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# Coccolithophore community response to ocean acidification and warming in the Eastern Mediterranean Sea: results from a mesocosm experiment

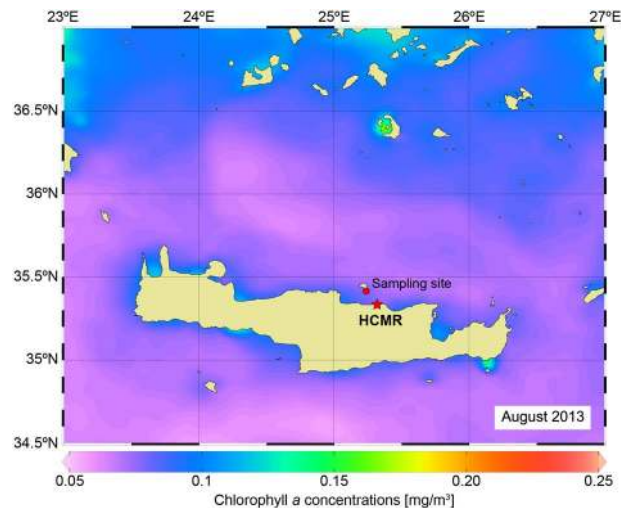
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Mesocosm experiments have been fundamental to investigate the effects of elevated CO<sub>2</sub> and ocean acidification (OA) on planktic communities. However, few of these experiments have been conducted using naturally nutrient-limited waters and/or considering the combined effects of OA and ocean warming (OW). Coccolithophores are a group of calcifying phytoplankton that can reach high abundances in the Mediterranean Sea, and whose responses to OA are modulated by temperature and nutrients. We present the results of the first land-based mesocosm experiment testing the effects of combined OA and OW on an oligotrophic Eastern Mediterranean coccolithophore community. Coccolithophore cell abundance drastically decreased under OW and combined OA and OW (greenhouse, GH) conditions. *Emiliana huxleyi* calcite mass decreased consistently only in the GH treatment; moreover, anomalous calcifications (i.e. coccolith malformations) were particularly common in the perturbed treatments, especially under OA. Overall, these data suggest that the projected increase in sea surface temperatures, including marine heatwaves, will cause rapid changes in Eastern Mediterranean coccolithophore communities, and that these effects will be exacerbated by OA.

CO<sub>2</sub> anthropogenic emissions into the atmosphere have been increasing since the industrial revolution, especially in the last decades. This process alters the climate system and the ocean uptake of anthropogenic CO<sub>2</sub>, causing shifts in marine carbonate chemistry (i.e. ocean acidification, OA)<sup>1</sup>. Moreover, the extra heat trapped in the atmosphere by greenhouse gases is largely transferred to the ocean, causing ocean warming (OW), enhancing water column stratification (i.e. a process that hampers the supply of nutrients to the upper ocean layers)<sup>2,3</sup>, and increasing the frequency, intensity, extent and duration of marine heatwaves (i.e. periods of few days to few months characterized by extremely high surface ocean temperatures)<sup>4,5</sup>.

The Mediterranean region is considered particularly vulnerable to climate change<sup>6–9</sup>. Anthropogenic CO<sub>2</sub> has already invaded the whole Mediterranean basin<sup>10</sup> and a pH lowering of 0.245–0.457 units has been estimated for its surface waters by year 2,100 based on two IPCC atmospheric CO<sub>2</sub> scenarios<sup>11,12</sup>. Meanwhile, atmospheric warming is expected to proceed in the Mediterranean area 20% faster than the global average<sup>13</sup>. The results of a linear black box model suggest that this warming might induce a 5.8 °C increase in sea surface temperatures (SST) by the end of this century (compared to the average SST for the period 1986–2015)<sup>14</sup>. According to the IPCC RCP8.5 (a scenario consistent with the worst-case emissions<sup>15</sup>), the Mediterranean Sea will be subjected to long-lasting marine heatwaves, occurring at least once per year, by the end of the twenty-first century<sup>4</sup>. Interestingly, the Mediterranean Sea is already seasonally subject to vertical stratification, especially in its oligotrophic

