranean Sea seems mainly regulated by the variation of the length and calcification degree within the Type A morphotype. Along the studied transect, two morphotype groups were encountered: A and B/C; Type A being by far the most abundant (Fig. 2.3), as seen in Knappertsbusch (1993) and Oviedo et al. (2015). Type B/C constituted on average less than 5% of the *E. huxleyi* population, with the exception of the Atlantic−Gibraltar domain (Fig. 2.3), where coccolith mass values were the lowest. The concomitance of high Type B/C percentages, indicating Atlantic surface water influx into the Mediterranean Sea, and low average coccolith mass values, is likely not a coincidence since this morphotype is typically less calcified than Type A. However, the Spearman's analysis did not reveal a significant correlation between mass and Type A%. The existence of a link between average coccolith mass and *E. huxleyi* morphotypes distribution has been previously suggested, but never quantified in detail (Beaufort et al., 2011; Cubillos et al., 2007).

Emiliania huxleyi coccoliths displayed a wide range of distal shield lengths and calcification degrees within Type A. Our results (Fig. 2.5, Table A2) suggest that the proportion of Type A calcification varieties within the *E. huxleyi* assemblage can considerably influence the average coccolith mass of this species in the Mediterranean Sea. Several varieties of *E. huxleyi* Type A are reported in the literature (Young et al., 2003), mainly from the North Atlantic Ocean (Beaufort and Heussner, 2001; Berger et al., 2014; Smith et al., 2012) and the Mediterranean Sea (Alcober et al., 1994; Cros and Fortuño, 2002; Dimiza et al., 2008a, 2008b; Riaux-Gobin et al., 1995; Triantaphyllou et al., 2010). We identified a total of four calcification varieties, but only two of them were significantly correlated with the average coccolith mass: A1 and A3b. Overcalcified coccoliths included in A3b, were generally larger than the undercalcified coccoliths included in A1, as demonstrated by the relationships between coccolith length, A1% and A3b% (Table A2). Similar positive trends between size and calcification degree were noticed in oceanic communities of the North Atlantic (Young et al., 2014a).

Overall, the comparison between morphological variables suggests that *E. huxleyi* coccolith mass is controlled by the distribution of its morphotypes and Type A varieties, characterized by different size ranges and calcification degrees. The Eastern Mediterranean was especially rich in large, overcalcified specimens, explaining the high coccolith mass values calculated for this province (Fig. 2.3).

2.4.2 Ecology of *E. huxleyi* Type A varieties

The distribution of *E. huxleyi* morphotypes and of the four Type A calcification varieties in the Mediterranean Sea, might be linked primarily to carbonate chemistry, salinity and

temperature gradients. It has been previously suggested that, in natural settings, multiple factors modulate coccolith calcification, including the seawater carbonate chemistry, temperature and nutrient limitation (Berger et al., 2014; Oviedo et al., 2016; Poulton et al., 2014; Smith et al., 2012; Young et al., 2014a; Young and Westbroek, 1991), although it can not be excluded that the observed correlations reflect the geographical distribution of *E. huxleyi* morphotypes (Boeckel and Baumann, 2008; Horigome et al., 2014; Smith et al., 2012).

Emiliania huxleyi Type B/C tends to be more abundant in the SW than in the SE Mediterenean (Fig. 2.3); in correspondence of lower pH and lower [CO_3^{2-}] (Fig. S3; Oviedo et al., 2015). Beaufort et al. (2011) already proposed the existence of a connection between carbonate chemistry and the distribution of differently calcified species and morphotypes of Noëlaerhabdaceae. The importance of the carbonate system (pH, pCO_2, [CO_3^{2-}]) in relation to the distribution of *E. huxleyi* morphotypes and Type A calcification varieties is supported by both our CCA and Spearman's rank correlation analysis (Fig. 2.6, Table A3).

As suggested in Horigome et al. (2014), carbonate chemistry might have limiting effects on *E. huxleyi* coccolith morphology only when critical values are reached (e.g. pH lower than a certain threshold). The relationship between carbonate chemistry parameters and the relative abundances of the Type A varieties is not uniform: contrary to expectations, the percentage of low−calcified A1 increases in higher pH water, while that of high−calcified A3a decreases. Also, the two high−calcified varieties A3a and A3b are linked in different ways to the carbonate system. Possibly, the Type A varieties possess different sensitivities to changes in carbonate chemistry and the positive correlation we observe between the calcification degree (C_i) and pH is not straightforward, but likely derives from the calcification varieties distribution and their unique relationships with the carbonate system. Our interpretation is supported by the complexity of *E. huxleyi* genome, capable of plastic responses to changing environmental conditions (Read et al., 2013), and the proven presence in the Mediterranean Sea of at least two Type A clades (Bendif et al., 2014; Hagino et al., 2011). Besides, laboratory experiments have demonstrated that various strains of *E. huxleyi* can respond differently to carbonate chemistry perturbations (Hoppe et al., 2011; Langer et al., 2009; Meyer and Riebesell, 2015). Field studies have also proved the occurrence of overcalcified coccoliths, including one corresponding morphologically to A3a, in relatively low pH, calcite saturation state and [CO_3^{2-}] (Beaufort et al., 2011; Smith et al., 2012).

The correlations between A3a and the carbonate system (Table A3), suggest that this Type A calcification variety is preferably distributed in lower [CO_3^{2-}], pH and higher pCO_2

waters within the Mediterranean Sea. Remarkably, we did not encounter A3a coccoliths in the Eastern Mediterranean, and this calcification variety could be not only influenced by the carbonate system, but by additional environmental gradients like temperature and nutrients. These results seem to agree with previous studies, which showed a higher abundance of morphologically equivalent coccoliths in winter (colder, nutrient-richer waters), in both the Mediterranean and the North Atlantic Ocean (Smith et al., 2012; Triantaphyllou et al., 2010). Another important environmental parameter, which apparently explains the differential distributions of A1 and A3b, is salinity: while the percentage of A1 coccoliths decreases with salinity, the percentage of A3b changes in the opposite direction. These environmental correlations reflect a preferential distribution of A1 within superficial, western Mediterranean waters, and of A3b within relatively deeper, eastern Mediterranean waters. Salinity has been suggested as a factor that influences coccolith production rate, size, degree of calcification and shape (Fielding et al., 2009; Green et al., 1998; Paasche et al., 1996; Saruwatari et al., 2016); yet, these physiological effects have been verified only for larger salinity ranges than those characterizing our Mediterranean transect (36 − 39 PSU), or for very low salinities, typical of coastal areas (Paasche et al., 1996). It is still uncertain if salinity could have some effect on the distribution of *E. huxleyi* populations, but strong correlations between this environmental parameter and the abundance of *E. huxleyi* morphotypes have been found in the past, perhaps linked to salinity-driven changes in water density (Poulton et al., 2011). Coccoliths morphologically equivalent to A3b had been reported in the Aegean Sea within the winter assemblage, although a direct effect of salinity on their abundance could not be observed, since this environmental parameter was rather constant in the area during the year (Triantaphyllou et al., 2010). Our results highlight the existence of some relationship between salinity and the relative distribution of two *E. huxleyi* Type A calcification varieties in the Mediterranean Sea (A1, A3b); however, the nature of this relationship is doubtful: maybe salinity simply reflect the density change along the water column, and hence simply the preference of the A1 variety for shallow waters, and of the A3b variety for deeper waters; in parallel, it seems to reflect also their preferential distribution along the transect (relative abundance in Western and Eastern Mediterranean). Finally, seasonal unimodal patterns of calcification for *E. huxleyi* Type A in the Aegean Sea (Triantaphyllou et al., 2010) and the discovery of occasional 'mixed' coccospheres and intergradational coccolith varieties (Smith et al., 2012), suggest that single coccolithophore cells of this morphological group might produce different varieties during their life span. It is important to take into account that our results cannot prove any direct environmental effect on the *E. huxleyi* Type A

varieties: to test any direct effect, specimens of selected varieties should be selected and subjected to different conditions.

2.5 Conclusions

Emiliania huxleyi coccolith mass in the Mediterranean Sea seems to be explained by the relative abundance of several Type A calcification varieties, in particular: the large, heavily calcified specimens (A3b) that are more common in the SE basin, and the small, lightly calcified specimens (A1) that are more common in the SW basin. Our results suggest that carbonate chemistry gradients could influence the distribution of different *E. huxleyi* Type A calcification varieties, together with temperature, nutrient concentrations and water density.

CHAPTER 3

Coccolithophore haploid and diploid distribution patterns in the Mediterranean Sea: can an haplo-diploid life cycle be advantageous under climate change?

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Abstract

Coccolithophores are unicellular pelagic algae, capable of calcification. In the Mediterranean Sea, several species have a well-known haplo-diploid life cycle, alternating the production of different types of calcite plates, the holo- and hetero-coccoliths. We analyzed the distribution of both phases along a W-E Mediterranean transect during April 2011 and May 2013 (spring season), following strong environmental gradients. The proportion of holococcolithophores:heterococcolithophores of selected species varies not only vertically along the water column, but also longitudinally, following the overall gradient. Based on the environmental affinities of the coccolithophore life phases, we conclude that a dimorphic life cycle might provide the ability to adapt to the south-eastern (SE) Mediterranean environment, in conditions characterized by surface water with relatively high calcite saturation state, high temperature, stratification and nutrient limitaton, and support the survival of species whose diploid phases are in contrast adapted to Atlantic or south-western (SW) Mediterranean conditions. Thus, an haplo-diploid life cycle could provide a way to adapt to rapid and adverse environmental changes.

3.1 Introduction

Coccolithophores are unicellular pelagic calcifying phytoplankton belonging to the phylum Haptophyta and the class Prymnesiophyceae. The products of their calcification consist of minute and elaborated calcite plates, known as coccoliths, which constitute the cell exoskeleton (coccosphere). Coccolithophores are usually outcompeted by other groups of phytoplankton in high productivity areas of the ocean, but they thrive in low productivity areas, where they can constitute the majority of the phytoplankton population (Lessard et al., 2005; Tyrrell and Merico, 2004). They have a poorly documented life cycle, characterized by the secretion of two different types of coccolith: the heterococcoliths and the holococcoliths, originally considered two different species. The discovery of their alternation was first detected in *Coccolithus pelagicus*, having a motile and a non-motile

stage (Parke and Adams, 1960), with heterococcoliths produced during the diploid phase, and holococcoliths during the haploid phase (Billard, 1994). Thought to be a transition between the two life phases (Geisen et al., 2004), the presence of both stages on a single coccosphere cell have been repeatedly found in natural populations, especially in the Mediterranean Sea (Alcober and Jordan, 1997; Cortés, 2000; Cortés and Bollmann, 2002; Cros et al., 2000; Cros and Fortuño, 2002; Frada et al., 2009; Geisen et al., 2002; Karatsolis et al., 2014; Kleijne, 1991; Malinverno et al., 2008; Samtleben and Schröder, 1992; Šupraha et al., 2014; Thomsen et al., 1991; Triantaphyllou et al., 2004, 2009, 2014; Triantaphyllou, 2010; Triantaphyllou and Dimiza, 2003). The haplo-diploid nature of hetero-holococcolith alternation was later confirmed, through DNA content analysis by flow-cytometry, for four coccolithophore species (Houdan et al., 2004). The triggers behind coccolithophore phase change are still under discussion. It may be stimulated by exposure to extreme environmental conditions and guarantee the survival of coccolithophore species in a changing oligotrophic habitat (Cros et al., 2000; Cros and Estrada, 2013; Houdan et al., 2005, 2006; Valero et al., 1992). Holococcolithophores are especially abundant in the upper photic zone of subtropical oceans (Kleijne, 1991; Winter et al., 1979). Based on their distribution, it has been hypothesized that this phase exhibits a preference for warm, oligotrophic, stratified waters (Cros and Estrada, 2013), and in fact they are common in the Mediterranean Sea during summer (Dimiza et al., 2008b; Malinverno et al., 2009; Šupraha et al., 2016). Only a few previous studies have focused on the distribution of two life phases of the same coccolithophore species (Cros and Estrada, 2013; Dimiza et al., 2015; Šupraha et al., 2016). In the last decades sea surface temperature has been increasing over the Mediterranean Sea (Belkin, 2009; Calvo et al., 2011), in association with water column stratification and a deepening of the thermocline (Lejeusne et al., 2010). Moreover, since the beginning of the industrial era, anthropogenic CO_2 released into the atmosphere has been sequestered by surface waters, contaminating the whole basin and lowering the pH by 0.055 – 0.156 units (Hassoun et al., 2015a). Such changes are expected to impact the marine community, including coccolithophores, but the response involves complex mechanisms on multiple trophic layers (Portner et al., 2014). The environmental control on haploid and diploid phases in coccolithophores is still largely unknown and field studies can shade light on their ecology. The present work is the first attempt to characterize the distribution of holococcolithophores along a large biochemical and physical gradient in the Mediterranean Sea, in order to underpin the potential triggers of coccolithophore life phase change in the natural environment. General observations, based on seven selected species (*Calcidiscus leptoporus*, *Coccolithus pelagicus*, *Coronosphaera mediterranea*,

Syracosphaera bannockii, *S. histrica*, *S. molischii*, *S. pulchra*), were reinforced analyzing the distribution of both life phases of *Coccolithus pelagicus* subsp. *braarudii*, *Calcidiscus leptoporus*.

3.1.1 Oceanographic setting

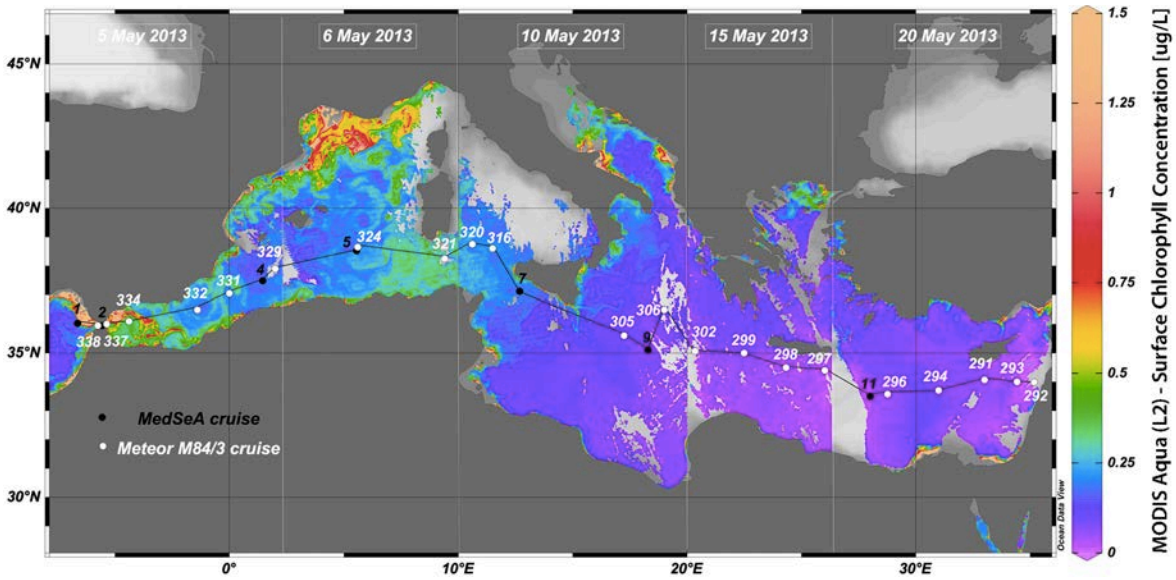

Fig. 3.1 Surface Chlorophyll Concentration during May 2013, at the time of the MedSeA cruise. Superimposed are the Meteor M84/3 and MedSeA stations considered for this work.

This study focuses on data collected during two oceanographic cruises, in April 2011 and May 2013 in the southern basins of the Mediterranean Sea, including the Gibraltar Strait, the Alboran Sea, the southern Alguero-Balearaic Basin, the Sicily Strait, the southern Ionian Sea and the Levantine Basin. The Mediterranean is a semi-enclosed basin, connected to the Atlantic Ocean and the Black Sea through respectively the Gibraltar and the Dardanelles Straits; and artificially to the Red Sea through the Suez Canal. The main water exchange occurs in the west, through the Gibraltar Strait, where the low-salinity and nutrient-rich Atlantic waters enter the Mediterranean Sea. The Atlantic waters flow superficially, due to their low density, and propagate towards the Levantine Basin, gradually losing nutrients and increasing their salinity and temperature (Malanotte-Rizzoli and Hecht, 1988; Wüst, 1961). The Mediterranean is characterized by strong longitudinal environmental gradients (Crombet et al., 2011; Ribera d'Alcalà, 2003; Skliris, 2014; Tanhua et al., 2013a, 2013b). In April 2011 and May 2013, waters in the south-eastern basins were much warmer, saltier and oligotrophic than those in the south-western basins (Oviedo et al., 2015), see also Fig.S1, S2. Carbonate ion (CO_3^{2-}) concentration and pH (Fig.S3), as well as total alkalinity, were all higher in the Eastern Mediterranean during

both cruises (Oviedo et al., 2015); Álvarez et al., 2014; Gemayel et al., 2015; Hassoun et al., 2015; Tanhua et al., 2013). Finally, an eastward deepening of the Deep Chlorophyll Maximum (DCM) and of the 0.1 % limit of Photosynthetic Available Radiation (PAR) was observed during the 2013 MedSeA cruise (Fig.S12).

3.2 Material and methods

Twenty-seven oceanographic stations, distributed along a west-east (W-E) transect extending between 36.03°N – 6.64 ºW and 33.99°N – 35.17°E, were considered in this study (Fig. 3.1, Table S5). Such stations were surveyed during the Meteor M84/3 (6 – 28 April 2011) and the 2013 MedSeA (1 – 31 May 2013) cruises. A profiling CTD (Sea-Bird Electronics 991) recorded temperature, salinity, fluorescence and dissolved oxygen concentration for each station during both cruises. Data relative to the 2013 MedSeA cruise CTD data can be downloaded from PANGAEA (Ziveri and Grelaud, 2015). Nutrient concentrations during this last cruise were measured with a Bran + Luebbe3 AutoAnalyzer, according to Grasshoff et al., 1999, after onboard filtration through glass fiber filter (Whatman GF/F; 0.7 µm) and storage at - 20°C. Detection limits were 0.02 µM and 0.01 µM for nitrate plus nitrite (NO_3^-+NO_2^-) and phosphate (PO_4^{3-}) concentrations respectively. The quality of the nutrient measurements was confirmed by the results of the inter-comparison exercises (QUASIMEME). Total alkalinity and dissolved inorganic carbon data for this same cruise are available in PANGAEA (Goyet et al., 2015). CO2Sys (Lewis and Wallace, 1998) was used to calculate the remaining variables of the carbonate system: equilibrium constants (Mehrbach et al., 1973) were refitted (Álvarez et al., 2014; Dickson and Millero, 1987). The methods used during the M84/3 cruise to obtain nutrient and carbonate system variables have been previously described (Oviedo et al., 2015; Rahav et al., 2013; Tanhua et al., 2013a); all CTD and bottle data regarding this cruise are also available (http://cchdo.ucsd.edu/cruise/06MT20110405; http://cdiac.ornl.gov/oceans/Coastal/Meteor_Med_Sea.html).

A total of 107 seawater samples were analyzed for coccolithophores. Sample depth ranged between 0 and 175 m (as described in Table S5). During both cruises, seawater was collected with Niskin bottles; 2.5 – 5 L were filtered per sample on cellulose acetate-nitrate filters (Millipore, Ø 47 mm, 0.45 µm) using a hydraulic vacuum pump (Eyela, A-1000S), and oven-dried at 40 º C for 8 – 12 hours. From each phytoplankton sample, a piece of filter was radially cut, mounted on a stub and gold-coated, before analysis at 3000 X magnification with a Zeiss EVO MA 10 scanning electron microscope (SEM). A minimum of 80 – 100 coccospheres (maximum 420) were counted per sample, along a longitudinal transect of at least 5 mm, for an average of 4.1 mL of water.

Heterococcospheres and holococcospheres were identified down to species level (Cros and Fortuño, 2002; Young et al., 2003), based also on the Nannotax3 website (http://ina.tmsoc.org/Nannotax3/). Upper and lower confidence limits were calculated (Bollmann et al., 2002); corresponding errors oscillated between 149 – 1258 cells L^{-1} for samples containing, respectively, 5.6 x 10^3 and 4.1 x 10^5 cells L^{-1}. Only values that exceeded the maximum counting error per sample were subsequently considered for statistical analysis and interpretation. Coccolithophore absolute abundance data concerning the M84/3 cruise were previously published (Oviedo et al., 2015). In order to express the prevalence of the holococcolithophore phase, we used the HOLP index (Cros and Estrada, 2013): HOLP = 100 x (Total holococcolithophores / Total HHLC)

where Total_HHLC is the number of coccolithophores (of both life phases) that belong to species with an established life cycle, excluding those species with a potentially non-calcified haploid phase (e.g. *E. huxleyi*). In the calculation of the HOLP index were included the most abundant holo-heterococcolithophore species, listed in Table S6. The HOLP index can vary between values of 0 (absence of holococcolithophores) and 100 (absence of heterococcolithophores).

PAST 3.14 (Hammer et al., 2001) was used to perform all statistical analysis. First, in order to characterize the coccolithophore life cycle phases along the transect, we performed a hierarchical cluster analysis on the 2013 MedSeA and M84/3 cruise stations. The analysis was constrained by longitude, and based on the average HOLP index calculated between 5 and 100 m, excluding a few stations for which only one sample depth was available. We assigned the 2013 MedSeA and M84/3 cruise stations to the SW or to the SE Mediterranean (Fig. 3.3, 3.5, 3.6; Table S5) based on the dendogram of the cluster analysis (Fig. 3.2). The mean coccolithophore abundances and HOLP indices in the SW and SE Mediterranean were compared through t-test; means were considered statistically different at *p*-values ≤ 0.05.

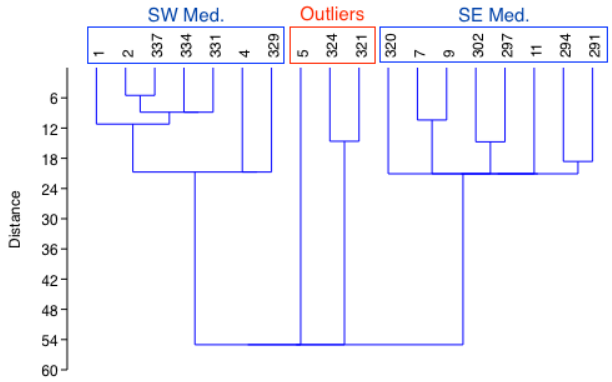

Fig. 3.2 Dendogram from the cluster analysis of MedSeA and M84/3 stations, based on the average HOLP index between 5 and 100 m.

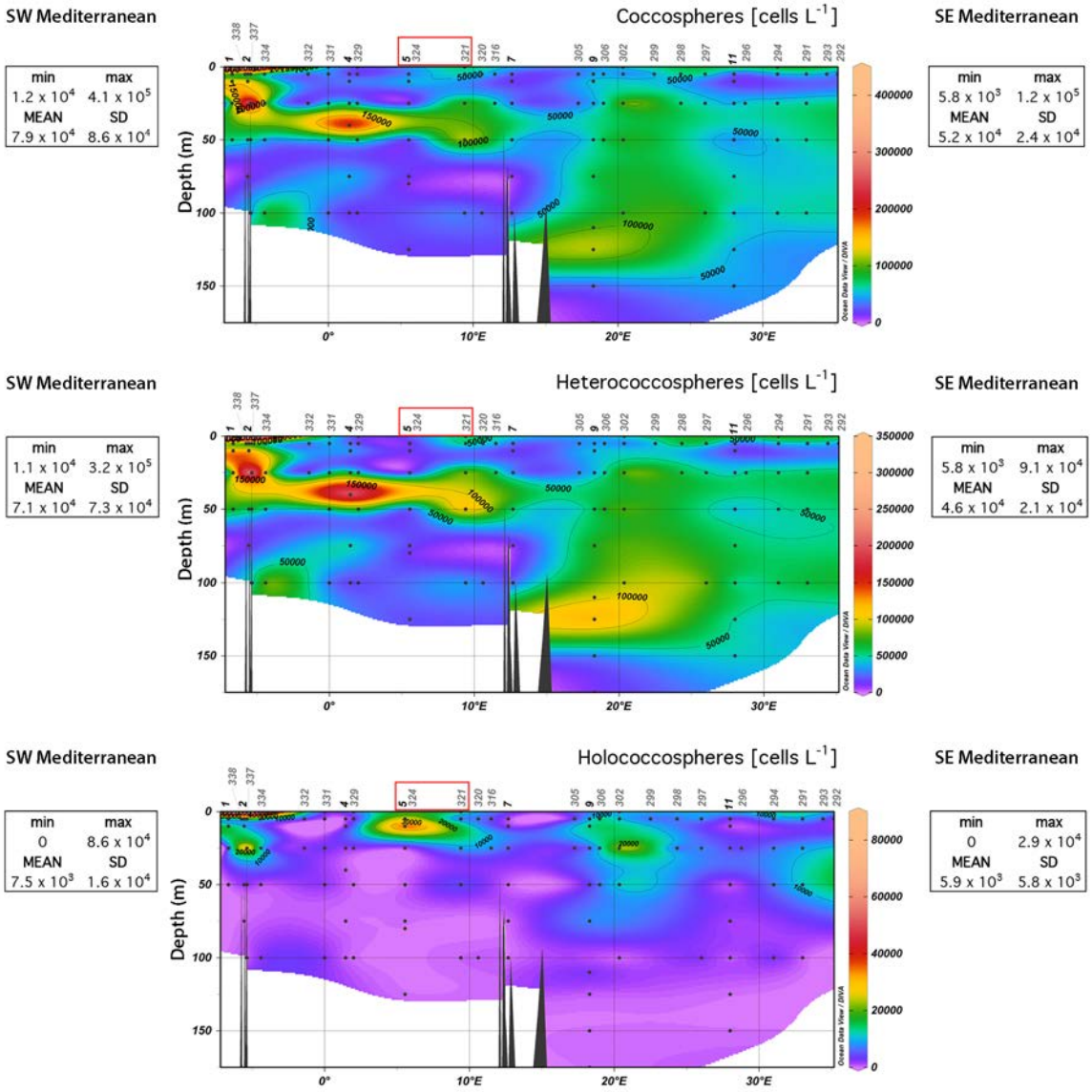

Fig. 3.3 MedSeA and M84/3 combined data on absolute abundance of total coccospheres, heterococcosphere and holococcospheres. The minimum (min), maximum (max), mean (MEAN) and standard deviation (SD) values refer to the first 100 m of the water column in the Western and Eastern Mediterranean. Stations 5, 321 and 324 are treated as outliers and excluded from the calculations.

Then, the biological and environmental data were compared through Spearman correlation analysis. A first analysis was run between the total holococcolithophore population, the HOLP index and the environmental parameters (Table 3.1). A second analysis was run between the absolute abundance of seven selected species, for both the holococcolithophore (HOL) and heterococcolithophore (HET) forms (*Calcidiscus leptoporus*, *Coccolithus pelagicus*, *Coronosphaera mediterranea*, *Syracosphaera bannockii*, *S. histrica*, *S. molischii*, *S. pulchra*) and the environmental parameters (Table 3.2). Those species were selected for having an established taxonomy (both hetero- and

holococcoliths forms are known) and a high average relative abundance in our samples (Table S6). Correlation coefficients (r) with *p*-values ≤ 0.05 were considered statistically significant. Abundance data for all species are available on PANGAEA (doi: 10.1594/PANGAEA.875202; 10.1594/PANGAEA.875924).

	T	Sal	NOx	PO_4	pH	pCO_2	CO_3^{2-}	PAR%
Tot. HOL	****0.35**	0.00	****-0.45**	****-0.40**	****-0.26**	****-0.25**	****0.26**	****0.66**
HOLP index	****0.41**	0.03	****-0.52**	****-0.44**	-0.14	-0.11	****0.30**	****0.68**

Table 3.1 Environmental correlations of absolute total holococcolithophore abundance (Tot. HOL; N= 97) and of the HOLP index (N= 94). PAR% values were available only for the MedSeA cruise dataset (N= 41 in relation to Tot. HOL; N= 38 in relation to HOLP). Significant Spearman correlation coefficients are in bold (*$p \leq 0.05$, **$p \leq 0.01$).

3.3 Results

3.3.1 Absolute abundances and HOLP index

During the two W-E trans-Mediterranean samplings in 2011 and 2013, the total coccolithophore concentrations in the upper 100 m (including both holococcolithophore and heterococcolithophore specimens, Fig. 3.3) ranged between 5.7 x 10^3 cells L^{-1} and 4.1 x 10^5 cells L^{-1}. The lowest values were recorded at station 5 (southern Alguero-Balearic Basin, May 2013), while the highest values were recorded at station 337 (Gibraltar Strait, April 2011). The mean coccolithophore concentration in the first 100 m of the water column was significantly (t-test $p = 0.04$) higher in the SW than in the SE Mediterranean stations. Heterococcolithophores were the largely dominating coccolithophore life phase and their distribution drives the total coccolithophore population (Fig. 3.3) and were recorded in all samples from 0 to 175 m depth. Minimum and maximum abundances in the upper 100 m similar to those of the total population (5.7 x 10^3 cells L^{-1}; 3.2 x 10^5 cells L^{-1}). Their mean concentration in the first 100 m of the water column was significantly (t-test $p = 0.02$) higher in the SW than in the SE Mediterranean stations. Holococcolithophores had a patchy distribution, but were particularly abundant between 0 and 50 m depth (Fig. 3.3). Their highest concentration was recorded at station 337 (8.6 x 10^4 cells L^{-1}, April 2011). Their mean concentration was higher in the SW than in the SE Mediterranean, but the difference was not statistically significant (t-test $p = 0.52$). Overall, heterococcolithophores largely dominated the coccolithophore assemblage. Two coccolithophore species (*Calcidiscus leptoporus* and *Coccolithus pelagicus* subsp. *braarudi*) were represented mainly by their holococcolithophore stage along the transect (Fig. 3.4).

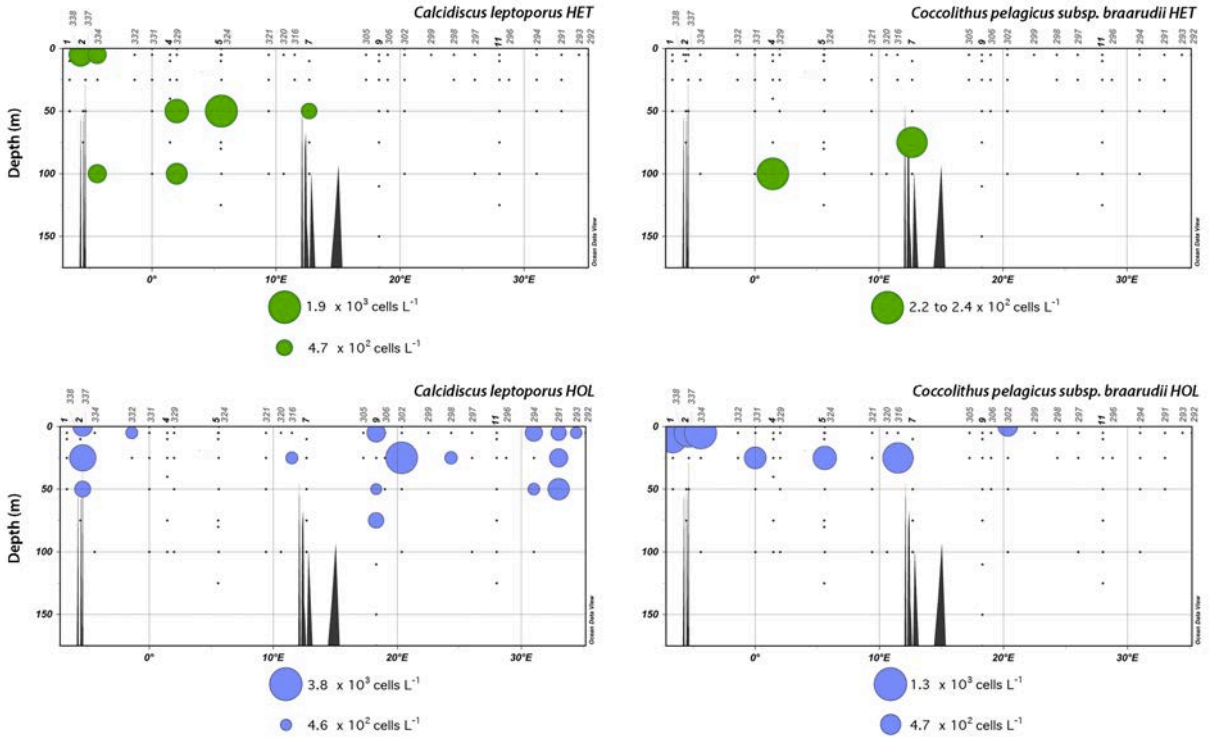

Fig. 3.4 Absolute abundance of the HET and HOL forms of *Calcidiscus leptoporus* (left) and *Coccolithus pelagicus* subsp. *braarudii* (right).

Calcidiscus leptoporus HET and *Coccolithus pelagicus* subsp. *braarudii* HET were found only in the SW Mediterranean, where they reached respectively a maximum of ≈ 2000 cells L^{-1} and ≈ 250 cells L^{-1}. *Coccolithus pelagicus* subsp. *braarudii* HOL was found prevalently in the SW Mediterranean, reaching a maximum of 1300 cells L^{-1} at M84/3 cruise station 334. On the other hand, *Calcidiscus leptoporus* HOL was prevalently found in the SE Mediterranean, reaching a maximum of 4000 cells L^{-1} at M84/3 cruise station 302. The HOLP index, indicating the incidence of the holococcolithophore phase, varied greatly in our samples: between 0 (only heterococcolithophores) and 100 (only holococcolithophores) in both the SW and SE Mediterranean stations (Fig. 3.5).

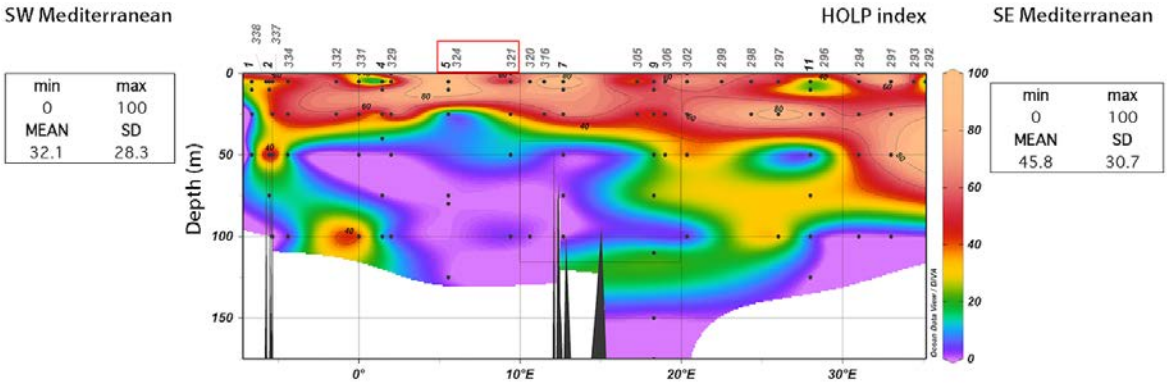

Fig. 3.5 HOLP index along the transect for the first 100 m. Stations 5, 321 and 324 are treated as outliers and excluded from the calculations.

The mean HOLP index calculated for the first 100 m of the water column was significantly higher (t-test p = 0.04) in the SE than in the SW Mediterranean. The anomalous stations highlighted by the cluster analysis (Fig. 3.2) were characterized by HOLP index results distant by other stations (outliers). The high values of station 5 (average = 89.13) contrasted with the lower values registered in the SW Mediterranean cluster (average = 34.11) and in stations 324 and 321 (average = 27.26). The average HOLP index in stations 324 and 321 was also considerably lower than that registered in the SE Mediterranean cluster (average = 53.86). In particular, very high HOLP index values (= 100) were registered within the first 10 m of station 5 (Fig. 3.5), due to an almost mono-specific bloom of *Syracosphaera bannockii* HOL (= 69 – 73% of the total coccospheres); this form is scarce in all other samples, oscillating between 0 and 16%. On the other hand, *S. bannockii* HET was not found in station 5 and it was rare along the remaining stations, never representing more than 2% of the total coccolithophore community.

Stations 5, 324 and 321 were adjacent to each other, defining an interval of high HOLP index variability between 5.55 and 9.40 °E; having being isolated as outliers, they were excluded from the calculations presented in Fig. 3.3, 3.5, 3.6, and from the correspondent t-tests.

3.3.2 Environmental correlations

Holococcolithophore absolute abundance (Tot. HOL) and the HOLP index were both negatively correlated with [NO_3^-+NO_2^-], [PO_4^{3-}], and positively with [CO_3^{2-}]. Other significant correlations included: temperature, pH, pCO_2 and PAR% for Tot. HOL; temperature and PAR% for the HOLP index (Table 3.1). Figure 3.6 illustrates the variability of the main environmental and coccolithophore parameters within the analyzed 2013 MedSeA cruise stations (excluding station 5).

A high HOLP layer was identified for the SW and SE Mediterranean basins; its lower limit corresponds to the depth above which the HOLP index is ≥ 45 (the average HOLP index drops dramatically from 45 to 0 between 10 and 25 m depth in the SW basin; Fig. 3.6) and where more than 75% of the total water column integrated holococcolithophores were found.

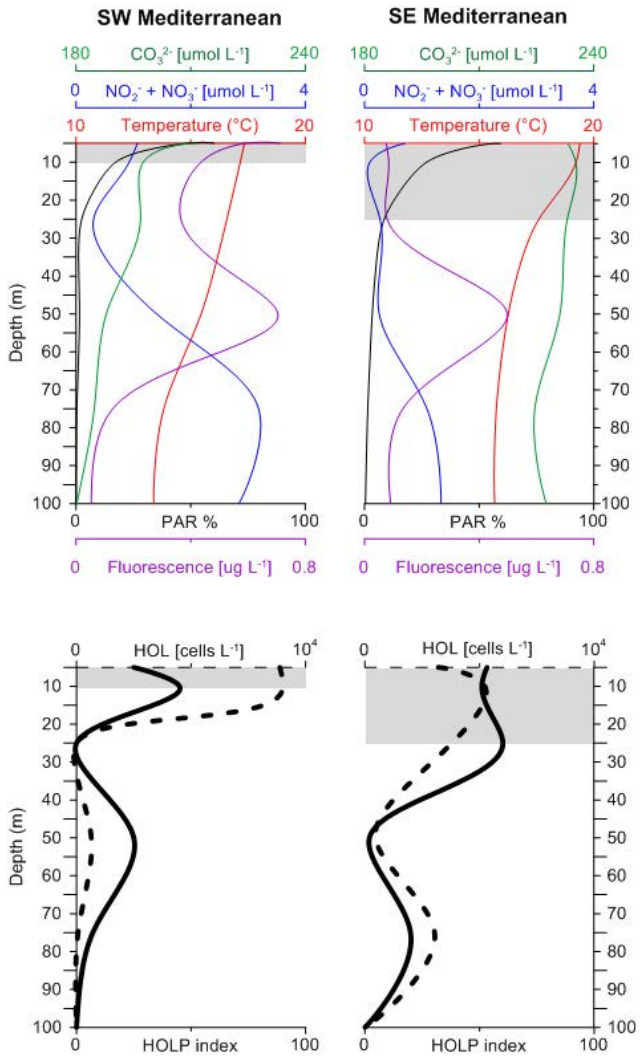

Fig. 3.6 Average [CO_3^{2-}], [NO_2^- + NO_3^-], temperature, PAR%, fluorescence, holococcolithophore absolute abundance (HOL, dotted line) and HOLP index (solid line) in the first 100 m of the SW (left) and SE Mediterranean (right) during the MedSeA cruise. The interval in grey delimits the high HOLP layer.

The high HOLP layers extended within the upper 10 m of the superficial water column in the SW basin, and within the upper 25 m in the SE basin. These depth intervals were characterized by the co-occurrence of low nutrient concentrations, high PAR%, high temperature and high [CO_3^{2-}]. The first 10 m of stations 1, 2 and 4 had on average [NO_3^- + NO_2^-] = 0.50 µmol L^{-1}, [PO_4^{3-}] = 0.04 µmol L^{-1}, temperature = 17.25°C, PAR% = 38.76 and [CO_3^{2-}] = 204.63 µmol kg^{-1}; below 10 m, the average values were the following: [NO_3^- +

NO_2^-] = 1.94 µmol L^{-1}, [PO_4^{3-}] = 0.12 µmol L^{-1}, temperature = 14.81°C, PAR% = 1.09 and [CO_3^{2-}] = 187.42 µmol kg^{-1}. There are not nutrient data available for station 5 at 10 m, however [NO_3^- + NO_2^-] and [PO_4^{3-}] at 5 m were 0.16 µmol L^{-1} and 0.02 µmol L^{-1} respectively. At 25 m or in the first deeper sample available for stations 1 and 5, [NO_3^- + NO_2^-] and [PO_4^{3-}] were 0.57 µmol L^{-1} and 0.02 µmol L^{-1} respectively. Between 5 - 10 m depth, the average temperature was 17.41°C, PAR% = 31.85 and [CO_3^{2-}] = 211.33 µmol kg^{-1}; below 10 m, [NO_3^- + NO_2^-] = 0.47 µmol L^{-1}, [PO_4^{3-}] = 0.02 µmol L^{-1}, temperature = 14.54°C, PAR% = 2.12 and [CO_3^{2-}] = 204.01 µmol kg^{-1}. The first 25 m of stations 7, 9 and 11 had on average [NO_3^- + NO_2^-] = 0.35 µmol L^{-1}, [PO_4^{3-}] = 0.02 µmol L^{-1}, temperature = 18.74°C, PAR% = 31.58 and [CO_3^{2-}] = 233.85 µmol kg^{-1}; below 25 m, the average values were the following: [NO_3^- + NO_2^-] = 0.89 µmol L^{-1}, [PO_4^{3-}] = 0.04 µmol L^{-1}, temperature = 15.90°C, PAR% = 1.69 and [CO_3^{2-}] = 227.73 µmol kg^{-1}. In the SE Mediterranean, the HOLP layer occurred above the DCM (positive peak of fluorescence around 50 m; Fig. 3.6) and within the upper part of the thermocline (interval characterized by a particularly strong decline in temperature with depth). In the SW Mediterranean, the HOLP layer occurred again above the DCM, but nonetheless in correspondence of a superficial positive peak in fluorescence; also, within this depth interval were not observed strong variations in temperature.

In conclusion, there seems to be a consist relationship between the position of the HOLP layer, the depth of the DCM and the thermocline, but only in the oligotrophic SE Mediterranean.

Cell concentrations, for both the diploid and haploid phases, of the 7 selected species (*Calcidiscus leptoporus*, *Coccolithus pelagicus*, *Coronosphaera mediterranea*, *Syracosphaera bannockii*, *S. histrica*, *S. molischii* and *S. pulchra*) show distinct correlations with the environmental parameters (Table 3.2).

	T	Sal	NO_x	PO_4	pH	pCO_2	CO_3^{2-}	PAR%
C.leptoporus HOL	***0.25**	****0.31**	****-0.30**	-0.17	0.01	-0.11	****0.34**	0.28
C.leptoporus HET	-0.17	****-0.28**	****0.29**	****0.35**	-0.19	0.15	****-0.38**	-0.25
C.pel.subsp.braarudii HOL	0.01	****-0.39**	-0.10	0.03	-0.07	****-0.27**	-0.15	0.31
C.pel.subsp.braarudii HET	***-0.21**	-0.05	****0.25**	****0.26**	0.13	****0.26**	***-0.25**	-0.24
C.mediterranea HOL	0.02	-0.11	****-0.28**	-0.09	-0.23	***-0.23**	0.04	****0.45**
C.mediterranea HET	0.12	-0.13	-0.15	0.04	-0.15	-0.01	-0.01	0.28
S.bannockii HOL	0.19	***-0.25**	0.09	0.00	0.12	0.01	-0.05	****0.51**
S.bannockii HET	-0.09	-0.05	-0.08	-0.07	0.04	***-0.20**	0.02	-0.05
S.histrica HOL	0.03	-0.06	-0.05	-0.10	0.12	-0.14	0.05	0.16
S.histrica HET	0.11	0.20	***-0.22**	-0.14	0.10	-0.18	***0.23**	0.09
S.molischii HOL	0.12	****0.29**	****-0.32**	****-0.41**	****-0.32**	-0.17	****0.27**	0.20
S.molischii HET	****-0.31**	****-0.32**	***0.20**	****0.26**	-0.15	-0.03	****-0.36**	-0.07
S.pulchra HOL	****0.26**	0.19	****-0.57**	****-0.59**	-0.09	****-0.25**	****0.35**	****0.47**
S.pulchra HET	****0.26**	****0.31**	****-0.36**	****-0.32**	***0.23**	****-0.35**	****0.45**	0.09

Table 3.2 Environmental correlations of selected species, calculated for the absolute abundance of each phase. Significant Spearman correlation coefficients are in bold (N= 91; *$p \leq 0.05$, **$p \leq 0.01$). PAR% values were available only for the MedSeA cruise dataset (N= 37).

It is important to note that for a given species, the correlation with the environmental parameters exhibits opposite sign whether the haploid or the diploid phase is considered, with the exception of *S. pulchra*: its heterococcolithophore and holococcolithophore stages were similarly correlated with temperature, $[NO_3^- + NO_2^-]$, $[PO_4^{3-}]$, pCO_2 and $[CO_3^{2-}]$.

Among the outliers (Fig. 3.2), station 5 had the highest average HOLP index (89.13), while to stations 324 and 321 corresponded lower values (respectively 34.57 and 19.95). The gradual decrease in HOLP index of the stations in the outlier group stations, corresponds to gradual increases in the range of $[NO_3^- + NO_2^-]$, $[PO_4^{3-}]$ and slight decreases in $[O_2]$ and pH. In station 5, $[NO_3^- + NO_2^-]$ = 0.02 – 0.70 µmol L^{-1}, $[PO_4^{3-}]$ = 0 – 0.02 µmol L^{-1}, $[O_2]$ = 236.60 – 249.07 µmol kg^{-1}, pH = 8.09 – 8.14; in station 324, $[NO_3^- + NO_2^-]$ = 0 – 2.00 µmol L^{-1}, $[PO_4^{3-}]$ = 0 – 0.10 µmol L^{-1}, $[O_2]$ = 216.00 – 234.50 µmol kg^{-1}, pH = 7.90 – 8.00; in station 321, $[NO_3^- + NO_2^-]$ = 0.10 – 5.20 µmol L^{-1}, $[PO_4^{3-}]$ = 0 – 0.20 µmol L^{-1}, $[O_2]$ = 180.00 – 238.50 µmol kg^{-1}, pH = 7.90 – 8.00.

3.4 Discussion

Holococcolithophores are particularly abundant in the Mediterranean Sea, more than in adjacent regions like the Atlantic or the Red Sea (Kleijne, 1991), especially in summer (Cros and Estrada, 2013; Dimiza et al., 2008b; Malinverno et al., 2009; Šupraha et al., 2016). Our results from a Mediterranean longitudinal transect, characterized by strong biogeochemical differences (in nutrients, temperature, carbonate system), showed that

they reached maximum density always at shallower depths than the heterococcolithophores (Fig. 3.3, 3.6) and had a wider vertical distribution in the SE Mediterranean. Holococcolithophores' preferential distribution in shallow waters has been previously noticed in the Mediterranean (Cros et al., 2000; Cros and Estrada, 2013; Dimiza et al., 2004, 2015; Malinverno et al., 2009; Oviedo et al., 2015; Triantaphyllou et al., 2002), and might be justified by an affinity for nutrient-poor, high-light conditions. In fact, haploid cell replication involves lower energetic costs than that of diploid cells (Lewis, 1985); the former also tend to be smaller, meaning higher surface-volume ratios and enhanced nutrient absorption (Karl et al., 2001); in the case of *E. huxleyi*, it has been demonstrated that the haploid phase is more resistant to phosphorus limitation (Rokitta et al., 2016), a condition which is accentuated in the upper photic zone of the Mediterranean Sea, particularly in the SE basin (Fig. S2). Due to their ultra-oligotrophic character, the waters of the photic zone in the SE basin

tend to be more transparent than those in the SW Mediterranean, allowing a deeper light penetration and the formation of a DCM (see fluorescence and 0.1% PAR limit in Fig. S12). Holococcolithophores might have an advantage over heterococcolithophores there, being better adapted to high light intensities: the crystalline structure of some holococcoliths has been proven to be extremely effective in backscattering harmful UV radiations (Quintero-Torres et al., 2006). Holococcolithophores' preference for the upper photic zone is supported also by the distributions of *C. pelagicus* subsp. *braarudii* and *C. leptoporus*. The holococcospheres and heterococcospheres of these species had different vertical distributions, reflecting the general trends we just discussed (Fig. 3.4). However, while *C. pelagicus* subsp. *braarudii* was found almost exclusively in the SW basin, together with the diploid form of *C. leptoporus*, the haploid form of *C. leptoporus* was prevalently found in the SE Mediterranean. Under controlled culture conditions, both *C. pelagicus* subsp. *braarudii* HOL and *C. leptoporus* HOL grow faster than their diploid counterparts under nutrient depletion (Houdan et al., 2006), while *C. pelagicus* subsp. *braarudii* HET and *C. leptoporus* HET are considered high-productivity indicators, typical of temperate zones (Cachão and Moita, 2000; Renaud et al., 2002; Renaud and Klaas, 2001; Saez et al., 2003). Thus, the longitudinal extension of these species and their life phases might reflect the negative W-E gradient in $[NO_3^-+NO_2^-]$ and $[PO_4^{3-}]$ (Fig. S2): *C. pelagicus* subsp. *braarudii* and *C. leptoporus* seem to cope better with the Mediterranean environment when in their haploid forms; also, our data suggest that, among them, only the haploid phase of *C. leptoporus* might be able to reach significant concentrations in the SE basin. The idea that the two coccolithophore life phases might exploit separate ecological niches (Coelho et al., 2007; Cros and Estrada, 2013; Oviedo et al., 2015;

Šupraha et al., 2016) is supported by the distributions we observed along the transect (Fig. 3.3, 3.4), as well as by their distinct environmental correlations (Table 3.2). The environmental correlations relative to the abundance of the haploid stage agreed in sign among species, but varied in intensity, perhaps due to slightly different ecological requirements (Cros, 2001). In this work, the HOLP index was used (Cros and Estrada, 2013; Šupraha, 2016; Šupraha et al., 2016) to understand the triggers of coccolithophore life phase change. We found that the HOLP index increased eastward (Fig. 3.5); moreover, the absolute holococcolithophore abundance and the HOLP index were tested against the same set of environmental variables and showed similar correlations (Table 3.1), although the only significant carbonate system parameter to which the HOLP index was correlated was carbonate ion concentration. The environmental correlations of the HOLP index reflect natural longitudinal gradients that co-vary and therefore it is difficult to disentangle the real triggers of phase change solely from our data. An haplo-diploid life cycle is considered to be advantageous in a temporally and spatially variable environment (Hughes and Otto, 1999; Nöel et al., 2004; Stebbins and Hill, 1980; Valero et al., 1992). Also, genomic evidence suggests that some strains of *E. huxleyi* adapted to low environmental variability and low biotic pressure may have totally abandoned this life strategy (von Dassow et al., 2015). Although such observations have been done solely on *E. huxleyi* (Noelaerhabdaceae), other coccolithophore species (Isochrysidales) may adapt to similar environmental conditions might have lost their ability to change phase.