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**Figure 1.** Aqua MODIS annual composite of Chlorophyll *a* concentration (L-3) for year 2013. The approximate locations of the sampling site and the Hellenic Centre for Marine Research (HCMR) (where the mesocosm experiment was carried out) are also indicated.

eastern regions<sup>16–18</sup>. Such conditions might be exacerbated by OW<sup>19–22</sup>, with serious consequences on marine biodiversity and productivity<sup>23,24</sup>.

Coccolithophores are a group of unicellular eukaryotic phytoplankton and the vast majority of them produce small elaborate calcite plates (*i.e.* coccoliths) covering their cell<sup>25</sup>. These organisms exercise a significant role in the Earth's biogeochemical cycles, contributing to ~50% of the total CaCO<sub>3</sub> pelagic sedimentation<sup>26,27</sup>, and are distributed globally, including the Mediterranean Sea<sup>28,29</sup>. Many laboratory experiments have investigated the response of coccolithophores to OA<sup>30–33</sup>, but only a minority of them have focused on the combined effects of multiple environmental variables<sup>34–43</sup>. Meanwhile, most of the previous CO<sub>2</sub> perturbation mesocosm experiments involving coccolithophores have been conducted under naturally eutrophic conditions, or contemplated the addition of nutrients to stimulate cell growth (see review<sup>33</sup>): only a few of them were performed under nutrient limitation<sup>44–47</sup>.

The response of Mediterranean coccolithophores to OA and OW can be seasonal, species- and strain-specific<sup>48–51</sup>. This fact, combined with the occurrence of highly diverse coccolithophore communities in the Mediterranean Sea<sup>28,52,53</sup> and other oligotrophic systems<sup>54</sup>, complicates the prediction of their overall response to climate change. Any projection is further complicated by the fact that several coccolithophore species possess a haplo-diploid life cycle: during the haploid (holococcolithophore, HOL) and diploid (heterococcolithophore, HET) life stages, a single cell can change its calcification process and produce different kinds of coccoliths, likely adapting to different environmental conditions<sup>55–57</sup>.