Life phase transformations are likely triggered by exogenous factors (environmental stimuli), but endogenous mechanisms might avoid this energetically costly process for short-termed environmental changes (Houdan et al., 2004). The Mediterranean Sea exhibits seasonal changes, strong biogeochemical gradients and mesoscale structures like gyres and eddies (Bergamasco and Malanotte-Rizzoli, 2010; Millot, 1987, 1991; Shaltout and Omstedt, 2014), and can therefore be considered a variable environment.

Stations 5, 321 and 324 of the 2013 MedSeA and M84/3 cruises could not be assigned to either the SW nor to the SE Mediterranean based on their average HOLP index (Fig. 3.2), corresponding perhaps to areas of marked environmental instability. Extremely high HOLP index values (100) were registered at 5 and 10 m depth in the MedSeA 2013 cruise station 5. This station stood out for its low superficial nutrient concentrations (phosphate and nitrate plus nitrite), lower salinity, higher dissolved oxygen, higher [CO_3^{2-}] and higher pH than other stations of the MedSeA (Fig. S1, S2, S3). When compared with the other two outlier stations (324 and 324), the most striking environmental differences consisted again in lower nutrient concentrations and in slightly higher dissolved oxygen and pH. The maximum fluorescence was found deeper in station 5 than in adjacent stations of the

same cruise. Such environmental conditions suggest that the water column at station 5 during the sampling was likely stratified, perhaps due to the persistence of an anticyclonic eddy in this area, slightly detectable in the satellite derived Chl *a* concentration data (Fig. 3.1), although no similar structures were recognizable from altimetric data. In the core of an anticyclonic eddy, water stratification can take place creating negative anomalies in superficial nutrient concentrations and a DCM (Brenner et al., 1991; Krom et al., 1992). This water mass stays relatively isolated from its surroundings, and can serve as a distinct ecological niche (Bracco et al., 2000; Margalef, 1978), regulating the local coccolithophore distribution (Cokacar et al., 2004; Garcia-Soto et al., 1995; Read et al., 2007; Vaillancourt et al., 2003). The positive HOLP index anomaly in station 5 was due to a monospecific bloom of *S. bannockii* HOL (= 69 - 73% of the total assemblage). Both life phases have been identified in North, South Atlantic (Balestra et al., 2004; Boeckel and Baumann, 2008; Charalampopoulou et al., 2011; Daniels et al., 2014a; Poulton et al., 2010) and Mediterranean (Cros et al., 2000; Geisen et al., 2002; Malinverno, 2003; Oviedo et al., 2015; Šupraha et al., 2016) living assemblages. A relative abundance of *S. bannockii* similar to that in station 5, was registered previously only in the Bay of Biscay, during April 2010 (Daniels et al., 2014a), but in that case it was represented by a mix of the two phases, with a dominance of heterococcolithophores. During the MedSeA cruise (station 5), the diploid phase was completely absent, and it was very rare in the closest Meteor M84/3 cruise samples (station 324), occurring only at 25 m with a concentration of 678 cells L^{-1}. The presence of both life phases during the bloom of *S. bannockii* in the Bay of Biscay, was interpreted either as a signal of similar ecologies for the two phases, or as an ongoing adaptation of cells to lower nutrient conditions (Houdan et al., 2006). Based on our observations for the MedSeA and Meteor M84/3 cruises, we suggest that the two life phases of this species should have different ecologies, and that probably the contemporary observation of *S. bannockii* HET and HOL in the Bay of Biscay was conducted during the adaptation of this species to a seasonal environmental change.

A high absolute abundance of *S. bannockii* HOL has been previously reported in summer in the Mediterranean, positively correlated with temperature and negatively with nutrient concentration (Šupraha et al., 2016). During our samplings in 2011 and 2013, *S. bannockii* did not show any strong correlations with these same variables (Table 3.2), likely due to its low occurrence along the remaining transect; however, there was a strong negative anomaly in nutrient concentration in station 5. Adding to the absence of any evident temperature anomaly between this location and the adjacent stations (Fig. S1), we think that the bloom of *S. bannockii* HOL was mainly triggered by nutrient limitation. Although there is no apparent influence of temperature on the *S. bannockii* HOL bloom we

observed, this parameter might still influence the main vertical and longitudinal trends in abundance (Cros and Estrada, 2013; Dimiza et al., 2008; Oviedo et al. 2015, 2016; Šupraha et al., 2016) and affect the HOLP index. Another possible factor explaining the dominance of *S. bannockii* HOL in station 5 might be the stable conditions (low turbulence) of the water column in this area. Experimental evidence indicates in fact that the haploid phases of coccolithophores tend to be inhibited by turbulence (Houdan, 2003; Houdan et al., 2005, 2006). The carbonate system also helps to explain both the general HOLP index trends and the anomaly in station 5. Carbonate ion (CO_3^{2-}) concentration was the only carbonate system parameter to show significant correlations with the HOLP index (Table 3.1). It tended to increase eastward and upward in the water column; it was also visibly higher in station 5 than in adjacent stations of the SW Mediterranean, but lower than in the SE basin (Fig. S3), indicating that, likely, it has a secondary effect. The interaction between the coccolithophore life cycle and the carbonate system has been scarcely studied until present. Coccolithophores produce coccoliths made of calcium carbonate in an intracellular vesicle through mechanisms which are still not fully understood (Taylor et al., 2017). Holococcolithophores could have been negatively affected by low carbonate saturation levels during the Paleocene Eocene Thermal Maximum (PETM), but only when coupled with exceedingly high, non-optimal temperatures, which could have increased the energetic cost of extra-cellular calcification (Gibbs et al., 2015). Likely, the cell need to absorb both CO_3^{2-} and HCO_3^- to calcify in seawater, at pH > 7 (Ziveri et al., 2012). A connection between holococcolithophore distribution and the carbonate system was suspected earlier (Oviedo et al., 2015), but it was difficult to differentiate the role of the different components; on the other hand, based on the preference of some heterococcolithophore species for the Eastern Mediterranean, an enhanced ability to utilize CO_3^{2-} for calcification in respect to Western Mediterranean species was hypothesized (Oviedo et al., 2015). Culture experiments have additionally demonstrated species-specific and strain-specific effects of the carbonate system on coccolithophore calcification (Fiorini et al., 2011a, 2011b; Langer et al., 2006, 2009), dependant on the collection site of the strains (Rickaby et al., 2016). Calcification itself is a highly energy consuming process (Monteiro et al., 2016), and its function can change between holococcolithophores and heterococcolithophores, which seem to inhabit very different nutrient regimes. It is thus plausible that the haploid phase of coccolithophores might be more efficient in calcifying in high [CO_3^{2-}] conditions, such as those of the upper photic zone of the SE Mediterranean, rather than the diploid phase. Light sensibility seems to explain the vertical distribution of holococcolithophores, but it does not seem to be the best explanation for the longitudinal differences in HOLP index along the 2013

MedSeA cruise transect (the only cruise for which PAR% values were available, Fig. 3.6): the average PAR% in the first 100 m was very similar for the two main basins. The same was probably valid for the Meteor M/84 cruise. Finally, it is worth noticing that some environmental and biological dynamics which were not measured during the cruises might also have an important role, such as the rate and quality of viral infection (Frada et al., 2008, 2012): haploid cells of *E. huxleyi* can appear in post-bloom conditions, after heterococcolithophores have been decimated by virus infections. Overall, this study contributes to the understanding of the coccolithophore haploid and diploid distribution patterns in a changing ocean. Ocean warming, associated to surface water column stratification and nutrient limitation, might further stimulate the haploid phase over the diploid phase, raising the relative abundance of holococcolithophores in the Mediterranean Sea. The frequency of diploid-haploid transformations could increase in a warming scenario, constituting a survival strategy for species whose diploid phases are adapted to cooler and nutrient-richer conditions; however, ocean acidification and the associated decrease in seawater carbonate ion concentration might negatively affect holococcolithophores. More studies are needed to confirm the potential influence of the carbonate system on the coccolithophore life cycle.

3.5 Conclusions

Coccolithophores' haploid and diploid natural life cycle is a successful ecological strategy in the Mediterranean Sea, probably crucial for the eastward propagation of species whose diploid forms are adapted to North Atlantic or SW Mediterranean conditions (e.g. *Calcidiscus leptoporus*, *Coccolithus pelagicus* subsp. *braarudii*). Holococcolithophores tend to concentrate in the upper photic zone (first 10 – 25 m) and usually constitute a minority of the total population, but their number can locally increase and surpass that of heteroccolithophores, even within the SW basin. The distribution of total holococcolithophores and their prevalence index (HOLP) suggest an adaptation of this life phase to oligotrophic, warm waters, rich in carbonate ions; light intensity is also a potential factor explaining their superficial distribution. The unusually high HOLP indices (≈100) registered in the upper 10 m of one SW Mediterranean station, caused by a mono-specific bloom of *S. bannockii* HOL, indicate that nutrient-limitation might be sufficient to trigger a diploid-haploid transformation, at least in this species.

Based on the IPCC projections (IPCC, 2007; Pachauri et al., 2014), by the year 2100 the Mediterranean Sea may experience a pH reduction of 0.245 – 0.462 in the Western Basin and of 0.242 – 0.457 in the Eastern Basin (Goyet et al., 2016), while regionally averaged temperatures could increase by 6°C (Sakalli, 2017). These changes are expected to

cause shifts in the carbonate system, including a drop in carbonate ion concentration, and a decrease in superficial nutrient concentrations (Gruber, 2011). Overall, a haplo-diploid life cycle might support the survival of coccolithophore species under warming and increasingly water-stratified conditions; however, the influence of the carbonate system on their life cycle should be clarified to make realistic projections.

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Data Archiving

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CHAPTER 4

Hetero- and holococcolithophore diversity patterns in the Mediterranean Sea

Abstract

Coccolithophores are highly diverse in the Mediterranean Sea. Their total species diversity has been linked in the past to an array of environmental parameters; however, relatively little is known of the patterns and environmental controls. In particular, no detailed information is available on the two calcified life stages, heterococcolithophore (diploid) and holococcolithophores (haploid). The present study demonstrates that the diversity of the two life cycles follows distinct patterns.

When the upper 100 m of the photic zone are considered, heterococcolithophore species diversity tends to be higher in the Western Mediterranean, while that of holococcolithophore tends to be higher in the Eastern Mediterranean. Data considered in this study were collected analysing samples from two research cruises (Meteor M84/3; MedSeA), which took place during the spring season. The stations of these two cruises encompass a Mediterranean-wide longitudinal transect, extending from the west of the Strait of Gibraltar (- 6.64 °E) to the Levantine Sea (35.17 °E). We calculated the total coccolithophore, heterococcolithophore and holococcolithophore diversity indexes for all stations, considering also the vertical distribution. The diversity patterns and main environmental controls were considered for the Western and Eastern Mediterranean basins. Our results indicate that presently heterococcolithophores drive the total coccolithophore diversity in the Mediterranean Sea, although holococcolithophore diversity is high in the Eastern Mediterranean, and occasionally exceeds that of heterococcolithophores. Heterococcolithophore and holococcolithophore diversities showed overall opposite environmental correlations and their variability seems strictly related to their ecological preferences. Climate change, through oceanic warming and acidification, might increase the number of ecological niches for holococcolithophores in the Mediterranean Sea, and therefore their relative diversity in respect to the diploid life stage.

4.1 Introduction

The calcifying algae known as coccolithophores are a major phytoplankton group, whose representatives can be found worldwide in the oceans, between equatorial and sub-polar latitudes (Brown, 1995; McIntyre and Bé, 1967; Winter and Siesser, 1994). These

organisms have a central role in the carbon cycle, participating to both the organic carbon pump and the calcium carbonate pump (Iglesias-Rodriguez et al., 2002; Rost and Riebesell, 2004). Their contribution to the carbon cycle depends not only on the total cell abundance, but also on the species assemblage, which can include a wide range of cell sizes producing variable amounts of calcite (Young and Ziveri, 2000). Shifts in the relative abundance of species are expected to influence the PIC : POC 'rain ratio', and therefore the exchange of CO_2 between atmosphere and ocean (Baumann et al., 2004; Daniels et al., 2014b; Rost and Riebesell, 2004; Ziveri et al., 2007). At present, about 280 living coccolithophore species have been defined based on the morphology of their calcitic exoskeleton (the coccospheres; Young et al., 2003), although the total number of living coccolithophore species is estimated to be between 200 and 500 (Young et al., 2005a). Total coccolithophore diversity tends to be higher in sub-tropical and equatorial waters rather than at high latitudes or close to coasts (Honjo and Okada, 1974; O'Brien et al., 2016; Poulton et al., 2017; Winter and Siesser, 1994); these trends have been related to irradiance, temperature, surface water stratification, nutrient concentrations and total chlorophyll, showing also strong seasonal variability at subtropical latitudes (Kahn and Aubry, 2012; O'Brien et al., 2016; Okada and Honjo, 1973; Thierstein et al., 2004; Winter et al., 1979).

In the Mediterranean Sea, coccolithophores are highly diverse (Cros, 2001; Kleijne, 1991, 1993; Knappertsbusch, 1993; Oviedo et al., 2015), especially during summer (Dimiza et al., 2008b; Knappertsbusch, 1993; Malinverno et al., 2009; Šupraha et al., 2016). Additionally, the two life phases of the coccolithophore, the heterococcolithophore (HET) corresponding to the diploid phase and the holococcolithophore corresponding (HOL) to the haploid phase, present dissimilarities in their distribution at basin-scale (Oviedo et al., 2015; D'Amario et al., 2017, under review). In a context of changing climate, we do not know yet how warming and/or ocean acidification might affect coccolithophore diversity in the Mediterranean Sea, as the question may be complicated by the distinct diversity patterns observed for the HET and HOL (Oviedo et al., 2015). Starting from phytoplankton data collected along the same longitudinal Mediterranean transect in April 2011 and May 2013, we discuss the relative contribution of HET and HOL to the total coccolithophore diversity and their distinct distributions by taking into account the natural environmental correlations along the transect. Finally, we speculate about the effects of warming and ocean acidification on the diversity of coccolithophores, including both the haploid and diploid phases, in the Mediterranean Sea.

4.1.1 Oceanographic settings

The Mediterranean Sea is a semi-enclosed sea, located in a transitional climate zone, between sub-tropical and temperate areas. It is usually divided into two main provinces: the Western Basin and the Eastern Basin, separated by the Strait of Sicily (sill at about 400 m depth). The Western Basin is directly connected to the Atlantic Ocean through the Strait of Gibraltar (sill at about 350 m depth), allowing the inflow of relatively fresh and eutrophic waters and the initiation of a thermohaline, anti-estuarine circulation. This large scale circulation is complicated by the geography and bathymetry of the Mediterranean Sea, which includes numerous sub-basins and extensive coastal zones, leading to the occurrence of sub-currents and mesoscale structures (Pinardi et al., 2015; Rohling et al., 2009; Tanhua et al., 2013b).

Strong longitudinal environmental gradients were repeatedly observed between the western and the eastern areas of the Mediterranean Sea (Crombet et al., 2011; Ribera d'Alcalà, 2003; Skliris, 2014; Tanhua et al., 2013b) and confirmed during both the MedSeA and Meteor M84/3 cruises (Goyet et al., 2015; Tanhua et al., 2013a), which provided the data of the present work. The large-scale longitudinal gradients consist in eastward increases of sea surface temperature, salinity, dissolved oxygen (Fig. S1), carbonate ion concentration, pH and alkalinity (Fig. S3). Parallel decreases in nutrients (nitrate, nitrite, phosphate; Fig. S2) were accompanied, at least during the MedSeA cruise (no correspondent data available for the Meteor M84/3 cruise), by a deepening of both the Deep Chlorophyll Maximum (DCM) and the 0.1 % limit of Photosynthetic Available Radiation (PAR; Fig. S12).

4.2 Material and methods

The phytoplankton samples included in the present study were collected during the Meteor M84/3 (6 – 28 April 2011) and the MedSeA (1 – 31 May 2013) cruises, extending longitudinally between 6.64 °E and 35.17°W (Fig. 4.1).

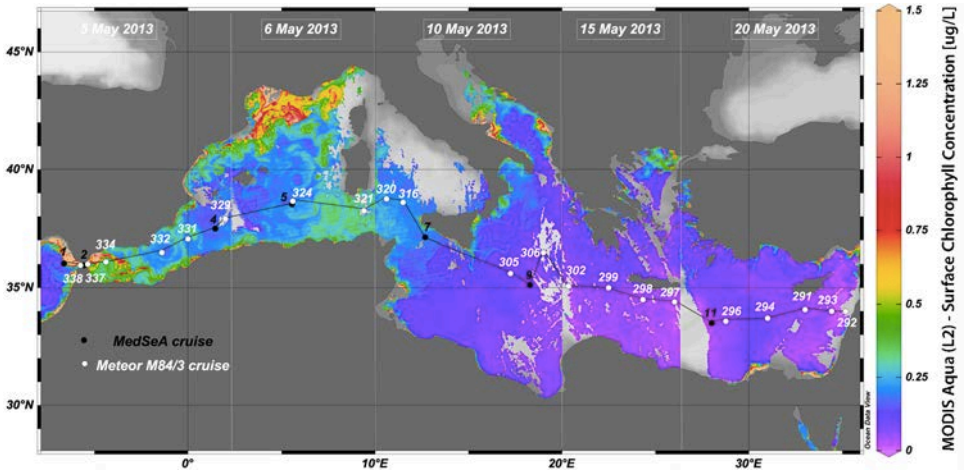

Fig. 4.1 Analysed stations, superimposed on the Surface Chlorophyll Concentration measured in May 2013 during the MedSeA cruise (D'Amario et al., 2017 under review).

Temperature, salinity, fluorescence and dissolved oxygen concentration data were recorded in both cases using a profiling CTD (Sea-Bird Electronics 991): 2013 MedSeA CTD data can be retrieved from PANGAEA (Ziveri and Grelaud, 2015), while 2011 M84/3 cruise CTD data can be downloaded at the following webpage http://cchdo.ucsd.edu/cruise/06MT20110405.

Nutrient concentrations for the 2013 MedSeA cruise were obtained on-board by water filtration through glass fibre filters (Whatman GF/F; 0.7 µm), storage at - 20°C, and measurements through Bran + Luebbe3 AutoAnalyzer, following the method of Grasshoff et al. (1999). Detection limits corresponded to 0.02 µM for nitrate plus nitrite (NO_3^-+NO_2^-), and to 0.01 µM for phosphate (PO_4^{3-}); measurement quality was confirmed by inter-comparison exercises (QUASIMEME). Directly measured total alkalinity and dissolved inorganic carbon are available in PANGAEA (Goyet et al., 2015); other carbonate system variables were derived for the first two using CO2Sys (Lewis and Wallace, 1998), refitting the equilibrium constants (Álvarez et al., 2014; Dickson and Millero, 1987; Mehrbach et al., 1973). Nutrients concentrations and carbonate system parameters for the 2011 Meteor M84/3 cruise have been already described (Oviedo et al., 2015; Rahav et al., 2013; Tanhua et al., 2013a); also bottle data can be downloaded from http://cdiac.ornl.gov/oceans/Coastal/Meteor_Med_Sea.html.

Long. (°E)	Lat. (°N)	Day-month-year	Station	Cruise	Depth (m)												
					0	5	10	25	40	50	75	80	100	110	125	150	175
-6.64	36.03	2-May-13	1	MedSeA		x	x	x		x							
-5.75	35.95	25-Apr-11	338	M84/3		x											
-5.56	36.12	3-May-13	2	MedSeA		x	x			x	x						
-5.36	36.00	25-Apr-11	337	M84/3	x	x		x		x			x				
-4.40	36.10	24-Apr-11	334	M84/3		x		x		x			x				
-1.40	36.50	24-Apr-11	332	M84/3		x		x									
0.00	37.05	23-Apr-11	331	M84/3	x	x		x		x			x				
1.45	37.49	7-May-13	4	MedSeA		x	x	x	x		x		x				
2.00	37.90	23-Apr-11	329	M84/3	x	x		x		x			x				
5.55	38.52	8-May-13	5	MedSeA		x	x	x		x	x	x			x		
5.60	38.65	21-Apr-11	324	M84/3	x		x	x		x			x				
9.40	38.25	20-Apr-11	321	M84/3		x		x		x			x				
10.61	38.75	20-Apr-11	320	M84/3		x							x				
11.50	38.60	19-Apr-11	316	M84/3		x		x									
12.68	37.12	11-May-13	7	MedSeA		x	x	x		x	x		x				
17.25	35.60	14-Apr-11	305	M84/3		x		x									
18.29	35.11	12-May-13	9	MedSeA		x	x	x		x	x			x	x	x	x
19.00	36.50	15-Apr-11	306	M84/3		x		x		x							
20.35	35.07	13-Apr-11	302	M84/3	x	x		x		x			x				
22.50	35.00	12-Apr-11	299	M84/3		x											
24.33	34.50	12-Apr-11	298	M84/3		x		x									
26.02	34.40	11-Apr-11	297	M84/3		x		x					x				
28.00	33.50	15-May-13	11	MedSeA		x	x	x		x	x		x		x	x	
28.77	33.58	11-Apr-11	296	M84/3				x									
31.00	33.70	10-Apr-11	294	M84/3	x	x		x		x			x				
33.00	34.07	8-Apr-11	291	M84/3		x		x		x			x				
34.42	34.00	9-Apr-11	293	M84/3		x											
35.17	33.99	9-Apr-11	292	M84/3		x											

Table 4.1 On the left: details concerning the stations analysed in this work; on the right: sample depths analysed for coccolithophore abundance and diversity.

Coccolithophores data have been collected from a total of 107 samples (Table S5). Seawater was first collected in Niskin bottles at depth ranging between 0 and 175 m, transferred in sterile plastic containers, and 2.5 to 5 L per depth were filtered on cellulose acetate-nitrate filters (Millipore, Ø 47 mm, 0.45 µm) using a hydraulic vacuum pump (Eyela, A-1000S); filters were finally dried in the oven at 40°C for 8 - 12 hours. For each sample, a radial portion of the filter was mounted on a stub, coated with gold-platinum and observed at a magnification of 3000 X using a Zeiss EVO MA 10 scanning electron microscope (SEM). Coccosphere counting was done along a transect of at least 5 mm, corresponding on average to 4.1 mL of filtered water, and reaching a minimum of 80 – 100 coccospheres (420 maximum). Coccospheres were differentiated between HET and HOL and identified at species level according to the literature (Cros and Fortuño, 2002; Young et al., 2003) and the Nannotax3 website (http://ina.tmsoc.org/Nannotax3/). The species list for HET and HOL are in Tables S7 and S8. All coccolithophore abundances have been previously published in PANGAEA (D'Amario et al., 2017; Oviedo et al., 2017). PAST 3.14 (Hammer et al., 2001) was used for all statistical analyses and to calculate coccolithophore diversities in each sample. Coccolithophore diversity was based on the

proportion of species and expressed as Shannon index (H'); separate calculations were done to express HET diversity, HOL diversity and the total coccolithophore diversity (HET + HOL). Notice that if when a species was found in both its diploid and haploid life phases, its heterococcolithophore and holococcolithophore abundances were kept separated and treated separately in the calculation of the total coccolithophore, heterococcolithophore and holococcolithophore diversity indexes (H').

A hierarchical cluster analysis constrained by longitude and based on the environmental parameters was performed on the stations of the two cruises. To this aim, the environmental parameters (temperature, salinity, $[NO_2^-]$, $[NO_3^-]$, $[PO_4^{3-}]$, pH, pCO_2 and $[CO_3^{2-}]$) were averaged between 5 and 100 m for all stations. Also, the average HET and HOL diversity between 5 and 100 m was calculated for all stations in which coccolithophore data for this depth interval were available (18 out of 28), and plotted to highlight the longitudinal gradients of the two indexes.

A set of three Principal Component Analyses (PCA) were performed on total coccolithophore diversity, HET diversity, HOL diversity and on the environmental parameters previously used in the cluster analysis: PCA 1 included all samples from both cruises, PCA 2 included only the samples from Cluster A, and PCA 3 included only the samples from Cluster B.

4.3 Results

Retrieved from the two cruises, a total of 122 coccolithophore taxa were identified, including 74 HET and 48 HOL. Generally, *Emiliania huxleyi* dominated the total coccolithophore population (average = 48 ± 19 %). The majority of HET (52 ± 17 %) were represented by *Emiliania huxleyi*; the remaining 48 ± 17 % of the HET assemblage was represented by 73 species, whose individual contributions never exceeded 6 ± 10 %. The contribution of taxa to the HOL assemblage was more uniform: on average, *Corisphaera gracilis* (12 ± 23 %) had the highest relative abundance, followed by *Syracosphaera arethusae* HOL (9 ± 15 %), *S. pulchra* HOL oblonga type (9 ± 16 %), *S. molischii* HOL (9 ± 16 %), S*. bannockii* HOL (8 ± 21 %), *S. histrica* HOL (7 ± 17 %), *Helladosphaera cornifera* (6 ± 14 %); other HOL taxa had on average relative abundances ≤ 5%.

The total coccolithophore, HET and HOL diversity all showed a particularly patchy distribution along the transect (Fig. 4.2, 4.3). Total coccolithophore diversity oscillated between 0.30 in station 4 (25 m depth) and 2.74 in station 324 (0 m); HET diversity varied between 0.30 in station 4 (25 m) and 2.34 in station 337 (25 m); HOL diversity varied between 0 (samples where HOL were actually absent) and 2.53 in station 302 (50 m). Total coccolithophore and HET diversities were generally higher in the upper 100 m of the

water column, and rapidly decreased below 25 m in the SE Mediterranean basin. In the case of HOL diversity, high values concentrated within the upper 25 m in the western portion of the transect (with the only exception of station 331), while in the eastern portion the highest values were reached between 50 and 75 m depth.

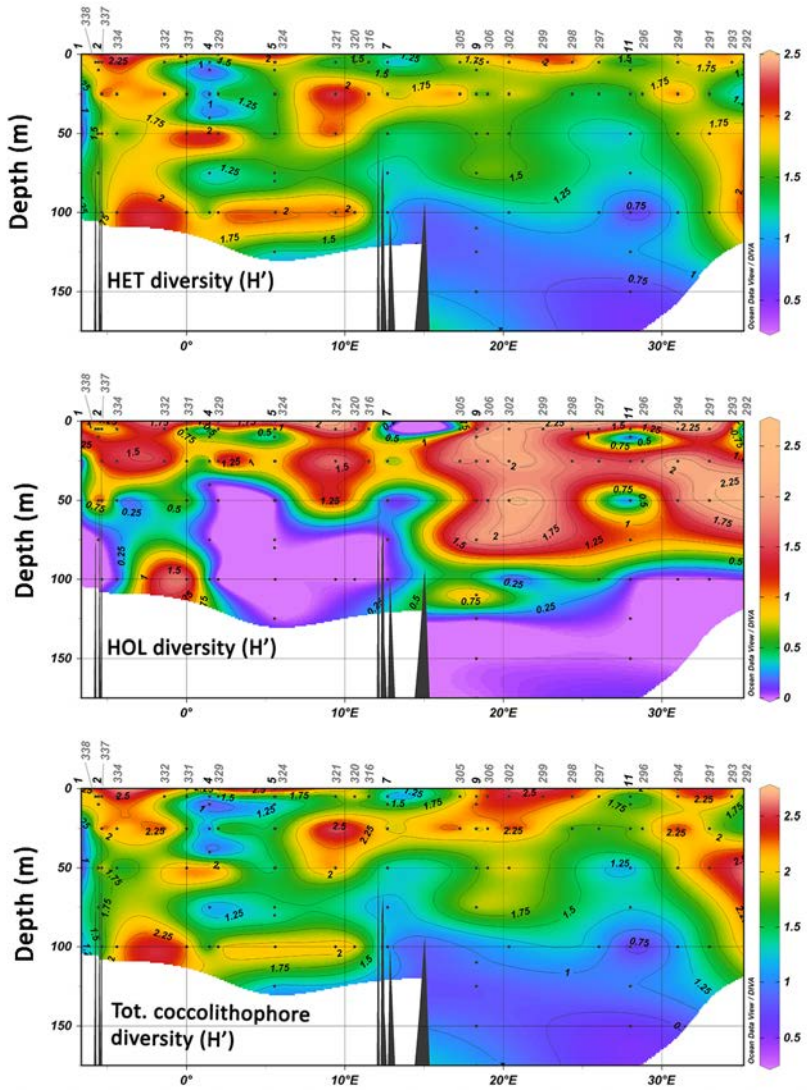

Fig. 4.2 MedSeA and M84/3 data combined: total coccolithophore, heterococcolithophore (HET) and holococcolithophore (HOL) diversity.

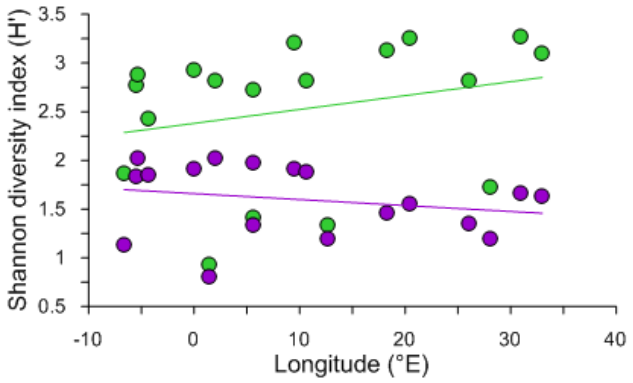

Fig. 4.3 Average Shannon diversity index (H') for the upper 100 m of the water column in each station, plotted again longitude. HOL diversity values and corresponding linear trend in green; HET diversity and corresponding linear trend in violet.

Two clusters were obtained from the hierarchical cluster analysis (Fig. 4.4): Cluster A (SW Mediterranean), included the stations located between -6.64 to 12.68° E; Cluster B (SE Mediterranean), included the stations located between 17.25 and 35.17° E. The average HET and HOL diversity plotted against longitude (Fig. 4.3) showed opposite trends for the two calcified life phases: HET diversity tends to slightly decrease eastward, while HOL diversity tends to increase. Moreover, the relationship between HET and HOL diversity is different in the stations included in Cluster A (SW Mediterranean) and those included in Cluster B (SE Mediterranean): while the general trend for the 2 groups is quite similar along the W-E transect, the diversities of HOL are always lower than those of the HET in the SW Mediterranean, while in the SE Mediterranean the HOL diversity reaches values close to, or even higher, than those of HET.

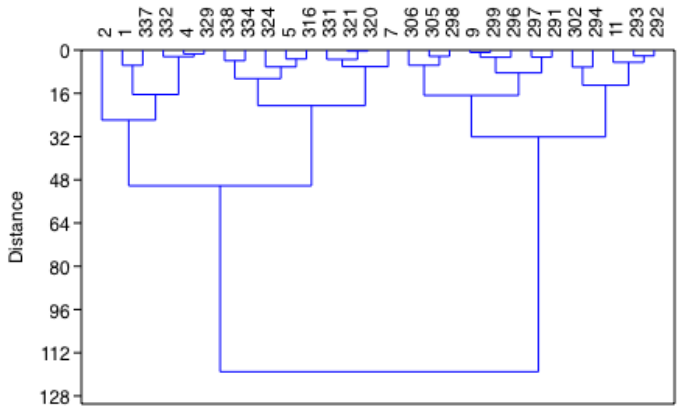

Fig. 4.4 Cluster analysis dendograms, based on the average HOL diversity between 5 and 100 m depth. Two clusters were identified, having significantly different ($p \leq 0.05$) averages.

The three PCA helped to visualize the total coccolithophore, HET and HOL diversity patterns with respect to the main environmental parameters. The 1st PCA conducted on the whole Mediterranean transect, (Fig. 4.5, Table 4.2) revealed that more than 99 % of the variance is explained by 2 factors. The first component (PC1) explains 74.41 % of the variance; it is characterized by positive loadings of [CO_3^{2-}], temperature, salinity, pH, and by negative loadings of pCO_2, [PO_4^{3-}] and [NO_2^-+NO_3^-]. The 2nd component (PC2) explains 25.12 % of the variance; it is characterized by positive loadings of salinity, pCO_2, [CO_3^{2-}], temperature, [NO_2^-+NO_3^-], [PO_4^{3-}], and the negative loading of pH. Taking into account the data presented in Fig. 4.5 and Table 4.2, the PC1 is mainly related to pCO_2, [CO_3^{2-}], [PO_4^{3-}] and [NO_2^-+NO_3^-], while the PC2 is mainly related to salinity, pCO_2, [CO_3^{2-}]. HET diversity was negatively correlated to both PC 1 and PC 2, while HOL diversity was positively correlated to both PC 1 and PC 2; no correlations were found for the total coccolithophore diversity (correlation coefficient in relation to PC 1 and PC 2 = 0).

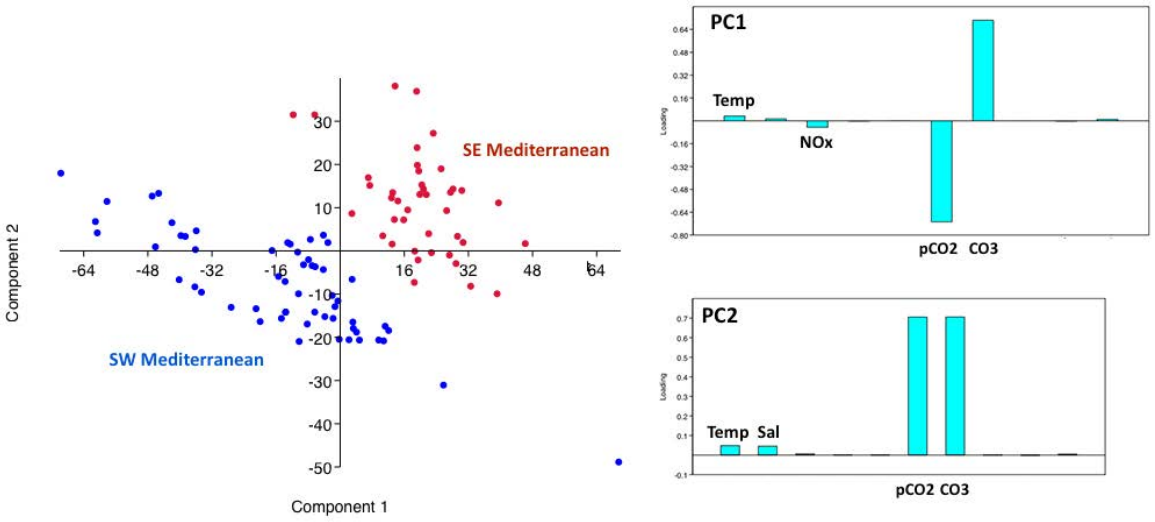

Fig. 4.5 Plot of the 1st PCA. SW and SE Mediterranean data points are respectively represented in blue and red. On the right, loading plots for Components 1 and 2.

PC	Eigenvalue	% variance		PC 1	PC 2
1	669.55	74.41	H' Tot. Coccos	0.52	0.44
2	225.97	25.12	H' HET	0.37	0.73
			H' HOL	-0.73	0.06
			Temp	-0.78	0.03
			Sal	0.29	-0.08
			NO2+NO3	-0.87	0.50
			PO4	0.86	0.50
			pH	0.00	0.00
			pCO2	-0.13	-0.07
			CO3	0.33	0.08

Table 4.2 PCA 1 results.

The 2nd PCA (Fig. 4.6, Table 4.3), conducted on the samples of the SW Mediterranean, revealed that more than 93 % of the variance is explained by only one factor. PC 1 explains 93.57 % of the variance; it is characterized by positive loadings of pCO_2, [NO_2^- +NO_3^-], salinity, and by negative loadings of [CO_3^{2-}] and temperature. Taking into account the data presented in presented in Fig. 4.6 and Table 4.3, the PC1 is related mainly to pCO_2 and [CO_3^{2-}]. HOL diversity was negatively correlated with PC1, while the HET and total coccolithophore diversity did not show any correlation (correlation coefficients = 0).

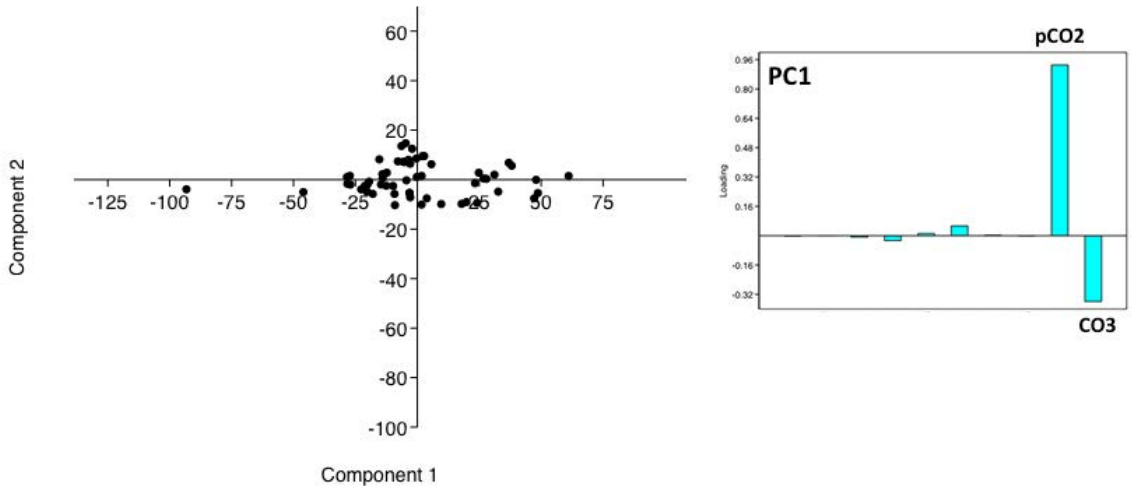

Fig. 4.6 Plot of the 2nd PCA. On the right, loading plots for Component 1.

PC	Eigenvalue	% variance		PC 1	PC 2
1	672.21	93.57	H' Tot. Coccos	0.00	-0.02
2	41.44	5.77	H' HET	0.00	-0.02
			H' HOL	-0.01	0.00
			Temp	-0.03	0.06
			Sal	0.01	0.05
			NO2+NO3	0.05	-0.06
			PO4	0.00	0.00
			pH	0.00	0.00
			pCO2	0.93	0.36
			CO3	-0.36	0.93

Table 4.3 PCA 2 results.

Finally, the 3rd PCA (Fig. 4.7, Table 4.4), conducted on the samples of the SE Mediterranean, more than 99 % of the variance is explained by 2 factors. The first component (PC1) explains 73.21 % of the variance; it is characterized by positive loadings of pCO_2, temperature, $[CO_3^{2-}]$, and no negative loadings. The 2nd component (PC2) explains 26.14 % of the variance; it is characterized by positive loadings of $[CO_3^{2-}]$, temperature, salinity, and by negative loadings of pCO_2. Taking into account the data presented in presented in Fig. 4.7 and Table 4.4, the PC1 may be mainly related to pCO_2, while the PC2 may be mainly related to $[CO_3^{2-}]$. The HET and total coccolithophore diversity were positively correlated with PC 1 and negatively with PC 2; HOL diversity did not show any correlation with PC 1, but still was negatively correlated with PC 2.

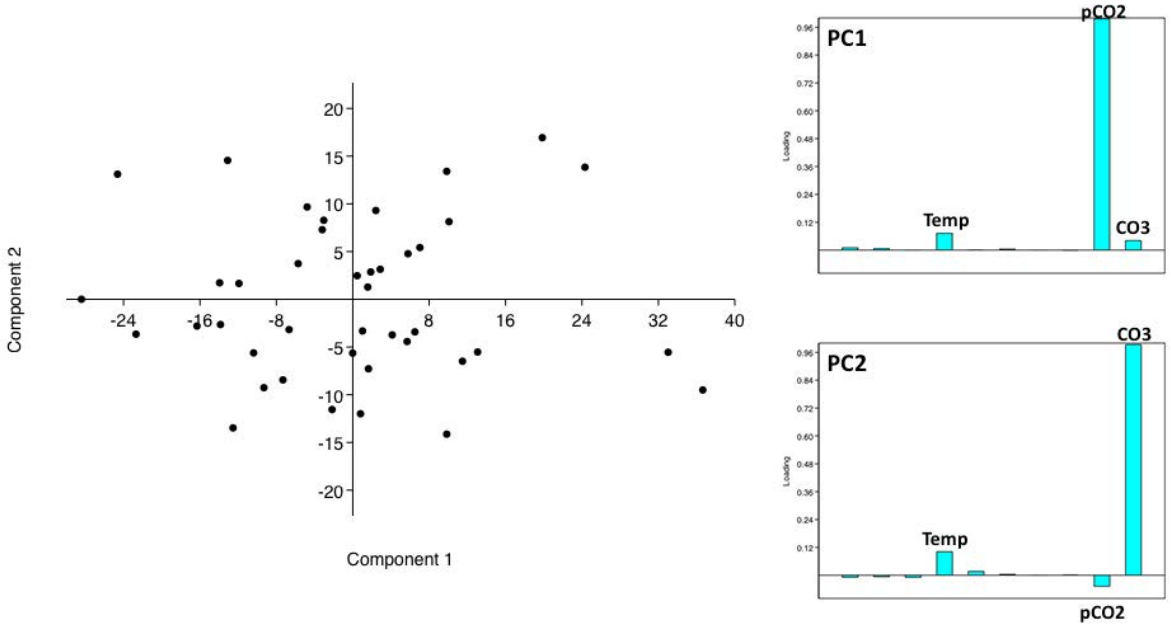

Fig. 4.7 Plot of the 3rd PCA. On the right, loading plots for Components 1 and 2.

PC	Eigenvalue	% variance		PC 1	PC 2
1	191.00	73.21	H' Tot. Coccos	0.01	-0.01
2	68.19	26.14	H' HET	0.01	-0.01
			H' HOL	0.00	-0.01
			Temp	0.07	0.10
			Sal	0.00	0.02
			NO2+NO3	0.00	0.00
			PO4	0.00	0.00
			pH	0.00	0.00
			pCO2	1.00	-0.05
			CO3	0.04	0.99

Table 4.4 PCA3 results.

4.4 Discussion

The high number of species morphotypes (sum of all HET and HOL) along the W-E transect during April 2011 and May 2013 is in line with previous studies (Cros, 2001; Kleijne, 1991, 1993; Knappertsbusch, 1993; Oviedo et al., 2015) and reflect the high diversity characterizing the Mediterranean Sea. It is interesting to note that the diversity was slightly higher during spring 2013 than during spring 2011 (Oviedo et al., 2015).

Many living coccolithophore species are thought to possess a dimorphic life cycle, alternating between a haploid and a diploid life phase. In several species, the change in life phase corresponds to a change in the type of coccoliths produced: from heterococcoliths to holococcoliths (Houdan et al., 2004; Nöel et al., 2004; Parke and Adams, 1960; Taylor et al., 2017). However, the recognized HET-HOL pairs described until present correspond to only a small fraction of the living species, the situation being further complicated by the eventual occurrence of cryptic speciation and polymorphism (Geisen et al., 2002, 2004; Saez et al., 2003; Young et al., 2005b). Hence, in a basin rich in holococcolithophores like the Mediterranean Sea (Cros et al., 2000; Cros and Estrada, 2013; Dimiza et al., 2004, 2015; Kleijne, 1991; Malinverno et al., 2009; Oviedo et al., 2015; Triantaphyllou et al., 2002), it is not yet possible to provide a realistic picture of the total coccolithophore species diversity. To identify the specimens (Table S7, S8) we followed the current nomenclature (Cros and Fortuño, 2002; Young et al., 2003; Nannotax3 website http://ina.tmsoc.org/Nannotax3/), but actually worked on the number of morphotypes rather than the real number species, meaning that HOL and HET of the same species have been considered separately in the calculations of the total coccolithophore, HET and HOL diversities, as explained also in the Methods section.

From Fig. 4.3, we can infer that the total coccolithophore diversity was mainly controlled by the HET; this is further supported by the fact that the HET represent on average more than 80 ± 16 % of the total number of morphotypes.

As presented by Oviedo et al. (2014), HET and HOL diversities showed distinct spatial variability. The HOL diversity increased eastward and reached its maxima at different depths in the western and eastern basins (Fig. 4.2), apparently following the deepening of the DCM and of the 0.1 % PAR (Fig. S12); in the other hand, HET diversity slightly decreased eastward, and did not show clear differences in vertical distribution between the western and eastern basins.

The reason behind the distinct diversity patterns for the HET and HOL might lie in their distinct ecological affinities. The HOL seem to have quite specific environmental affinities: HOL taxa are common in stratified waters, characterized by a deep nutricline and thermocline, well-lighted and rich in carbonate ions (Cros and Estrada, 2013; Oviedo et al., 2014; Šupraha et al., 2016; D'Amario et al., 2017, under review). These conditions are typically found in the Eastern Mediterranean, where we observe the highest HOL diversity (Fig. 4.2, 4.3). On the other hand, HET are known to be adapted to a wider range of conditions: western and eastern Mediterranean communities were previously identified (Oviedo et al., 2015), their distribution being highly correlated to pH, carbonate ion, and phosphate concentration. We infer that HET community can maintain similar levels of

diversity in the Western and Eastern Mediterranean, at least in spring, thanks to the presence of species adapted to a wide range of environmental conditions (e.g. from eutrophic to oligotrophic). HOL taxa have instead more specific environmental requirements, and hence show clearer longitudinal and vertical patterns than the HET, whose morphotypes simply concentrated in the upper 100 m. In the Eastern basin, HOL seem to inhabit a larger portion of the water column (D'Amario et al., 2017, under review). Several HOL species seem to have characteristic depth distributions (Cros, 2001; Triantaphyllou et al., 2002); hence, the existence of wider habitable depth interval in the Eastern basin could encourage a fine scale subdivision of their ecological niche. The presence of HOL diversity maxima at 50 - 75 m in the Eastern basin rather than at 25 m as in the Western Basin, could be explained by the parallel deepening of the nutricline and thermocline (Fig. S1, S2). A similar relationship between a maximum in HOL diversity (45 m depth) and the position of the nutricline was suggested by Triantaphyllou et al. (2002) for a summer community in the Gulf of Korthi, within the Aegean Sea.

The PCA analyses reveal that the environmental affinities of HOL diversity are similar to those concerning the total abundance and the HOLP index (D'Amario et al., 2017, under review). The idea that increase in coccolithophore diversity co-occurs with favourable growth conditions and perhaps competitive advantages (O'Brien et al., 2016), can be adapted to explain the variability of HOL diversity along our transect: HOL diversity might increase where the ideal conditions for certain HET taxa are not met anymore (e.g. eutrophic HET species that encounter oligotrophic conditions), and thus a change in life phase could hypothetically be induced.

Warming and ocean acidification are occurring in the Mediterranean Sea, and projections for the next century (IPCC, 2007; Pachauri et al., 2014) indicate that this trend will continue. Considering the environmental correlations observed in this and a previous study along the same transect and for the same samples (D'Amario et al., 2017 under review), it is possible that under rising temperatures and enhanced stratification of the upper water column the HOL ecological niche will expand westward in the Mediterranean Sea and that their diversity will increase. The relationship between HOL diversity and the carbonate system is more difficult to interpret: probably the most important variable in this context is the carbonate ion concentration, but more studies are needed to confirm its role.

4.5 Conclusions

Our observations along a W-E Mediterranean transect confirm that the Mediterranean Sea is a region of high coccolithophore diversity. At present, most of the morphotype diversity

is driven by heterococcolithophores, which represent more than 80% of the total. HET diversity tends to decrease eastward, from the Western to the Eastern Mediterranean, whole HOL diversity increases. However, the decrease in HET diversity is less pronounced than the increase in HOL diversity, probably due to their wide range of environmental affinities demonstrated by heterococcolithophore taxa (e.g. from oligotrophic to eutrophic). Additionally, HOL diversity reaches maximum values at deeper depth in the Eastern than in the Western Mediterranean, perhaps as a consequence of their more specific environmental affinities (e.g. apparently all oligotrophic). Overall, HOL diversity seems to increase in correspondence of well-lighted, oligotrophic and warm waters, rich in carbonate ions and low in pCO_2; these correlations suggests that HOL diversity is strictly related to HOL ecology. In this view, enhanced stratification and oligotrophy might not only expand the ecological niche of HOL, but also stimulate an increase in their diversity; it is more difficult to predict what will be the net effect of ocean acidification.

CHAPTER 5

Coccolithophore response to warming and acidification: a mesocosm study in the Eastern Mediterranean Sea

Abstract

Mesocosm studies are a fundamental tool to test the possible effects of climate changes like warming and acidification on plankton communities, including coccolithophores. However, few of these experiments have been conducted in naturally nutrient-limited environments, such as the Eastern Mediterranean Sea. Here, we present a 12 days-long mesocosm experiment (September 2013) based on marine water collected offshore of Heraklion (Crete), from 10 m depth. The experiment took place under strong nutrient limiting conditions, testing the response of the pelagic population to warming, acidification and their combination ("green house"). Filtered water samples were analysed for coccolithophore abundance by light microscopy, and for $CaCO_3$ concentration by inductively coupled plasma optical emission spectrometry. Our results indicate that temperature and nutrient limitation were the strongest drivers of coccolithophore abundance variability. The effects of acidification in these conditions were overall negligible, or even stimulating for the growth of *Rhabdosphaera* spp., highlighting the resilience of this genus. The coccolithophore response showed moderate variability within mesocosm replicates subjected to the same treatment. Our observations suggest that the net coccolithophore community response to environmental perturbations is strongly influenced by the initial assemblage composition; species and strain specific growth optima within species should be taken into account. Ultimately, the assemblage composition can regulate the total concentration of coccolith $CaCO_3$ and the PIC:POC ratio.

5.1 Introduction

Global climate change is an undergoing process, primarily driven by carbon dioxide (CO_2) emissions into the atmosphere. In the worst-case IPCC scenario, atmospheric [CO_2] might reach > 1000 ppm by year 2100, inducing a reduction of surface ocean pH, carbonate ion concentration, aragonite and calcite saturation levels (Ciais et al., 2013; Doney et al., 2009; Feely et al., 2009; Pachauri et al., 2014). Over 90% of the extra heat caused by greenhouse gas accelerated emissions ends up in the ocean causing ocean warming (Collins et al., 2013), stratification of the surface water column, and decreased nutrient

supply to the upper layers (Sarmiento et al., 2004). The Mediterranean Sea is considered particularly vulnerable to climate change (Giorgi, 2006): anthropogenic CO_2, which has already invaded the whole basin (Schneider et al., 2007), is lowering the surface pH of 0.245 - 0.457 (Goyet et al., 2016), while atmospheric warming could increase sea surface temperatures by 6°C (Sakalli, 2017) by the end of this century. Moreover, the Mediterranean is an oligotrophic basin, seasonally subject to vertical stratification, especially in the eastern areas (D'Ortenzio and D'Alcalà, 2009; Krom et al., 1991; Tanhua et al., 2013b); a condition which might be exacerbated by climate change (Gruber, 2011; Irwin and Oliver, 2009; Polovina et al., 2008).

Coccolithophores are one of the major groups of phytoplankton in the Mediterranean; they can dominate the phytoplankton community in both the western and eastern basins (Oviedo et al., 2015) and contribute to ~ 80 – 90% of calcium carbonate sedimentation (Broecker and Clark, 2009). Many experiments have tackled coccolithophore response to acidification (see meta-analyses in Meyer and Riebesell, 2015), but only a minority focused on the combined effects of multiple environmental variables (Arnold et al., 2013; Benner et al., 2013; De Bodt et al., 2010; Fiorini et al., 2011a; Milner et al., 2016; Rouco et al., 2013; Schlüter et al., 2014; Sett et al., 2014; Zondervan, 2007). Additionally, most of the previous mesocosm experiments which tested coccolithophores were conducted under eutrophic conditions, or involved the addition of nutrients to stimulate cell growth (Maugendre et al., 2017). Overall, few mesocosms were performed under nutrient-limitation (Bach et al., 2016; Oviedo et al., 2016; Sala et al., 2016).

Coccolithophore response to acidification and warming, can be seasonal, species- and strain-specific (Langer et al., 2006, 2009; Oviedo et al., 2016); considered the high diversity of coccolithophore communities in the Mediterranean Sea (Cros, 2001; Ignatiades et al., 2009; Oviedo et al., 2015) and likely in other oligotrophic basins (O'Brien et al., 2016), specific responses are particularly important to assess.

In the present work are presented the results of a mesocosm experiment which tested, for the first time, the effects of acidification and warming on a natural coccolithophore community from the the Eastern Mediterranean. These potential environmental stressors were tested both separately and in combination. Comparing absolute and relative cell abundances, we sought to disentangle the response of the total coccolithophore community, the two calcified life stages (hetero-, holococcolithophores), and of the dominating species.

5.2 Material and methods

5.2.1 Experimental design

The experiment tested the effects of ocean warming and acidification on the Eastern Mediterranean pelagic ecosystem during 12 days (1st - 12th September; experimental days -1 to 10). Four treatments were included: unperturbed ambient conditions (control = C), ocean acidification (OA), warming (W), combined acidification and warming (green house = GH). Each treatment was tested on three replicates (each ~3 m^2), for a total of 12 mesocosms bags (Table 5.1).

Day/month/year	Day	Treatment	Samples (replicates)
01/09/13	-1	C	Co (3), Ca (1)
		OA	Co (1), Ca (1)
		GH	Co (1), Ca (1)
		W	Co (1), Ca (1)
02/09/13	0	C	Co (3), Ca (3)
		OA	Co (3), Ca (3)
		GH	Co (3), Ca (3)
		W	Co (3), Ca (3)
03/09/13	1	C	Co (3), Ca (3)
		OA	Co (3), Ca (3)
		GH	Co (3), Ca (3)
		W	Co (3), Ca (3)
05/09/13	3	C	Co (3)
		OA	Co (3)
		GH	Co (3)
		W	Co (3)
07/09/13	5	C	Co (3), Ca (3)
		OA	Co (3), Ca (3)
		GH	Co (3), Ca (3)
		W	Co (3), Ca (3)
09/09/13	7	C	Co (3)
		OA	Co (3)
		GH	Co (3)
		W	Co (3)
12/09/13	10	C	Co (3), Ca (3)
		OA	Co (3), Ca (3)
		GH	Co (3), Ca (3)
		W	Co (3), Ca (3)

Table 5.1 Samples collected and analysed during the mesocosm experiment for coccolithophores (Co) and $CaCO_3$ (Ca).

The mesocosm experiment was based on Eastern Mediterranean water (35° 24.957' N, 25° 14.441' E, site depth 170 m, sampling depth 10 m, sampling temperature 25°C) collected aboard the R/V Philia using a submersible pump on the 30th - 31st August 2013. About 36 m^3 of water were transferred into polyethylene tanks (1 m^3 each), previously filled with tap water (for about one week), washed with HCl 10% and rinsed with deionized water. The marine water reached the CRETACOSMOS (http://cretacosmos.eu) at the Hellenic Centre for Marine Research (HCMR) two hours after collection, having being maintained under constant temperature (25°C) during transportation. The water of each polyethylene tank was split equally among 12 polyethylene bags (individual diameter 1.32 m), positioned in two concrete tanks (350 m^3 and 150 m^3) filled with water. Temperature was regulated during the experiment by releasing cold or hot water into the concrete tanks, outside of the mesocosm bags. The target for the bigger tank (containing C and OA mesocosms) was of 25°C, while for the smaller tank (containing W and GH mesocosms) was of 28°C.

Three mesocosm bags in each concrete tank were acidified (OA1, OA2, OA3, GH1, GH2, GH3), dispersing 28.5 - 31 L of CO_2-saturated seawater in each bag. Such water had been separated from the original batch before the mesocosm filling, bubbled several minutes with CO_2 and transferred into plastic 10 L Nalgene containers. The acidification was implemented over three days (1st – 3rd September 2013) to minimize biological stress, until the target levels of pH (~ 7.8) were reached and left to evolve independently. No nutrients were added during the experiment.

Every day before sampling, the water in all mesocosms was mixed for about two minutes using clean spatulas; then, it was vacuum-forced through plastic tubing and finally into 10 – 20 L containers previously washed with Elix water.

5.2.2 Environment and particulate organic carbon

Salinity was measured from the centre of each mesocosm using an Aanderaa Conductivity-Temperature sensor 3919.

The changes in temperature were monitored by a system of sensors (HOBO UA-002-64 and Aanderaa Conductivity-Temperature sensor 3919), connected to a control panel (IKS Aquastar, IKS ComputerSysteme GmbH).

Seawater pH was obtained on total scale using a pH meter (Metrohm, 827 pH lab) fitted with a glass electrode (Metrohm, Aquatrode Plus), calibrated on the total hydrogen ion concentration scale with a Tri/HCl buffer solution (Dickson et al., 2007) at a salinity of 38.0 provided by A. Borges (University of Liege).

Nitrite and nitrate were measured following (Strickland and Parsons, 1972) at a detection limit of 0.017 µmol; phosphate was instead determined using the MAGIC25 method (Rimmelin and Moutin, 2005), at a detection limit of 0.8 nmol. The N:P is expressed in molar ratio; it was obtained from nitrite, nitrate and phosphate concentrations, taking into account their molar mass.

The concentration of particulate organic carbon (POC) was measured with an elemental analyser CHNS FLASH 2000 Thermo Scientific (Cutter and Radford-Knoery, 1991; Verardo et al., 1990). The PIC:POC ratio was found comparing the concentrations of the particulate inorganic carbon (PIC) (calculated from the $CaCO_3$ concentration, Section 2.3) and of the POC.

5.2.3 Coccolithophores and calcium carbonate

A total of 130 samples were obtained from the filtration of the sampled mesocosm water: 78 for coccolithophore analysis and 52 for the quantification of calcium carbonate. Sampling frequency was higher in the first three days of experiment (-1 to 1) and lower afterwards; moreover, three replicates per treatment were included, with the exception of day -1 (Table 5.1).

Coccolithophore and calcium carbonate samples were filtered separately through a vacuum pump system (Eyela, A-1000S): three to five litres on cellulose acetate-nitrate filters (Millipore, Ø 47 mm, 0.45 µm) for the former, and six to eight litres on polycarbonate filters (Millipore, Ø 47 mm, 0.80 µm) for the latter. All filters were rinsed with ammonia (63 ml NH_3 + 500 ml of distilled water) to dissolve salt residues and oven-dried at ~ 40°C for about eight hours.

A portion of each cellulose acetate filter was radially cut and mounted on a microscope slide using transparent immersion oil. Between 120 and 1895 fields of view (1 FOV = 0.05 mm^2) were observed per sample at x 1000, using a polarizing light microscope (Leica DM6000B). The observed area per sample varied depending on cell concentration, corresponding on average to 68 mL of water. The 95% confidence interval, assuming a Poisson distribution, oscillated between 21 and 139 for a concentration of 54 cells L^{-1}, up to between 1.74 x 10^4 and 2.13 x 10^4 for a concentration of 1.92 x 10^4 cells L^{-1}. Both cell density and confidence limits were calculated following (Bollmann et al., 2002). Coccolithophores were identified down to genus level (*Rhabdosphaera* spp., *Syracosphaera* spp., *Umbellosphaera* spp.), except for the species *Emiliania huxleyi* and *Gephyrocapsa muellerae*; holococcolithophore species were not differentiated.

Each polycarbonate filter was completely immersed in 10 mL of HNO_3 1% (Merck Suprapur), underwent ultrasonic bath for 10 minutes and was left to rest for one night. The resulting solution was measured through inductively coupled plasma optical emission spectrometry (ICP-OES, Perkin-Elmer, model Optima 4300DV) to measure the calcium content; this quantity was then converted into calcium carbonate concentration through molar mass calculations, assuming that all calcium atoms had been originally bounded to a molecule of $CaCO_3$.

5.2.4 Statistics

We used PAST 3.14 (Hammer et al., 2001) for all statistical analyses. The OA3 mesocosm showed anomalous salinity values after day 2 (Fig. 5.1), perhaps due to the damaging of the mesocosm bag, and was thus excluded from all the statistical analysis.

A series of Spearman correlation analyses, t-tests and two-way ANOVA tests were performed on the samples available between days 2 and 10, as both temperature and the carbonate system reached the target levels after day 2.

The t-tests were done to compare the average coccolithophore abundances in the four treatments; averages were considered significantly different for *p*-values ≤ 0.05. The Spearman correlations were done to test the occurrence of any significant correlations a) of the coccolithophore abundances in respect to the environmental parameters, and b) of CaCO3 concentration in respect to the PIC:POC, POC, and coccolithophore abundances; correlation coefficients with *p*-values ≤ 0.05 were considered significant. The two-way ANOVA tests were done to individuate any statistically significant effects of temperature (T) and pH, individually and combined, on the coccolithophore abundances; the effects were considered significant for *p*-values ≤ 0.05.

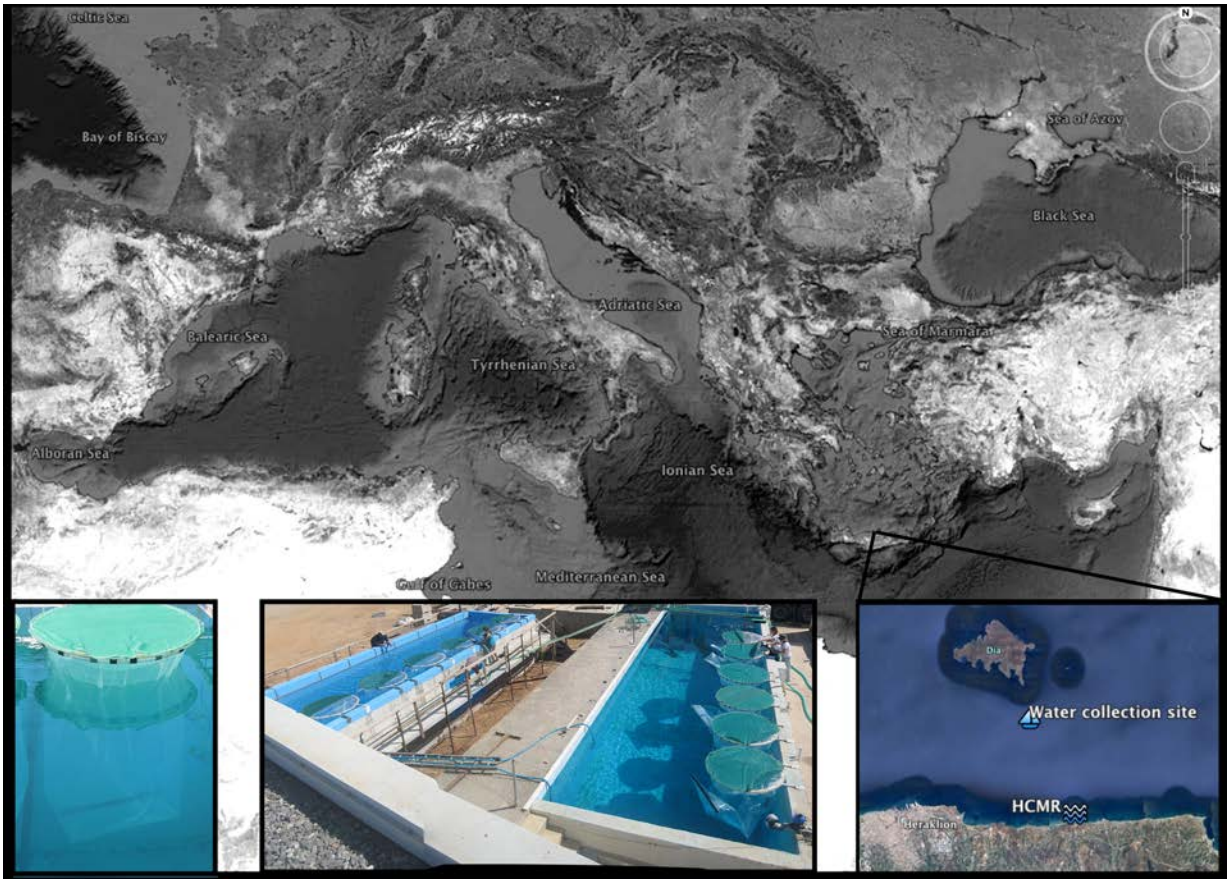

Fig. 5.1 Map showing the water collection and experimental sites. Bottom left: one of the mesocosm bags used during the experiment. Bottom centre: areal view of the experimental site; the two pools were set at different temperatures (25 and 28°C respectively).

5.3 Results

5.3.1 Environment

Temperature oscillated during the whole experiment between 24.95 and 25.32°C in the C and OA mesocosms. In the W and GH treatments, temperature increased during the first three day, reaching 27.04 – 27.94°C on day 1 and not exceeding 28.41°C for the rest of the experiment.

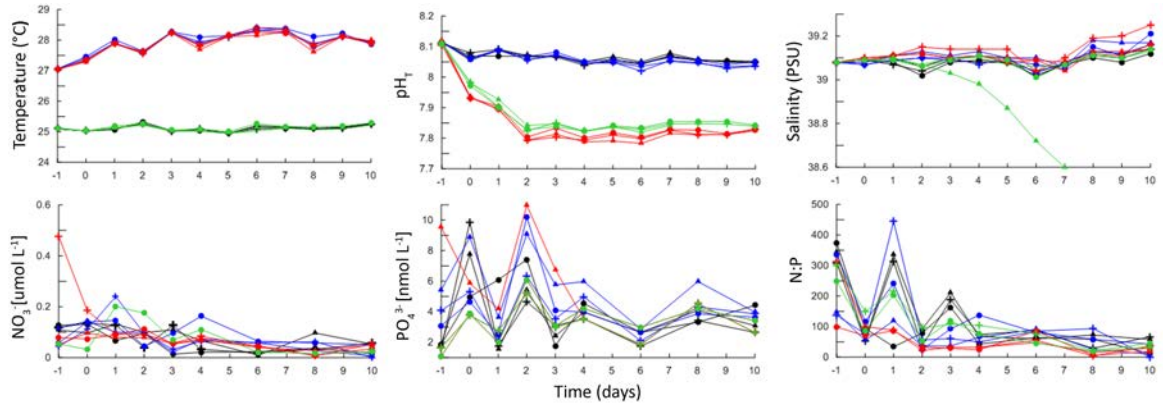

Fig. 5.2 Temporal dynamics of the main environmental parameters. The treatments are represented by different colours: C = black, W = blue, OA = green, GH = red. The replicates of each treatment are represented by different symbols: replicate 1 = circles, replicate 2 = cross, replicate 3 = triangle. In the salinity plot there is clear decline in the values of OA3 after the second experimental day.

The pH in the C and W mesocosms oscillated between 8.12 and 8.02 during the whole experiment; instead, the OA and GH treatments experienced three drops in pH in response to the addition of CO_2 saturated water, from 8.11 down to 7.80 – 7.83 on day 2, and growing again slightly on day 7, up to 7.83 – 7.85.

Salinity was maintained between 39.01 and 39.21 PSU in all treatments; apart from a small decline on day 6, there was a general tendency for this parameter to slightly increase with time, due to water evaporation. A big drop in salinity (from 39.07 to 38.60 PSU) was observed in OA3 after day 2, due to damaging of the mesocosm bag (Fig. 5.2); therefore, all results concerning this bag were not considered reliable for the purpose of this study.

Nitrate concentration (NO_3^-) declined with time in all treatments, oscillating overall between 0.00 and 0.20 µmol L^{-1}, except for mesocosm GH2, which on day -1 experienced a concentration of 0.48 µmol L^{-1}. Phosphate (PO_4^{3-}) concentration oscillated with no consistent pattern in all treatments, between 1.09 and 10.98 nmol L^{-1}. The N:P molar ratio temporal variability mainly reflects that of nitrate concentration: it generally decreased from day -1 to day 10 (from a maximum of 374.20 to a minimum of 0.24 among all treatments).

Between days 3 and 10, the mean pH was significantly (p = 0.00) lower in the OA and GH treatments (OA_{mean} = 7.84, GH_{mean} = 7.81), then in the C and W treatments (C_{mean} = 8.06, W_{mean} = 8.05); but also significantly (p = 0.00) higher in the OA than in GH mesocosms. Also, temperature in the W and GH treatments (W_{mean} = 28.13°C, GH_{mean} = 28.07°C) was higher (p = 0.00) than in the C and OA mesocosms (C_{mean} = 25.10°C, OA_{mean} = 25.11°C). At the same time, there were no significant differences among treatments in salinity, nutrient concentrations or N:P ratio.

5.3.2 Coccolithophores, $CaCO_3$ and PIC:POC

Initial coccolithophore abundances were not different among the four treatments (C1, C2, C3, OA1, W1, GH1): they oscillated overall within the same range of values on day -1 (Fig. 5.3). For the total coccolithophore community, values were comprised between 1.05 x 10^4 and 3.64 x 10^3 cells L^{-1}; for the HET between 1.00 x 10^4 and 3.48 x 10^3; for the HOL, between 4.57 x 10^2 and 1.64 x 10^2 cells L^{-1}; for *E. huxleyi* between 5.80 x 10^3 and 1.88 x 10^3 cells L^{-1}; for *Rhabdosphaera* spp. between 5.25 x 10^3 and 1.10 x 10^3 cells L^{-1}. The initial *Rhabdosphaera* spp. : *E. huxleyi* ratio was also similar among the treatments; values oscillated overall between 0.46 and 2.53.

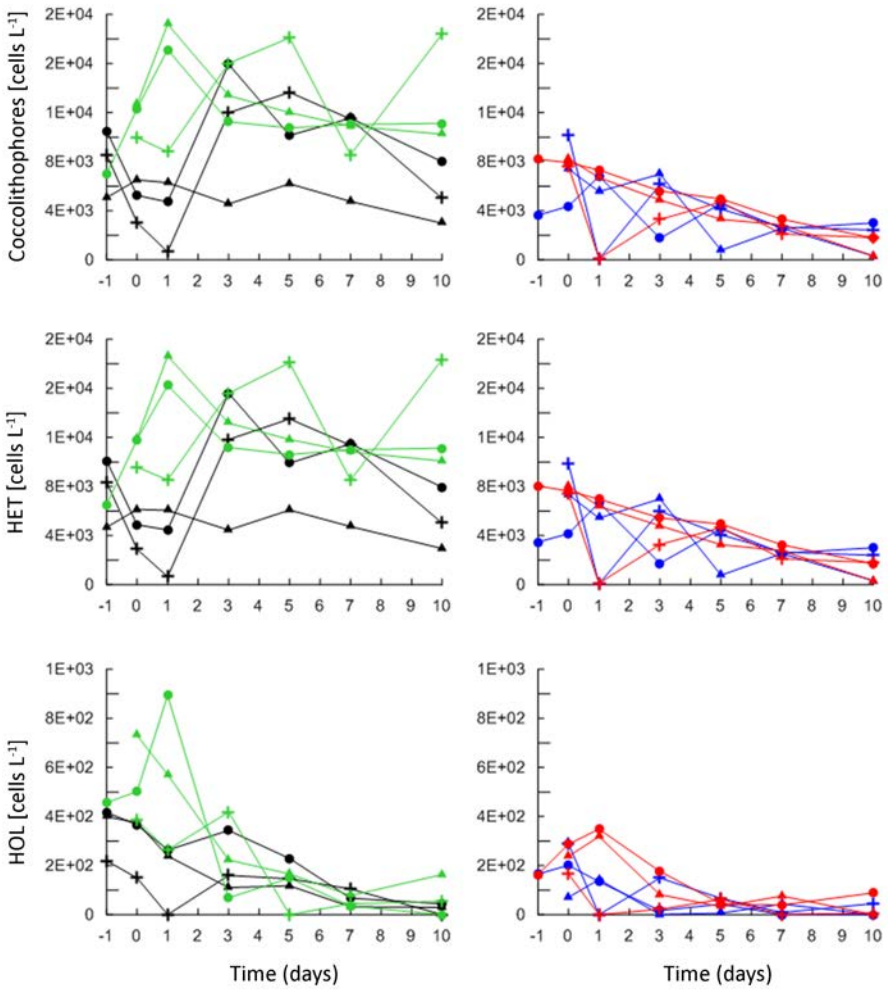

Fig. 5.3 Temporal dynamics of the total coccolithophore community, heterococcolithophores (HET) and holococcolithophores (HOL).

From day 3 to 10 (Fig. 5.3), the total coccolithophore abundance was maintained quite stable under the OA treatment, while it tended to decrease in the C, W and GH. HET constituted a major portion of the total coccolithophore community (82 - 99%) in all mesocosms, their temporal dynamics reflecting those of the total coccolithophores; HOL instead diminished in all treatments. Most heterococcolithophores (76 – 98%) were represented by *E. huxleyi* and *Rhabdosphaera* spp.. The absolute abundance of *Rhabdosphaera* spp. from day 3 to 10 (Fig. 5.4), was very variable in the OA mesocosms; in the C, W and GH treatments there were characterized instead by a quite uniform decreasing trend. *Emiliania huxleyi* absolute abundance followed similar trends compared to those of *Rhabdosphaera* spp., the decreasing trends being however more pronounced. The *Rhabdosphaera* spp.:*E. huxleyi* ratio between days 3 and 10 was very variable in the OA treatment, it increased slightly in C and remained fairly stable in the W and GH treatments. $CaCO_3$ concentration tended to increase between days 5 and 10 in all treatments, accompanied by increasing POC (Fig. 5.5).

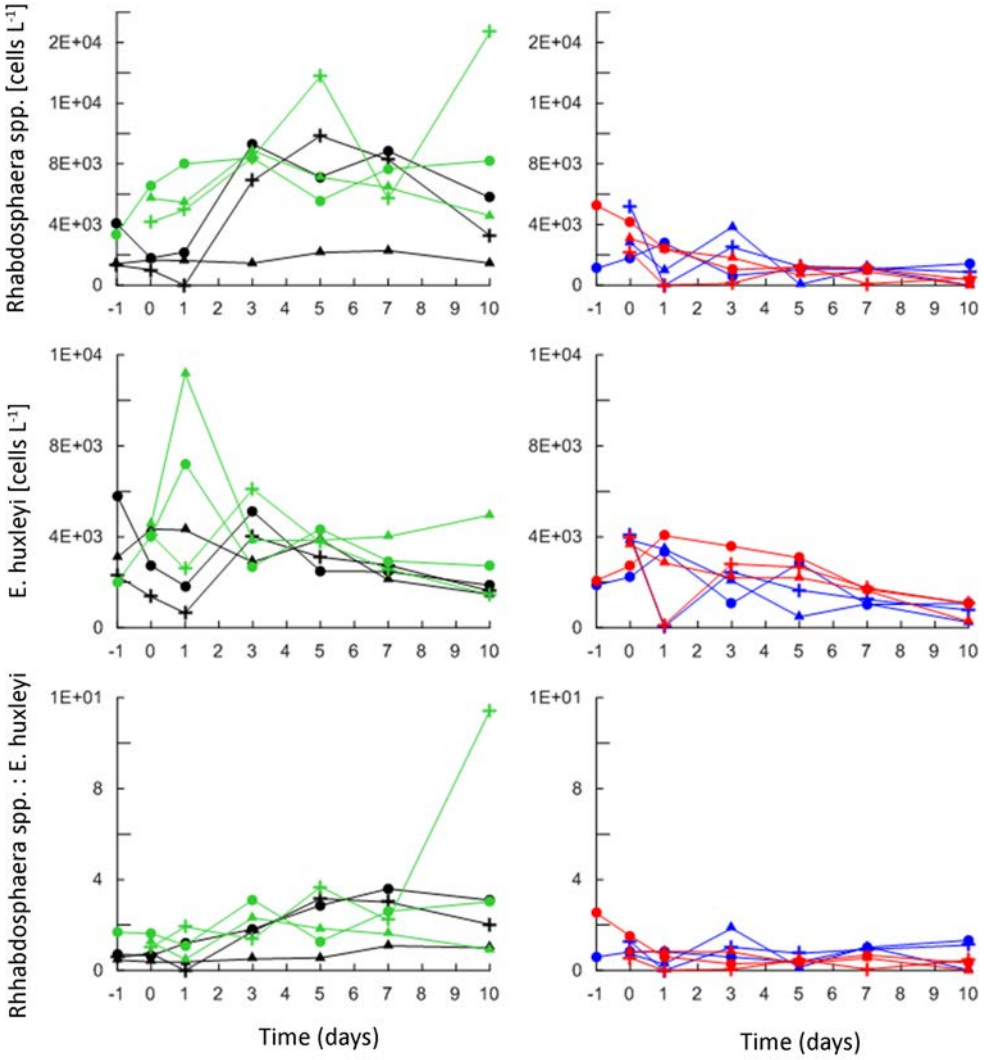

Fig. 5.4 Temporal dynamics of Rhabdosphaera spp., E. huxleyi, and of their abundance ratio.

The t-tests revealed that from day 3 to 10 the average total coccolithophore and HET abundances were significantly lower in the W and GH treatments than in the C and OA treatments (p = 0.00 – 0.01; W_{mean} = 0.32 and 0.31 x 10^4 cells L^{-1}; GH_{mean} = 0.32 and 0.32 x 10^4 cells L^{-1}; C_{mean} = 0.89 and 0.87 x 10^4 cells L^{-1}; OA_{mean} = 1.27 and 1.31 cells L^{-1}), while the average HOL never showed significant differences (p = 0.10 – 0.78). Both *E. huxleyi* and *Rhabdosphaera* spp. abundances were significantly lower in the W than in the C treatment (p = 0.00 – 0.04; W_{mean} = 0.13 and 0.12 x 10^4 cells L^{-1}; C_{mean} = 0.28 and 0.56 x 10^4 cells L^{-1}). *Rhabdosphaera* spp. was also less abundant in GH than in C (p = 0.00; GH_{mean} = 0.08 x 10^4 cells L^{-1}), and more abundant in OA than in C (p = 0.04; OA_{mean} = 0.93 x 10^4 cells L^{-1}). The *Rhabdosphaera* spp. : *E. huxleyi* ratio was similar in the OA and C treatments (p = 0.26; OA_{mean} = 3.59; C_{mean} = 2.04), but lower in W and GH (p = 0.00 –

0.01; W_{mean} = 0.85; GH_{mean} = 0.36). No significant differences were found in the $CaCO_3$ concentration among treatments between days 5 and 10 (p = 0.42 – 0.90).

Spearman analyses results showed that all coccolithophore abundances, including the *Rhabdosphaera* spp. : *E. huxleyi* ratio, were significantly correlated with temperature; other significant correlations were found in respect to salinity, $[NO_3^-]$ and the N:P ratio (Table 5.2). $CaCO_3$ concentrations were significantly correlated with the PIC:POC and the *Rhabdosphaera* spp. : *E. huxleyi* ratios (Table 5.3).

Response variable	Factor	df	Mean square	F	p (same)
Total coccolithophores	T	1	3.55	40.54	**0.00
	pH	1	0.03	0.40	0.53
	T x pH	1	0.11	1.29	0.26
HET	T	1	3.56	40.92	**0.00
	pH	1	0.03	0.40	0.53
	T x pH	1	0.12	1.35	0.25
HOL	T	1	2.44	3.96	*0.05
	pH	1	0.00	0.00	0.99
	T x pH	1	1.62	2.63	0.11
E. huxleyi	T	1	1.05	16.71	**0.00
	pH	1	0.11	1.79	0.19
	T x pH	1	0.05	0.82	0.37
Rhabdosphaera spp.	T	1	12.94	31.02	**0.00
	pH	1	0.17	0.42	0.52
	T x pH	1	0.93	2.24	0.14
Rhabdosphaera spp.:*E.huxleyi*	T	1	1.13	47.59	**0.00
	pH	1	0.01	0.58	0.45
	T x pH	1	0.20	8.49	**0.01

Table 5.2 Results of two-way ANOVA testing the effects of temperature (T), pH and their combination on the coccolithophore abundances (HET = heterococcolithophores, HOL = holococcolithophores). Significant correlations (*$p \leq 0.05$; **$p \leq 0.01$) are in bold.

	TOT	HET	HOL	*E. huxleyi*	*Rhabdosphaera* spp.	*Rhabdosphaera* spp.:*E. huxleyi*
T	**-0.65	**-0.64	**-0.51	**-0.53	**-0.63	**-0.44
pH	-0.03	-0.04	0.21	-0.02	-0.01	0.02

Table 5.3 Spearman correlation analysis results. Significant correlations (*$p \leq 0.05$; **$p \leq 0.01$) are in bold.

The ANOVA test (Table 5.4) indicates a significant effect of temperature on all coccolithophore abundances; temperature, plus combined temperature and pH affected the *Rhabdosphaera* spp. : *E. huxleyi* ratio.

	CaCO3
PIC:POC	****0.92**
POC	-0.35
TOT	0.20
E. huxleyi	-0.08
Rhabdosphaera spp.	0.38
Rhabdosphaera spp.:*E. huxleyi*	***0.49**
HET	0.20
HOL	-0.10

Table 5.4 Spearman correlation analysis results. Significant correlations (*$p \leq 0.05$; **$p \leq 0.01$) are in bold.

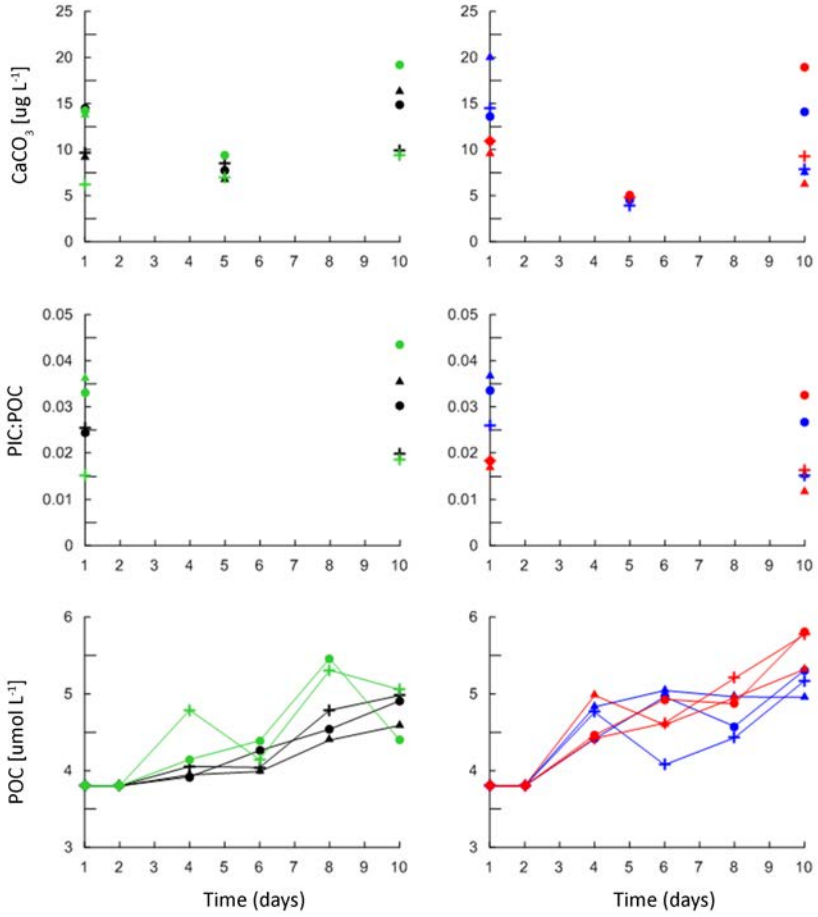

Fig. 5.5 Temporal dynamics of calcium carbonate concentration, PIC:POC ratio and POC concentration.

5.4 Discussion

In this mesocosm experiment different levels of pH and temperature were tested, to simulate the projected ocean acidification and warming for year 2100 by the IPCC. Temperature and pH target levels were reached on day 2 and maintained until the last day of the experiment (Fig. 5.2). Salinity and nutrient levels were not significantly different among the four treatments; still, a slight increase in salinity, and slight decreases in NO_3^-

and N:P ratio with time were identified in all treatments. The Spearman correlation analysis (Table 5.2) showed that temperature was the main factor correlated with coccolithophore abundances, followed by the N:P ratio, nitrate concentration and salinity. However, correlations relative to nitrate concentration and salinity very likely reflected the temporal evolution of the coccolithophore population. Salinity was negatively correlated with the abundance of both *E. huxleyi* and *Rhabdosphaera* spp., but we cannot ascertain if it really affected their growth. *Emiliania huxleyi* can survive under a wide range of salinities (Saruwatari et al., 2016; Tyrrell et al., 2008); such adaptability is possibly linked to high genome variability (Blanco-Ameijeiras et al., 2016; Read et al., 2013) and phenotypic plasticity (Langer et al., 2009; Paasche, 2002). Moreover, the relationship between salinity and *E. huxleyi* growth was found to be inconsistent in culture studies (Fielding et al., 2009; Paasche et al., 1996; Saruwatari et al., 2016). The response of Rhabdosphaeraceae to changing salinity has not yet been studied in detail; however, this family tends to be relatively more abundant in the Eastern than in the Western Mediterranean, under relatively high salinity conditions (Oviedo et al., 2015), and could be expected to be resilient to small increases in salinity such as those experienced during our experiment. These considerations suggest a very prudent interpretation of any correlation between salinity and coccolithophore abundance observed in our dataset.

The positive correlations found between HOL, nitrate and the N:P ratio are controversial, as the literature shows unanimously that this calcified coccolithophore life phase is abundant in oligotrophic environments and in areas characterized by low N:P ratios (e.g. Cros and Estrada, 2013; Dimiza et al., 2008; Kleijne, 1991; Šupraha et al., 2016). We thus believe that such correlations are very unlikely to represent a real cause-effect relationship. The variability in HOL concentrations was more probably driven by temperature. Although HOL inhabit preferentially superficial waters and increase during summer in the Mediterranean Sea (Cros, 2001; Kleijne, 1991; Oviedo et al., 2015; Šupraha et al., 2016), it is possible that the level of temperature stress faced during the experiment, even in the C and OA mesocosms, was excessive for the majority of the HOL. The negative correlation observed between *E. huxleyi*, nitrate concentration and the N:P ratio were instead in accord with recent studies highlighting a strong dependency of *E. huxleyi* growth on nitrate availability (Feng et al., 2016; Perrin et al., 2016). Under low phosphate concentrations, *E. huxleyi* is considered a good competitor with respect to other phytoplankton (Egge and Heimdal, 1994; Riegman et al., 2000), but still it could be outcompeted by other coccolithophore species better adapted to oligotrophic conditions. The inverse correlation between *Rhabdosphaera* spp.: *E. huxleyi* ratio, might be explained by the higher ecological affinity of *Rhabdosphaera* spp. for low nitrate concentrations and

N:P ratios; an affinity which can enable this species to outcompete even *E. huxleyi*. Rhabdosphaeraceae are in fact relatively abundant in the upper photic zone, under oligotrophic and warm conditions (Dimiza et al., 2008a; Malinverno et al., 2009); and *Rhabdosphaera clavigera* was found to inhabit preferentially the upper 50 m of Eastern Mediterranean Sea, under low PO_4 concentrations (Oviedo et al., 2015).

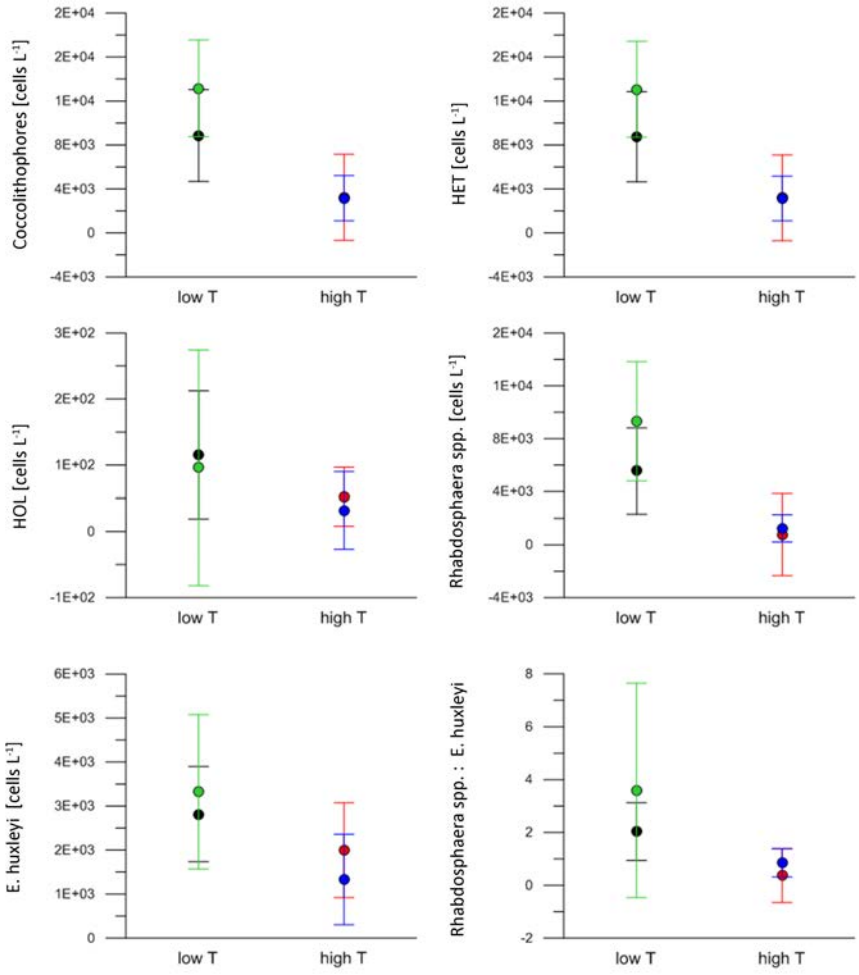

Fig. 5.6 Average coccolithophore abundances between days 3 and 10 in all treatments and replicates, excluding OA3. The standard deviations for each treatment refer to all data between days 3 and 10.

Between temperature and pH, only temperature showed significant correlations with respect to coccolithophore response: significant correlations were found for the total coccolithophore community, HET, HOL, *E. huxleyi*, *Rhabdopshaera* spp. abundances, and the *Rhabdosphaera* spp.: *E. huxleyi* ratio. The determining role of temperature on the coccolithophore community is supported also by the ANOVA test (Table 4) and by the marked tendency of coccolithophores in the W and GH treatments to maintain lower abundances than in the C and OA treatments (Fig. 6). Our results overall suggest that

temperature was the main driver of coccolithophore abundance variability, and that its effects override those of ocean acidification (low pH). *Emiliania huxleyi* and *Rhabdosphaera* spp., the main contributors to the analysed coccolithophore assemblage, were both negatively affected by warming; with *Rhabdosphaera* spp. being perhaps slightly more sensible to temperature, and more responsive than *E. huxleyi* to acidification (Fig. 2, t-tests). Coccolithophore growth rates can be influenced by temperature, but their mutual relationship cannot be described by a monotonic function; rather, coccolithophore growth can be stimulated by increasing temperatures up to a certain threshold, temperature optimum, which can be species and strain specific (Brand, 1982; Buitenhuis et al., 2008), and modulates the response of coccolithophore communities to acidification (Sett et al., 2014). *Emiliania huxleyi* has been observed to survive under a wide range of temperatures, up to 30°C (Winter and Siesser, 1994), and *Rhabdosphaera* spp. includes taxa commonly living in warm oceanic waters. The negative response of the coccolithophore community to warming, could possibly signal that the temperature optimum of the majority of coccolithophores (i.e. *Rhabdosphaera* spp., *E. huxleyi*, HOL) was overcome. *Rhabdosphaera* spp. seemed on average slightly stimulated over *E. huxleyi* in the ocean acidification treatment; however, when the replicates of these treatments are considered separately, it became clear that the effect of acidification was highly variable (Fig. 2). Such variability could reconcile our results with those obtained by Oviedo et al. (2016) during a summer mesocosm experiment in the NW Mediterranean Sea, which showed decreasing *R. clavigera* abundance in high pCO_2 conditions. Perhaps, the high variability encountered within our OA treatments derives from different specific/strain sensibilities of *Rhabdosphaera* spp. to low pH. In conclusion, as proposed by Oviedo et al., (2016), the effects of temperature and nutrient limitation might surpass those of acidification on coccolithophores inhabiting oligotrophic, stratified marine waters. It is important to take in account though that both their and our mesocosm experiment were short-termed (10 - 17 days), and thus potential plastic adaptations of the coccolithophore populations to the new conditions could not be observed.

The initial coccolithophore community composition seemed to influence the total coccolithophore response. In particular, it is probable that the *Rhabdosphaera* spp. : *E. huxleyi* ratio affected the total $CaCO_3$ concentrations and, consequently, the PIC:POC molar ratio (Table 3), due to the higher mass of *Rhabdosphaera* spp. coccoliths in respect to those of *E. huxleyi* (Young and Ziveri, 2000).

Although combined warming and acidification seem to decrease the total coccolithophore production in the oligotrophic, stratified Eastern Mediterranean Sea, future efforts should

concentrate on testing the effects of environmental changes on the dominant species and strains in a variety of marine regions and seasonal phases.

5.5 Conclusions

- Increasing oligotrophy with time likely favoured the growth of *Rhabdosphaera* spp. over that of *E. huxleyi* in the control treatment, and modulated the temporal response of the coccolithophore community to warming and acidification. Salinity showed an increasing trend with time, but its role in modulating the coccolithophore response to the tested environmental perturbations is highly uncertain.
- Temperature seems to drive the differences in coccolithophore abundance among treatments, inducing lower total coccolithophore, HET, HOL, *E. huxleyi*, *Rhabdosphaera* spp. concentrations and lower *Rhabdosphaera* spp.:*E. huxleyi* ratios. The effects of temperature apparently overcome those of ocean acidification (low pH).
- The response of *Rhabdosphaera* spp. to ocean acidification was highly variable, and could potentially reflect species and strain specific responses.
- Under warming and acidification, coccolithophore species and strains within species might respond differently to environmental perturbations, depending on their optimum growth requirements. Also, the resilience of different species to environmental perturbations can ultimately affect the total coccolithophore $CaCO_3$ production and the PIC:POC ratio in oligotrophic and ultra-oligotrophic settings.

CHAPTER 6

Conclusions and future directions

This thesis contributes to the understanding of the plankton ecology and calcification dynamics under climate change focusing on a specific group of abundant planktonic calcifying organisms. Natural coccolithophore communities from the Mediterranean Sea have been collected from field and mesocosm experiments and analysed. In the following paragraphs are summarized the main results and conclusions of the thesis, accompanied suggestions on future research.

The analysis of coccolithophore communities collected along a strong physicochemical gradient, resulted in new considerations about their species distribution, life cycle and calcification in relation to the main environmental parameters. *Emiliania huxleyi* coccolith mass was generally higher in the Eastern Mediterranean. The eastward increasing trend was explained by parallel increases in the average *E. huxleyi* coccolith size and calcification degree. The variability in coccolith size and calcification degree could not simply be justified by the relative abundance of different *E. huxleyi* mopho - Type A and - Type B/C coccoliths: the latter morphotype constituted normally less than 5 % of the total *E. huxleyi* population; Type A dominated, presenting a wide range of sizes and calcification degrees. Our data demonstrated that the average *E. huxleyi* coccolith mass was at least partly dependant on the relative abundance of A1 (undercalcified, smaller) and A3b (overcalcified with open central area, larger) coccoliths. The distribution of these and other Type A calcification varieties (A2, A3a) seems regulated by distinct sets of carbonate system variables, together with temperature, nutrients and salinity. We suspect that the calcification varieties described in our work represent a combination of genotypes and inter-gradational ecophenotypes. However, more studies centred on the morphology and genetics of *E. huxleyi* are needed to determine the proportion of strain selection and physiological adaptations taking place in the Mediterranean Sea. In order to progress our knowledge on this topic, future field work could continue to focus on the spatial and temporal variability of coccolith morphologies in natural settings, while culture experiments may characterize the genome of observed morphologies and test their stability under selected environmental pressures.

The complex coccolithophore life cycle was also addressed in this thesis. The HOLP index gives an indication of the relative proportion in the community of the haploid

(holococcolithophore) and diploid (heterococcolithophore) phase among well-described established species. HOLP index values were higher in the upper photic zone (0 to 10 - 25 m depth) and increased eastward, reaching their maximum average in the Levantine Basin. Our observations suggest that holococcolithophores are adapted overall to high light intensities, which can be found in the upper photic zone. The HOLP index was overall higher (33.84 – 89.13) in oceanographic stations whose upper 100 m were characterized by relatively low nitrate plus nitrite (0.08 – 2.75 μmol L^{-1}), low phosphate concentrations (0.00 – 0.11 μmol L^{-1}), and high temperatures (15.08 – 18.76 °C). In addition, this work highlights the possible role of dissolved carbonate ion in seawater: the co-occurrence of high carbonate concentrations (200.09 – 250.95 μmol L^{-1}) and high HOLP index, indicate that these conditions might be particularly favourable for the haploid phase of coccolithophores.

A haplo-diploid life cycle could support the introduction and eastward diffusion of coccolithophore species in the Mediterranean Sea (e.g. *Calcidiscus leptoporus*, *Coccolithus pelagicus* subsp. *braarudii*): a cell might change into its haploid phase under stressing conditions, and become able to exploit the new environment. Ocean warming, enhanced water column stratification and hence exhacerbated nutrient depletion in the upper photic zone are likely to stimulate the haploid phase over the diploid phase. However, the effects of combined ocean warming and acidification are more difficult to predict. Ocean acidification is projected to shift the carbonate system, reducing the concentration of dissolved carbonate ions. Until the role of carbonate ion concentration in respect with the coccolithophore life cycle will not be clarified, we cannot predict the net coccolithophore response. If the haploid coccolithophore phase will be found to depend on high carbonate ion concentrations, then ocean acidification could have a negative impact, and perhaps even prevent haplo-diploid transformations. Future studies should therefore test the role of several environmental parameters on the coccolithophore life cycle, their relative importance and net effects. The effects of carbonate chemistry on the coccolithophore cycle need to be investigated, and carbonate ion could be an interesting parameter to focus on. Taxonomic and genomic studies are also essential to identify the different life phases of single species, and quantify the diffusion of the haplo-diploid life strategy among coccolithophores, in the Mediterranean Sea as in other marine realms.

Coccolithophore diversity patterns were explored along the longitudinal Mediterranean transect, focusing on the different trends encountered for heterococcolithophores and holococcolithophores: heterococcolithophore diversity slightly decreased eastward, while that holococcolithophore diversity increased. Although heteroccolithophores apparently

drive most of the total coccolithophore diversity in the Mediterranean Sea (> 60% of all species). Still holococcolithophore contribution in imporntant to consider, especially in the superficial waters of the Eastern Mediterranean, characterized by high light intensities, oligotrophy, high carbonate ion concentrations and low pCO_2. The environmental correlations found with respect to the two calcified coccolithophore life phases indicate that their distinct distribution patterns could derive from their distinct ecological requirements. In fact, diploid-haploid transformations in coccolithophores are supposedly stimulated by the occurrence of stressing environmental conditions, the two life phases being able to exploit difference ecological niches.

Nutrient limitation in superificial waters, high temperatures and perhaps high carbonate ion concentrations, could favour holococcolithophores over their correspondent heterococcolithophores (the haploid over the diploid phase of the same species). In the Eastern Mediterranean, the proportion of the diploid phase over the haploid phase is statistically higher than in the Wester Mediterranean, and holococcolithophores can be found at deeper depths. Considering all of these results, we come to the conclusion that holococcolithophore diversity might be higher in the Eastern basin for two main reasons: i) a higher proportion of coccolithophore species might be induced to change into their haploid form, thus enhancing the total holococcolithophore diversity; ii) holococcolithophores can survive within a larger section of the water column, and species-dependant environmental sensibilities might encourage their vertical distribution within this depth interval. Ocean warming and related water column stratification could be hence expected to expand the overall ecological niche of holococcolithophores and enhance their diversity; still, the effects of ocean acidification (e.g. lowering in pH and carbonate ion concentration) are more complex and difficult to predict until their role on the coccolithophore life phase changes will not be clarified. Futures studies could address the relationship between the coccolithophore life cycle and diversity by keeping observations separated for the haploid and diploid life phases in natural samples; it would then be interesting to compare the results obtained with this method in the Mediterranean Sea with those from other oceanic basins, especially if conducted along pronounced environmental gradients.

The mesocosm experiment study provided the opportunity to test the response of eastern Mediterranean coccolithophore community to temperature and pH levels projected for year 2100. Temperature and acidification drivers were applied both separately and combined, and coccolithophore abundance patterns were analysed in detail. The experiment included a late summer communities collected in the Cretan Sea, where the

photic zone is oligotrophic to ultra-oligotrophic, coupled with relatively high temperatures and pH. The tested natural assemblage was collected from 10 m water depth and with the diploid phase was apparently dominating on the haploid phase (higher proportion of heterococcolithophores). Among the heterococcolithophores, *Emiliania huxleyi* and *Rhabdosphaera* spp. where the most abundant and drove the total coccolithophore response. All treatments experienced temporal trends: a decrease in nutrient concentrations (nitrate, phosphate) and a slight increase in salinity. Decreasing nutrient concentrations seemed to favour the growth of *Rhabdosphaera* spp. over that of *E. huxleyi*, affecting their proportion. The possibility that increasing salinity during the experiment affected the coccolithophore community exists, but is highly uncertain. Warming was likely the overall main driver affecting negatively the whole coccolithophore community, the total holococcolithophores and heterococcolithophores, including both *E. huxleyi* and *Rhabdosphaera* spp.. Seawater acidification did not seem to have important effects on the oligotrophic coccolithophore community. Only *Rhabdosphaera* spp. growth gave the impression to be slightly stimulated by low pH. When warming and acidification were combined, the detrimental effects of warming seem to overcome any effect of acidification. The response of *Rhabdosphaera* spp. to the environmental perturbations in each treatment was particularly variable. Such observations suggests that within *Rhabdosphaera* spp. could have been included species and strains with different growth optima, and that generalizations on the response of the coccolithophores to environmental perturbations, especially acidification, should be done cautiously. Such a concept is well discussed in the literature for *E. huxleyi*, but our results indicate that also the Rhabdosphaeraceae might include species and strains having different sensitivities and resilience to ocean acidification. Overall, our results indicate that the total coccolithophore response to environmental perturbations depends strongly on the initial community composition and on the growth requirements, especially nutrients and temperature, of the species and strains included. In the case of a eastern Mediterranean community dominated by *E. huxleyi* and *Rhabdosphaera* spp., nutrient concentrations and temperature are likely the main controls on population growth, while acidification has a negligible or even slightly stimulating effect on *Rhabdosphaera* spp.. Future investigations should address the response to combined warming and acidification of different communities, characteristic of distinct depths, marine realms and seasons, in order to quantify the variability of responses and provide solid bases for projections of the total coccolithophore feedback to climate change.

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SUPPLEMENTARY MATERIAL

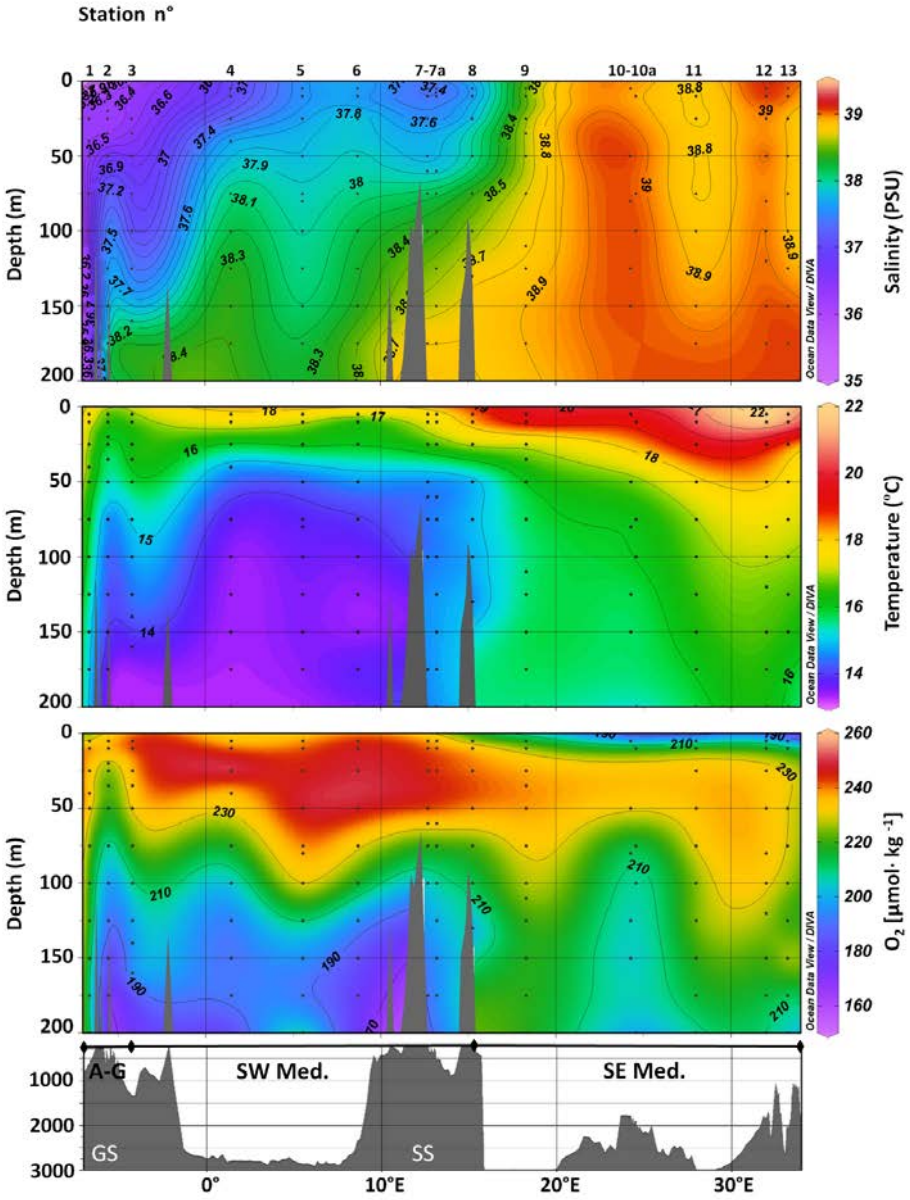

Fig. S1 Salinity, temperature and dissolved oxygen during the MedSeA 2013 cruise. A-G = Atlantic-Gibraltar province, SW Med. = SW Mediterranean province, SE Med. = SE Mediterranean province, GS = Gibraltar Strait, SS = Sicily Strait.

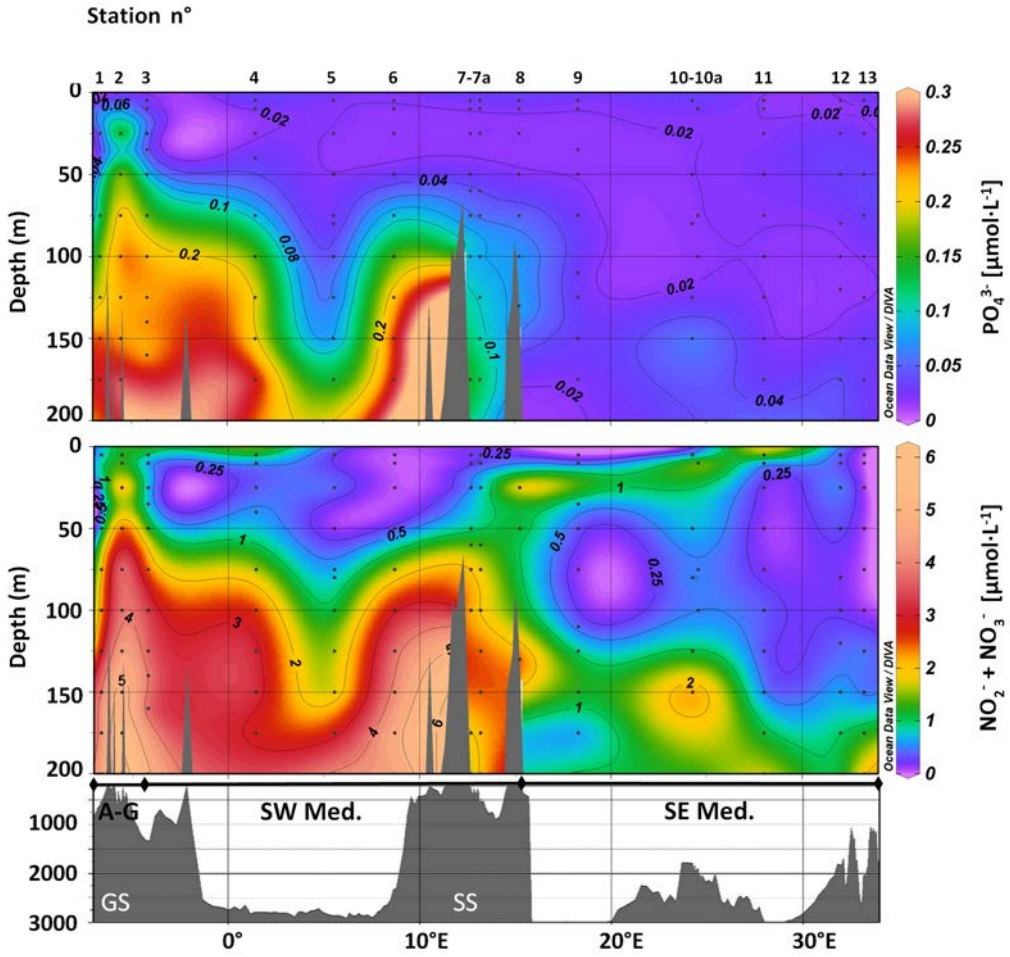

Fig. S2 Dissolved phosphate and nitrite + nitrate concentrations during the MedSeA 2013 cruise. A-G = Atlantic-Gibraltar province, SW Med. = SW Mediterranean province, SE Med. = SE Mediterranean province, GS = Gibraltar Strait, SS = Sicily Strait.

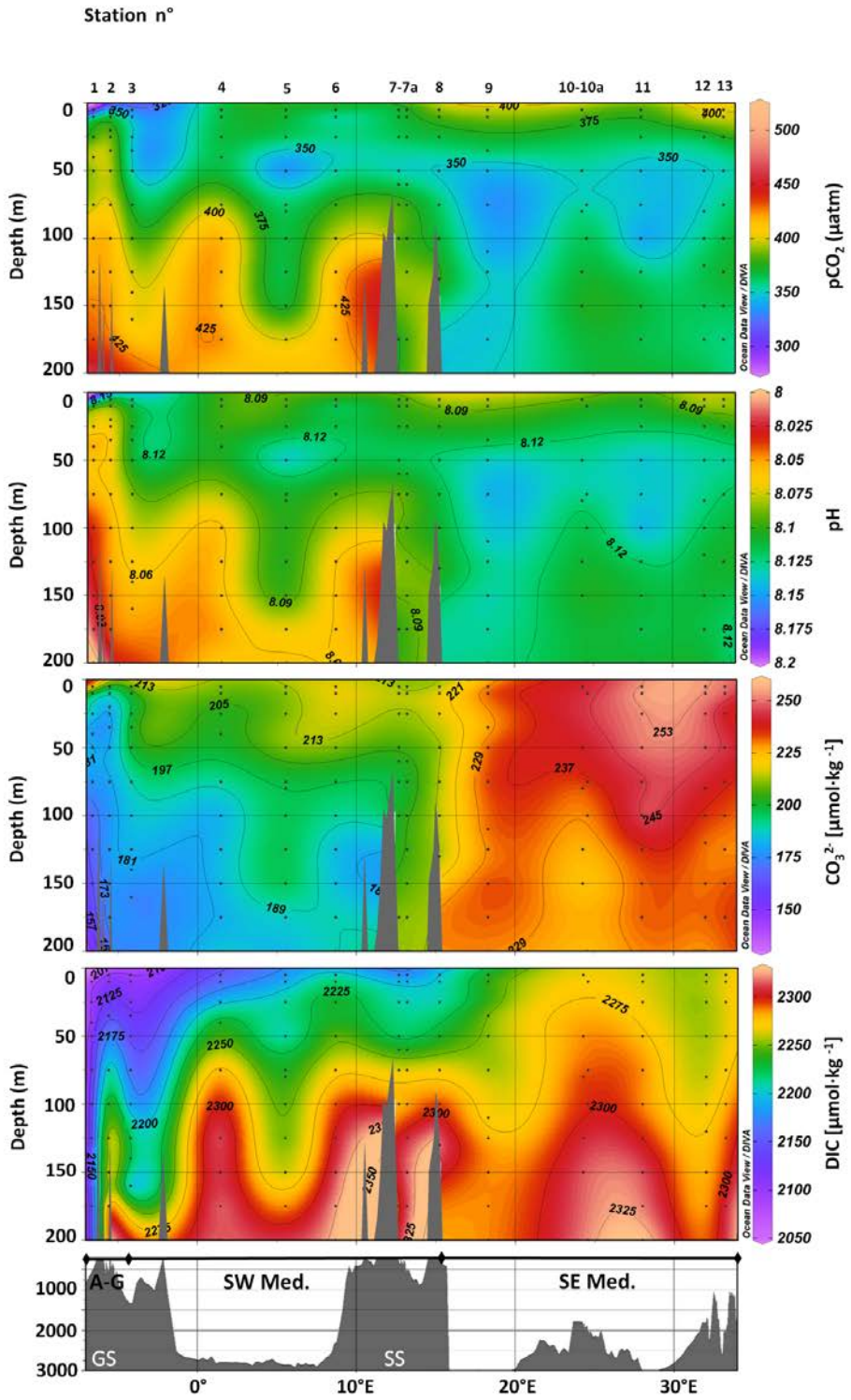

Fig. S3 Carbonate system during the MedSeA 2013 cruise (pH in total scale and showed in reversed color gradients). A-G = Atlantic-Gibraltar province, SW Med. = SW Mediterranean province, SE Med. = SE Mediterranean province, GS = Gibraltar Strait, SS = Sicily Strait.

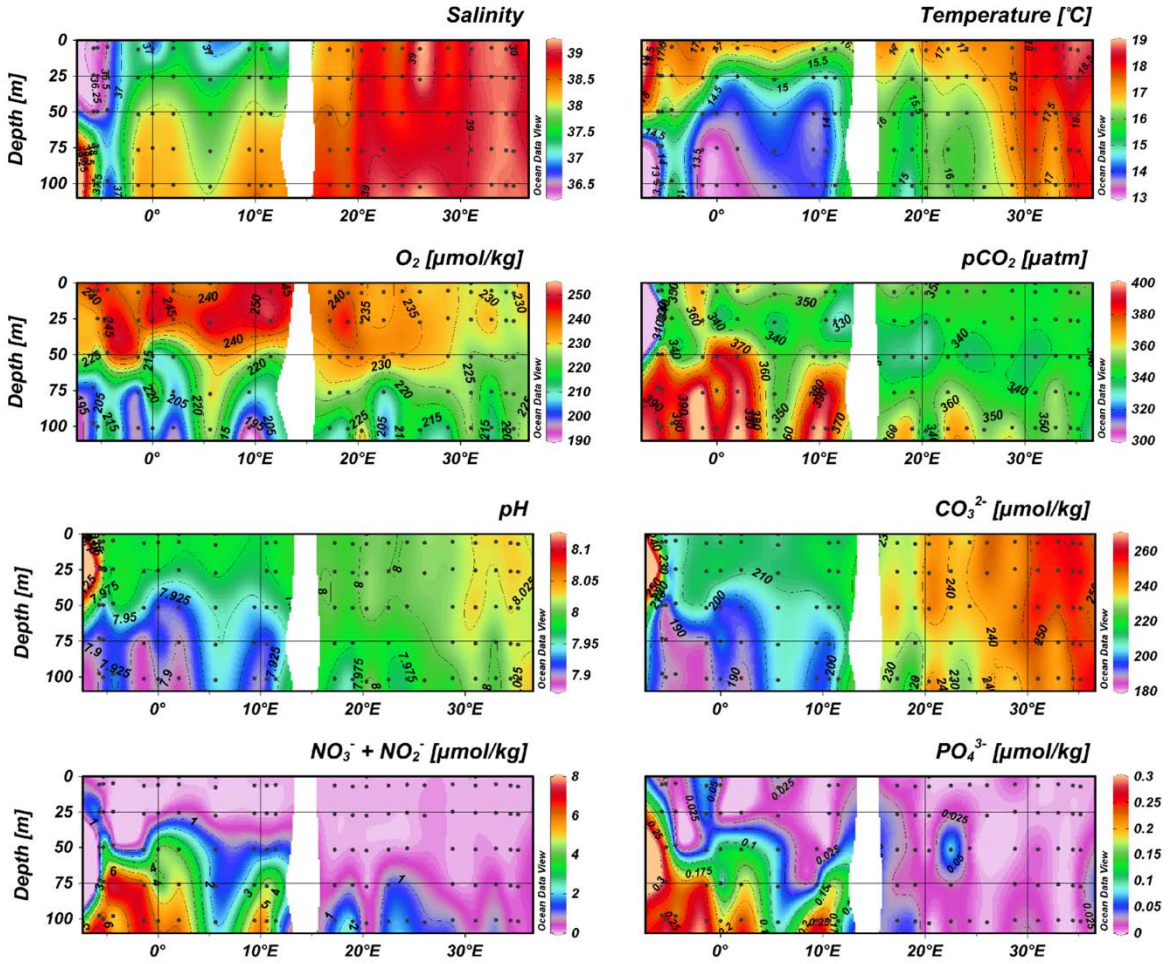

Fig. S4 Environmental parameters along the W-E transect during the Metero M84/3 cruise (from Oviedo et al., 2015).

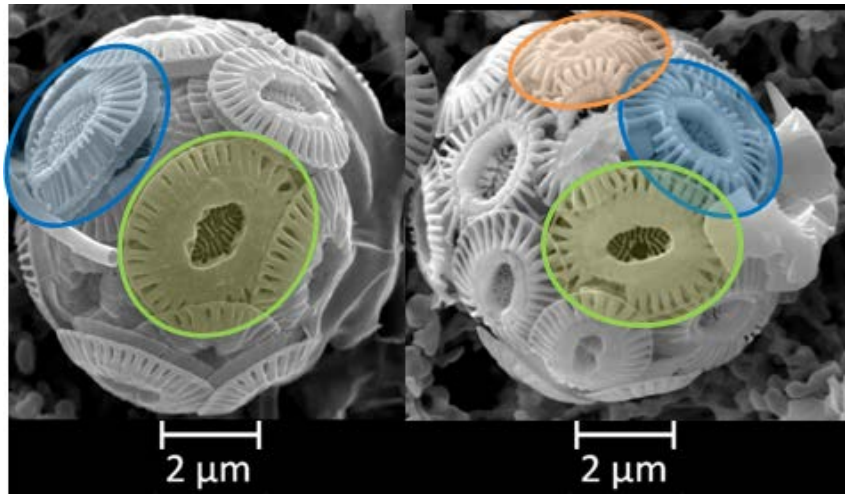

Fig. S5 Coccospheres composed by a mix of coccolith calcification varieties (blue = A1, green = A3b, orange = A3a).

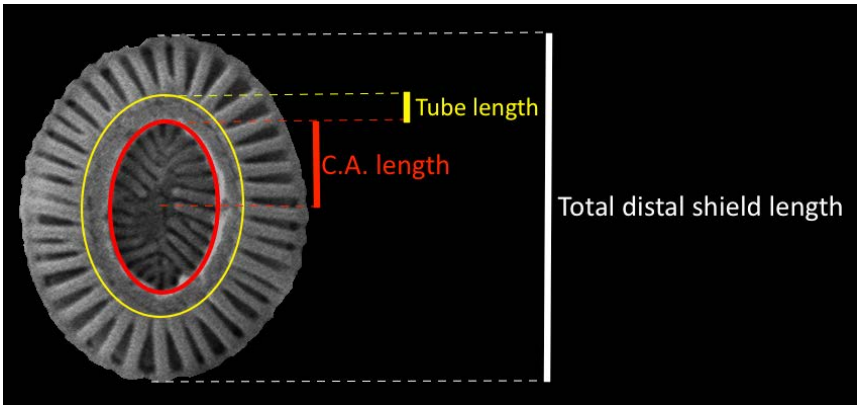

Fig. S6 Measurements of the central area (C.A. length) plus tube length, and of the total distal shield length for each coccolith.

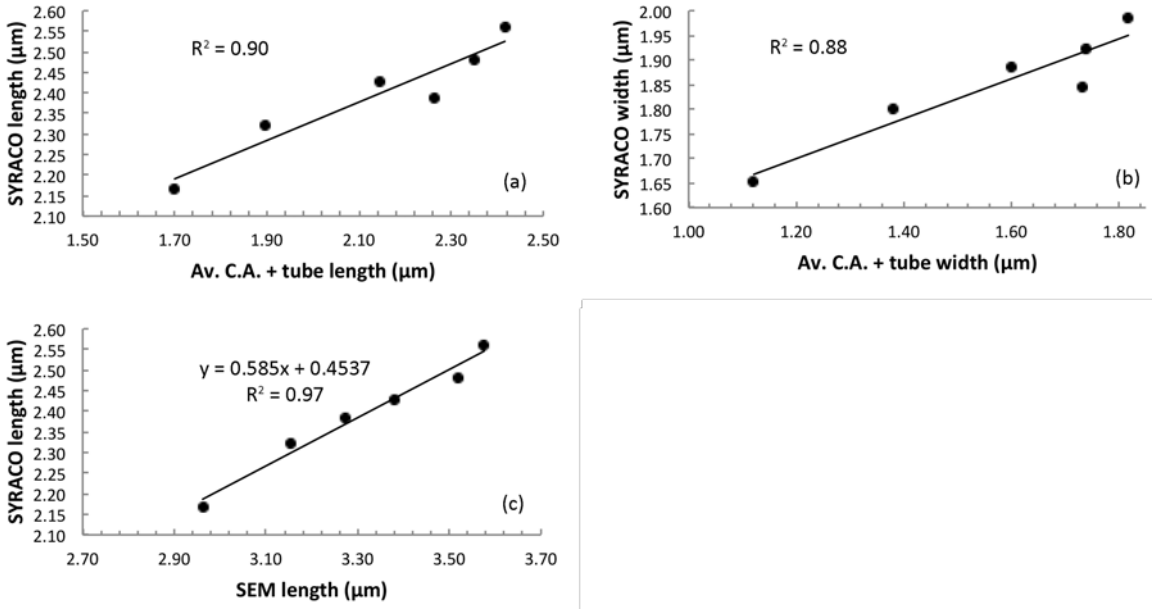

Fig. S7 Linear regressions between: coccolith length obtained from SYRACO, and coccolith central area plus tube length measured from SEM photos (a); coccolith width obtained from SYRACO, and coccolith central area plus tube width measured from SEM photos (b); coccolith length obtained from SYRACO, and length measured from SEM photos (c). The function in panel (c) was used to calculate the corrected length (L_c) for all our samples.

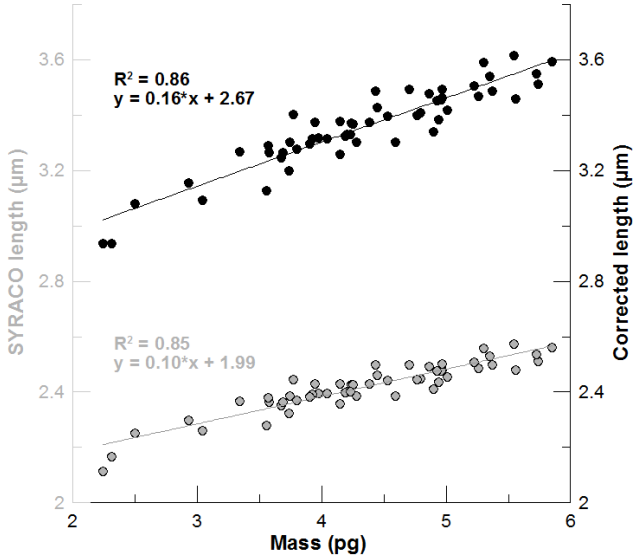

Fig. S8 Relationships between coccolith mass (M_s), the length measured with SYRACO and the corrected length (L_c). Note the different slopes of the linear regressions.

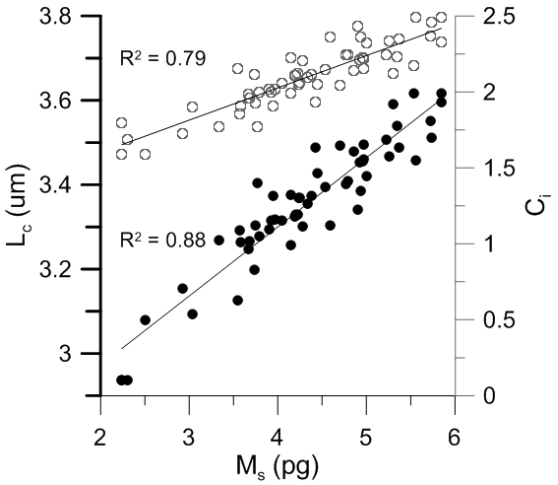

Fig. S9 Linear regressions between coccolith mass (M_s), corrected length (L_c) and calcification index (C_i).

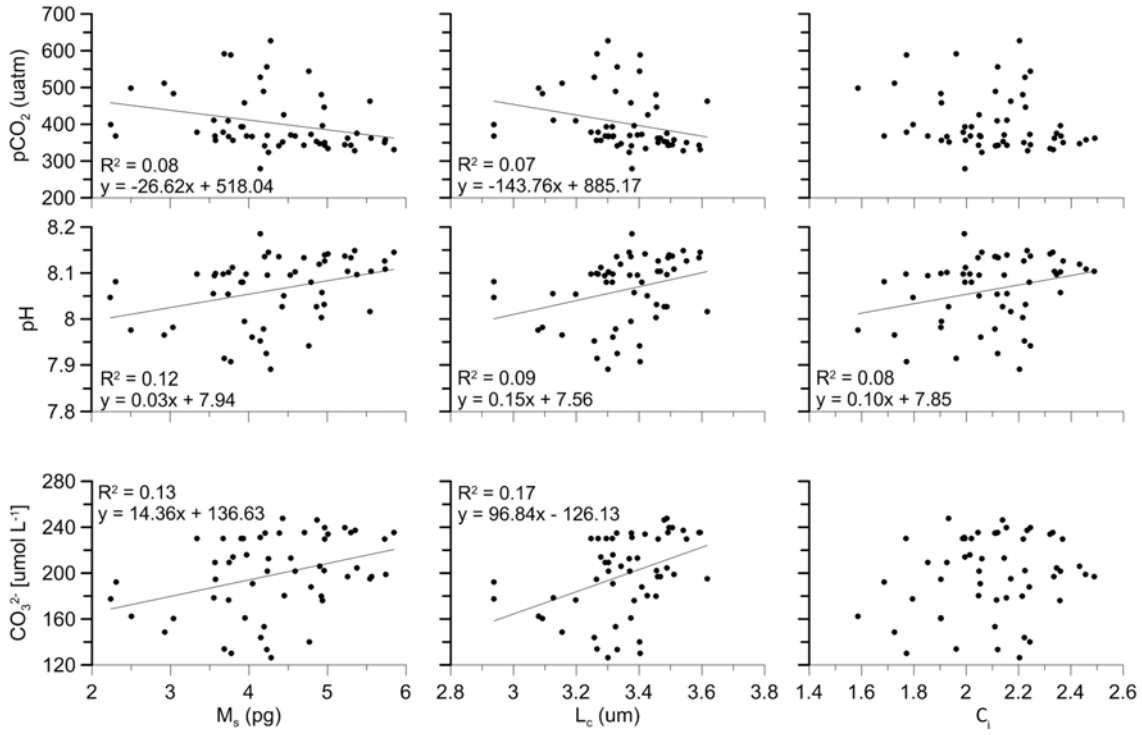

Fig. S10 Plots combining morphological (M_s, L_c, i) and carbonate system parameters. Linear regressions are shown only for combinations of parameters which have significant Spearman correlation coefficients ($p \leq 0.05$).

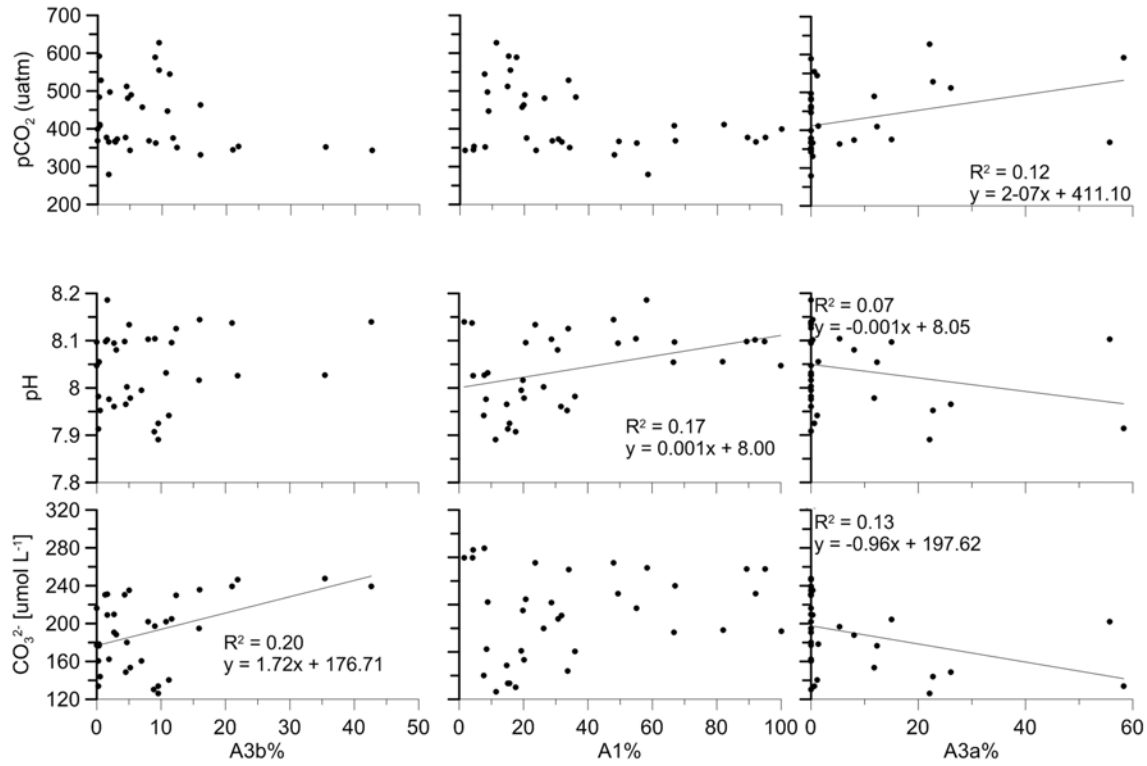

Fig. S11 Plots combining the percentage of three Type A calcification varieties and the carbonate system parameters. Linear regressions are shown only for combinations of parameteres which have significant Spearman correlation coefficients ($p \leq 0.05$).

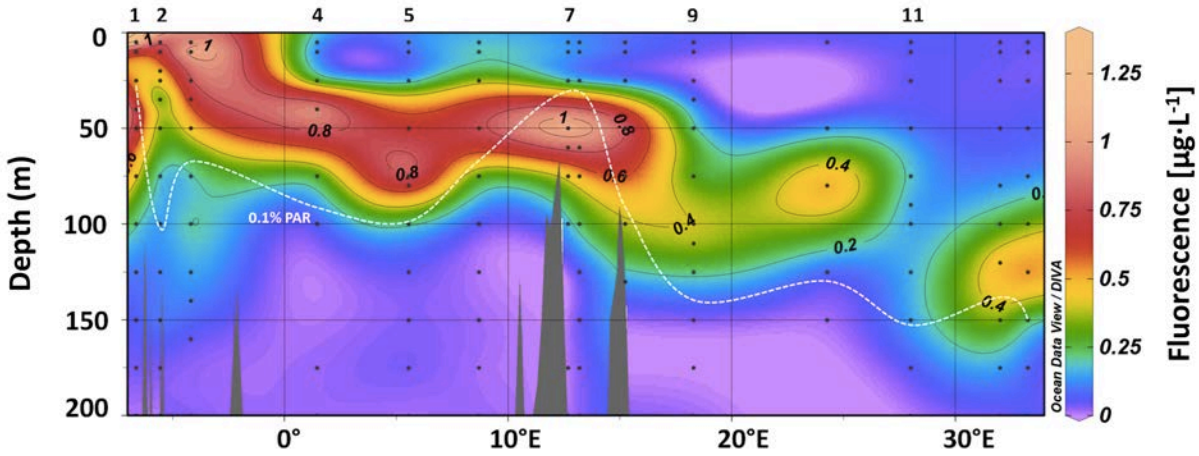

Fig. S12 Fluorescence (indicator of Chl *a* concentration) along the transect during the MedSeA cruise; superimposed is the limit of 0.1 % PAR.

	M_s	C_i
C_i	**0.86****	
L_c	**0.91****	**0.61****

Table S1. Spearman's correlation coefficients between morphological parameters (** ≤ 0.01).

	Type A%	A1%	A2%	A3a%	A3b%
M_s	0.22	**-0.33***	0.29	-0.03	**0.75****
C_i	0.03	-0.11	0.05	0.30	**0.45****
L_c	**0.29***	**-0.40***	**0.39***	-0.28	**0.81****

Table S2. Spearman's correlation coefficients between morphological parameters, Type A coccoliths (% in respect to the total E. huxleyi coccoliths), and the Type calcification varieties (% in respect to the total Type A coccoliths); ** $p \leq 0.01$, * $p \leq 0.05$.

	M_s	C_i	L_c	Type A%	A1%	A2%	A3a%	A3b%
Salinity	**0.61****	**0.37****	**0.65****	**0.53****	**-0.44****	0.19	-0.20	**0.67****
Temperature	**-0.28***	**-0.47****	-0.11	**0.29***	0.04	0.15	**-0.73****	0.03
Oxygen	-0.25	-0.24	**-0.29***	0.05	0.31	-0.14	-0.17	**-0.34***
$NO_2^-+NO_3^-$	-0.01	0.17	-0.08	**-0.42****	0.16	-0.16	**0.51****	-0.20
PO_4^{3-}	-0.12	0.10	-0.20	**-0.43****	0.25	-0.15	**0.44****	-0.33
pH	**0.41****	**0.29***	**0.39****	**0.54****	**0.36***	**-0.39***	**-0.36***	0.16
pCO_2	**-0.42****	-0.25	**-0.45****	**-0.50****	-0.20	0.22	**0.46****	-0.29
CO_3^{2-}	**0.37****	0.13	**0.47****	**0.68****	0.10	-0.17	**-0.55****	**0.37***

Table S3. Spearman's correlation coefficients between morphological parameters, Type A calcification varieties and environmental variables; ** $p \leq 0.01$, *p ≤ 0.05.

E. huxleyi Type A calcification variety	Similar morphology seen in	Coccolith/coccosphere original description	Sample collection details			
			Sample type	Location	Season	Year
A1	Triantaphyllou et al., 2010	"from high temperature water assemblage"	water	Aegean Sea (SE Med)	August-September	2011-2004
	Beaufort, Heussner, 2001	"open morphotype"	sediment trap	Bay of Biscay (N Atlantic)	June-September	1990
A3a	Beaufort, Heussner, 2001	"closed morphotype"	sediment trap	Bay of Biscay (N Atlantic)	June-September	1990
	Cros, Fortuño, 2002	"filled central area and overcalcified appearence"	water	Balearic Islands (NW Med)	September	1996
	Smith et al., 2012	"overcalcified"	water	Bay of Biscay (N Atlantic)	December-March	2008-2009
	Triantaphyllou et al., 2010	"from low temperature water assemblage"	water	Aegean Sea (SE Med)	January-March	2002-2008
A3b	Triantaphyllou et al., 2010	"from low temperature water assemblage"	water	Aegean Sea (SE Med)	January-March	2002-2008
	Dimiza et al., 2008b (Seasonality…)	"overcalcified specimen"	water	Aegean Sea (SE Med)	April	2002
	Dimiza et al., 2008a (Coccolithophores…)	-	water	Aegean Sea (SE Med)	February	2008

Table S4. Comparison of *E. huxleyi* Type A morphologies in the literature concerning the North Atlantic Ocean and the Mediterranean Sea (Med); A2 is not included being very common and considered the medium/normal calcified variety.

Long. (°E)	Lat. (°N)	HOLP province	Day-month-year	Station	Cruise	Depth (m)												
						0	5	10	25	40	50	75	80	100	110	125	150	175
-6.64	36.03	SW	2-May-13	1	MedSeA		x	x	x		x							
-5.75	35.95	SW	25-Apr-11	338	M84/3		x											
-5.56	36.12	SW	3-May-13	2	MedSeA		x	x			x	x						
-5.36	36.00	SW	25-Apr-11	337	M84/3	x	x		x		x			x				
-4.40	36.10	SW	24-Apr-11	334	M84/3		x		x		x			x				
-1.40	36.50	SW	24-Apr-11	332	M84/3		x		x									
0.00	37.05	SW	23-Apr-11	331	M84/3	x	x		x		x			x				
1.45	37.49	SW	7-May-13	4	MedSeA		x	x	x	x		x		x				
2.00	37.90	SW	23-Apr-11	329	M84/3	x	x		x		x			x				
5.55	38.52	outlier	8-May-13	5	MedSeA		x	x	x		x	x	x			x		
5.60	38.65	outlier	21-Apr-11	324	M84/3	x		x	x		x			x				
9.40	38.25	outlier	20-Apr-11	321	M84/3		x		x		x			x				
10.61	38.75	SE	20-Apr-11	320	M84/3		x							x				
11.50	38.60	SE	19-Apr-11	316	M84/3		x		x									
12.68	37.12	SE	11-May-13	7	MedSeA		x	x	x		x	x		x				
17.25	35.60	SE	14-Apr-11	305	M84/3		x		x									
18.29	35.11	SE	12-May-13	9	MedSeA		x	x	x		x	x			x	x	x	x
19.00	36.50	SE	15-Apr-11	306	M84/3		x		x		x							
20.35	35.07	SE	13-Apr-11	302	M84/3	x	x		x		x			x				
22.50	35.00	SE	12-Apr-11	299	M84/3		x											
24.33	34.50	SE	12-Apr-11	298	M84/3		x		x									
26.02	34.40	SE	11-Apr-11	297	M84/3		x		x					x				
28.00	33.50	SE	15-May-13	11	MedSeA		x	x	x		x	x		x		x	x	
28.77	33.58	SE	11-Apr-11	296	M84/3				x									
31.00	33.70	SE	10-Apr-11	294	M84/3	x	x		x		x			x				
33.00	34.07	SE	8-Apr-11	291	M84/3		x		x		x			x				
34.42	34.00	SE	9-Apr-11	293	M84/3		x											
35.17	33.99	SE	9-Apr-11	292	M84/3		x											

Table S5. Cruise, date of collection, station, geographical coordinates and sampling depth.

HETEROCOCCOLITHOPHORE SPECIES	SUB-SPECIES	%
Calcidiscus leptoporus (Murray & Blackman1898) Loeblich & Tappan, 1978		0.18
Coccolithus pelagicus (Wallich 1877) Schiller, 1930	*subsp. braarudii* (Gaardner 1962) Geisen et al., 2002	0.07
Coronosphaera mediterranea (Lohmann 1902) Gaarder, in Gaarder & Heimdal, 1977		0.44
Syracosphaera bannockii (Borsetti & Cati 1976) Cros et al., 2000		0.08
Syracosphaera histrica Kamptner, 1941		0.26
Syracosphaera molischii Schiller, 1925		1.61
Syracosphaera pulchra Lohmann, 1902		2.63
Subtotal		**5.27**
HOLOCOCCOLITHOPHORE SPECIES	**SUB-SPECIES AND FORMS**	**%**
Calcidiscus leptoporus (Murray & Blackman1898) Loeblich & Tappan, 1978 HOL	*subsp. leptoporus* (Murray & Blackman 1898) Loeblich &Tappan, 1978	0.12
	subsp. quadriperforatus (Kamptner 1937) Geisen et al., 2002	0.17
Coccolithus pelagicus (Wallich 1877) Schiller, 1930 HOL	*subsp. braarudii* (Gaardner 1962) Geisen et al., 2002	0.1
Coronosphaera mediterranea (Lohmann 1902) Gaarder, in Gaarder & Heimdal, 1977 HOL	*gracillima* type, *sensu* Geisen et al., 2002	0.15
	hellenica type, *sensu* Cros et al., 2000	0.36
	wettsteinii type, *sensu* Geisen et al., 2002	0.0
Syracosphaera bannockii (Borsetti & Cati 1976) Cros et al., 2000 HOL		1.82
Syracosphaera histrica Kamptner, 1941 HOL		0.39
Syracosphaera molischii Schiller, 1925 HOL		0.79
Syracosphaera pulchra Lohmann, 1902 HOL	*oblonga* type, *sensu* Young et al., 2003	0.87
	pirus type, *sensu* Young et al., 2003	0.26
Subtotal		**5.0**

Table S6. Heterococcolithophore and holococcolithophore species included in the HOLP index calculation; on the right, relative abundances in respect to the total coccolithophore assemblage.

Family ALISPHAERACEAE Young et al., 2003

Alisphaera capulata Heimdal in Heimdal & Gaarder, 1981
Alisphaera extenta Kleijne et al., 2002
Alisphaera gaudii Kleijne et al., 2002
Alisphaera ordinata (Kamptner 1941) Heimdal, 1973
Alisphaera unicornis Okada & McIntyre, 1977
Polycrater galapagensis Manton & Oates, 1980

Family CALCIOSOLENIACEAE Kamptner, 1927

Calciosolenia brasiliensis (Lohmann, 1919) Young *in* Young *et al.*, 2003
Calciosolenia murrayi Gran, 1912

Family CALCIDISCACEAE Young & Bown, 1997

Calcidiscus leptoporus (Murray & Blackman 1898) Loeblich & Tappan, 1978
Hayaster perplexus (Bramlette & Riedel 1954) Bukry, 1973
Umbilicosphaera foliosa (Kamptner 1963) Geisen in Sáez et al., 2003
Umbilicosphaera hulburtiana Gaarder, 1970
Umbilicosphaera sibogae (Weber - van Bosse 1901) Gaarder, 1970

Family CERATOLITHACEAE Norris, 1965; emend. Young & Bown, 2014

Ceratolithus cristatus Kamptner, 1950

Family COCCOLITHACEAE Poche, 1913; emend. Young and Bown, 1997

Coccolithus pelagicus subsp. braarudii (Gaarder 1962) Geisen et al., 2002

Family HELICOSPHAERACEAE Black, 1971

Helicosphaera carteri (Wallich 1877) Kamptner, 1954
Helicosphaera pavimentum Okada & McIntyre, 1977

Family NOELAERHABDACEAE Jerkovic 1970; emend. Young and Bown 1997

Emiliania huxleyi (Lohmann 1902) Hay & Mohler, in Hay et al. 1967
Gephyrocapsa ericsonii (McIntyre & Bé, 1967)
Gephyrocapsa muellerae Bréhéret, 1978
Gephyrocapsa oceanica Kamptner, 1943
Gephyrocapsa ornata Heimdal, 1973
Gephyrocapsa sp.
Reticulofenestra parvula (Okada & McIntyre 1977) Biekart, 1989

Family PAPPOSPHAERACEAE Jordan & Young, 1990; emend. Andruleit & Young, 2010

Pappomonas sp. type 2 Cros & Fortuño, 2002
Pappomonas sp. type 3 Cros & Fortuño, 2002
Papposphaera lepida Tangen, 1972
Papposphaera? sp. type 5 Cros & Fortuño, 2002

Family PONTOSPHAERACEAE Lemmermann, 1908

Pontosphaera japonica (Takayama 1967) Nishida, 1971
Scyphosphaera apsteinii Lohmann, 1902

Family RHABDOSPHAERACEAE Haeckel, 1894

Acanthoica acanthos Schiller 1925
Acanthoica quattrospina Lohmann, 1903
Algirosphaera cucullata (Lecal-Schlauder 1951) Young et al., 2003

Algirosphaera robusta (Lohmann 1902) Norris, 1984
Cyrtosphaera aculeata (Kamptner 1941) Kleijne, 1992
Discosphaera tubifera (Murray & Blackman 1898) Ostenfeld, 1900
Palusphaera vandelii Lecal, 1965
Palusphaera sp. 1 Cros & Fortuño, 2002
Rhabdosphaera clavigera var. *clavigera* Murray & Blackman, 1898
Rhabdosphaera clavigera var. *stylifera* (Lohmann, 1902) Kleijne and Jordan, 1990
Rhabdosphaera xiphos (Deflandre & Fert 1954) Norris, 1984

Family SYRACOSPHAERACEAE (Lohmann, 1902) Lemmermann, 1903

Calciopappus caudatus Gaarder & Ramsfjell, 1954
Calciopappus rigidus Heimdal in Heimdal & Gaarder, 1981
Coronosphaera mediterranea (Lohmann 1902) Gaarder, in Gaarder & Heimdal, 1977
Michaelsarsia elegans Gran, 191
Ophiaster formosus Gran, 1912
Ophiaster hydroideus (Lohmann 1903) Lohmann, 1913
Syracosphaera ampliora Okada & McIntyre, 1977
Syracosphaera anthos (Lohmann 1912) Janin, 1987
Syracosphaera bannockii (Borsetti & Cati 1976) Cros et al., 2000
Syracosphaera borealis Okada & McIntyre, 1977
Syracosphaera corolla Lecal, 1966
Syracosphaera dilatata Jordan et al., 1993
Syracosphaera hirsuta Kleijne & Cros, 2009
Syracosphaera lamina Lecal-Schlauder, 1951
Syracosphaera margíniporata Knappertsbusch, 1993
Syracosphaera molischii Schiller, 1925
Syracosphaera nana (Kamptner 1941) Okada & McIntyre, 1977
Syracosphaera nodosa Kamptner, 1941
Syracosphaera noroitica Knappertsbusch, 1993
Syracosphaera orbiculus Okada & McIntyre, 1977
Syracosphaera ossa (Lecal 1966) Loeblich & Tappan, 1968
Syracosphaera pirus Halldal & Markali 1955
Syracosphaera protrudens Okada & McIntyre, 1977
Syracosphaera pulchra Lohmann, 1902
Syracosphaera rotula Okada & McIntyre, 1977
Syracosphaera tumularis Sánchez-Suárez, 1990
Syracosphaera sp. Type D Klieijne, 1993

Family UMBELLOSPHAERACEAE, Young and Kleijne in Young et al. 2003

Umbellosphaera irregularis Paasche in Markali & Paasche, 1955
Umbellosphaera tenuis (Kamptner 1937) Paasche in Markali & Paasche, 1955

COCCOLITH GENERA *INCERTAE SEDIS*

Gladiolithus flabellatus (Halldal & Markali 1955) Jordan & Chamberlain, 1993

NANNOLITH GENERA *INCERTAE SEDIS*

Ericiolus sp.
Florisphaera profunda Okada & Honjo, 1973

Table S7. List of identified heterococcolithophore species.

Acanthoica quattrospina Lohmann, 1903 HOL
Anthosphaera lafourcadii (Lecal 1967) Kleijne, 1991
Anthosphaera periperforata Kleijne 1991
Anthosphaera sp. type A Cros & Fortuño, 2002
Anthosphaera sp. type C Cros & Fortuño, 2002
Calcidiscus leptoporus subsp. *leptoporus* (Murray & Blackman 1898) Loeblich & Tappan, 1978 HOL
Calcidiscus leptoporus subsp. *quadriperforatus* (Kamptner 1937) Geisen et al., 2002 HOL
Calicasphaera blokii Kleijne, 1991
Calicasphaera concava Kleijne, 1991
Calyptrolithina divergens (Halldal & Markali 1955) Heimdal, 1982
Calyptrolithina multipora (Gaarder in Heimdal & Gaarder 1980) Norris, 1985
Calyptrosphaera dentata Kleijne, 1991
Calyptrosphaera cialdii Borsetti & Cati, 1976
Calyptrosphaera heimdaliae Norris, 1985
Calyptrosphaera sphaeroidea Schiller, 1913 HOL
Coccolithus pelagicus subsp. *braarudii* (Gaarder 1962) Geisen et al., 2002 HOL
Corisphaera gracilis Kamptner, 1937
Corisphaera strigilis Gaarder, 1962
Coronosphaera mediterranea (Lohmann 1902) Gaarder, in Gaarder & Heimdal, 1977
HOL *gracillima* type, *sensu* Geisen et al., 2002
Coronosphaera mediterranea (Lohmann 1902) Gaarder, in Gaarder & Heimdal, 1977
HOL *hellenica* type, *sensu* Cros et al., 2000
Coronosphaera mediterranea (Lohmann 1902) Gaarder, in Gaarder & Heimdal, 1977
HOL *wettsteinii* type, *sensu* Geisen et al., 2002
Gliscolithus amitakareniae Norris, 1985
Helicosphaera HOL *confusus* type, informal = *Syracolithus confusus* Kleijne 1991
Helicosphaera HOL *catilliferus* type = *Syracosphaera catillifera* Kamptner 1937
Helladosphaera cornifera (Schiller 1913) Kamptner, 1937
Homozygosphaera arethusae HOL (Kamptner 1941) Kleijne 1991 = *Syracosphaera arethusae* (Kamptner 1941) Triantaphyllou et al. 2016 HOL
Homozygosphaera triarcha Halldal & Markali, 1955
Homozygosphaera vercellii Borsetti & Cati, 1979
Pontosphaera japonica (Takayama 1967) Nishida, 1971 HOL
Poricalyptra gaarderiae (Borsetti & Cati 1976) Kleijne, 1991
Poritectolithus sp. 2 Cros & Fortuño 2002
Scyphosphaera apsteinii Lohmann, 1902 HOL
Sphaerocalyptra adenensis Kleijne, 1991
Sphaerocalyptra quadridentata (Schiller 1913) Deflandre, 1952
Sphaerocalyptra sp. 1 Cros & Fortuño, 2002
Sphaerocalyptra sp. 3 Cros & Fortuño, 2002
Sphaerocalyptra sp. 6 Cros & Fortuño, 2002
Syracolithus sp. type A Kleijne, 1991
Syracosphaera anthos (Lohmann 1912) Janin, 1987 HOL
Syracosphaera arethusae (Kamptner 1941) Triantaphyllou et al. 2016 HOL
Syracosphaera bannockii (Borsetti & Cati 1976) Cros et al., 2000 HOL
Syracosphaera histrica Kamptner, 1941 HOL
Syracosphaera molischii Schiller, 1925 HOL
Syracosphaera nana HOL (Kamptner 1941) Okada & McIntyre, 1977 HOL
Syracosphaera pulchra Lohmann, 1902 HOL *oblonga* type, sensu Young et al., 2003
Syracosphaera pulchra Lohmann, 1902 HOL *pirus* type, sensu Young et al., 2003
Zygosphaera amoena Kamptner, 1937

Table S8. List of identified holococcolithophore species.