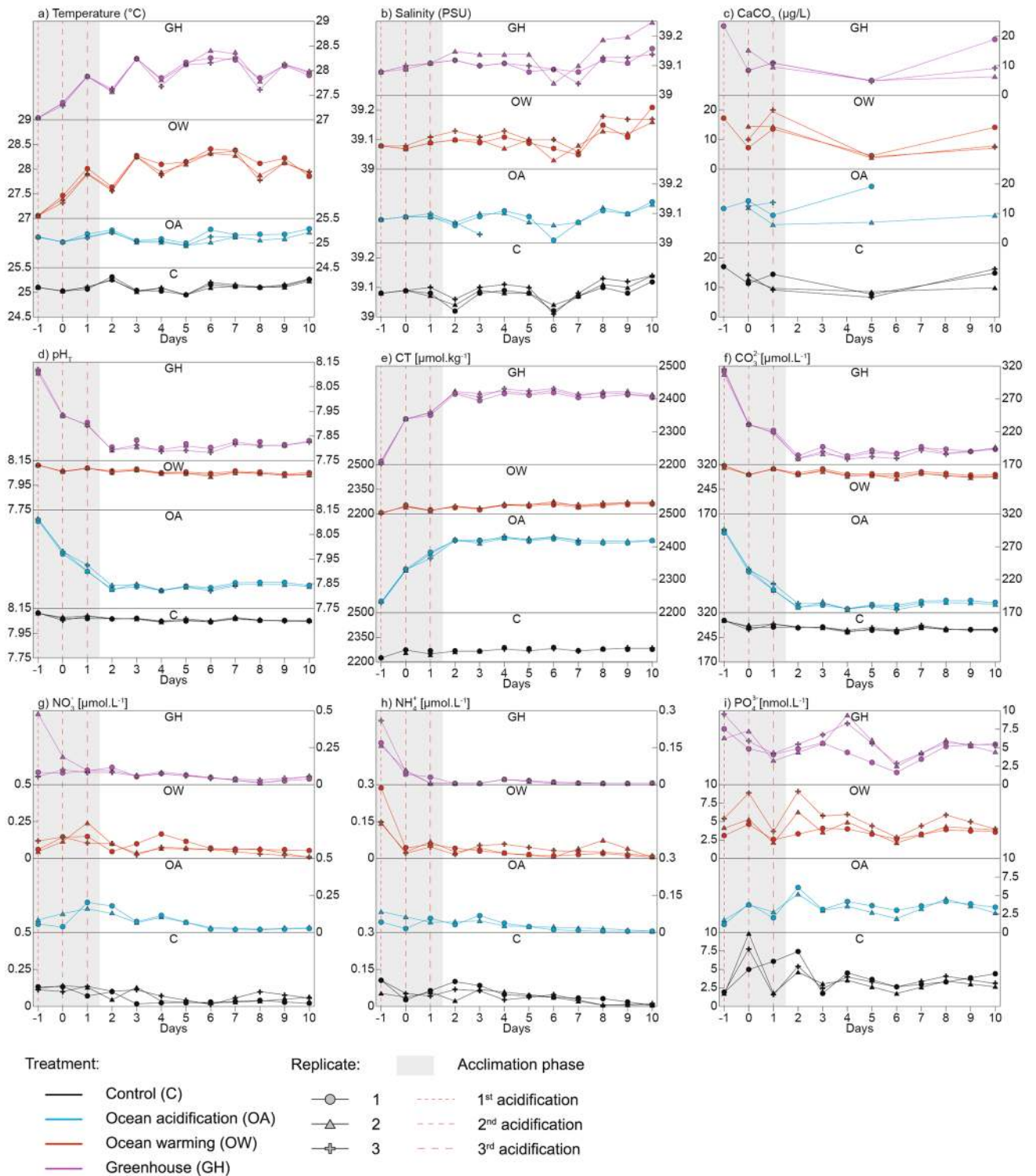
A previous mesocosm experiment<sup>58</sup> tested the effects of combined OA and OW on a phytoplankton community from the western Baltic Sea composed of non-calcifying species, highlighting a slight stimulating effect of OA and a strong negative effect of OW. In this work, we present the results of a mesocosm experiment that tested the combined effects of OA and OW on a natural Eastern Mediterranean coccolithophore community (collected in summer, offshore the island of Crete, Greece) (Fig. 1). The experimental data were analysed to disentangle the response of the local coccolithophore community and its dominant species to the environmental perturbations. Our working hypothesis was that the growth and calcification of local coccolithophores would be affected by both temperature and carbonate chemistry perturbations.

## Results

**Physico-chemical parameters during the mesocosm experiment.** The evolution of salinity, nutrient concentrations, temperature, carbonate chemistry and CaCO<sub>3</sub> concentrations during the experiment in all mesocosms is shown in Fig. 2a–i. The average salinity was  $39.10 \pm 0.01$  PSU in all mesocosms, and it slightly increased over time as a result of evaporation, reaching a maximum of 39.25 PSU on day 10 (in GH2). Salinity showed a significant drop in the 3<sup>rd</sup> replicate of the OA treatment (OA3) from day 3 onward (Fig. 2b), likely due to the presence of a hole in the bag. Notably, this salinity anomaly was accompanied by an unusual variability in bacterial production, primary production, and Chlorophyll *a*.

In order to avoid any bias in the interpretation of the data, we decided to exclude all the results obtained from OA3 from all the statistical analyses and averages presented hereafter.

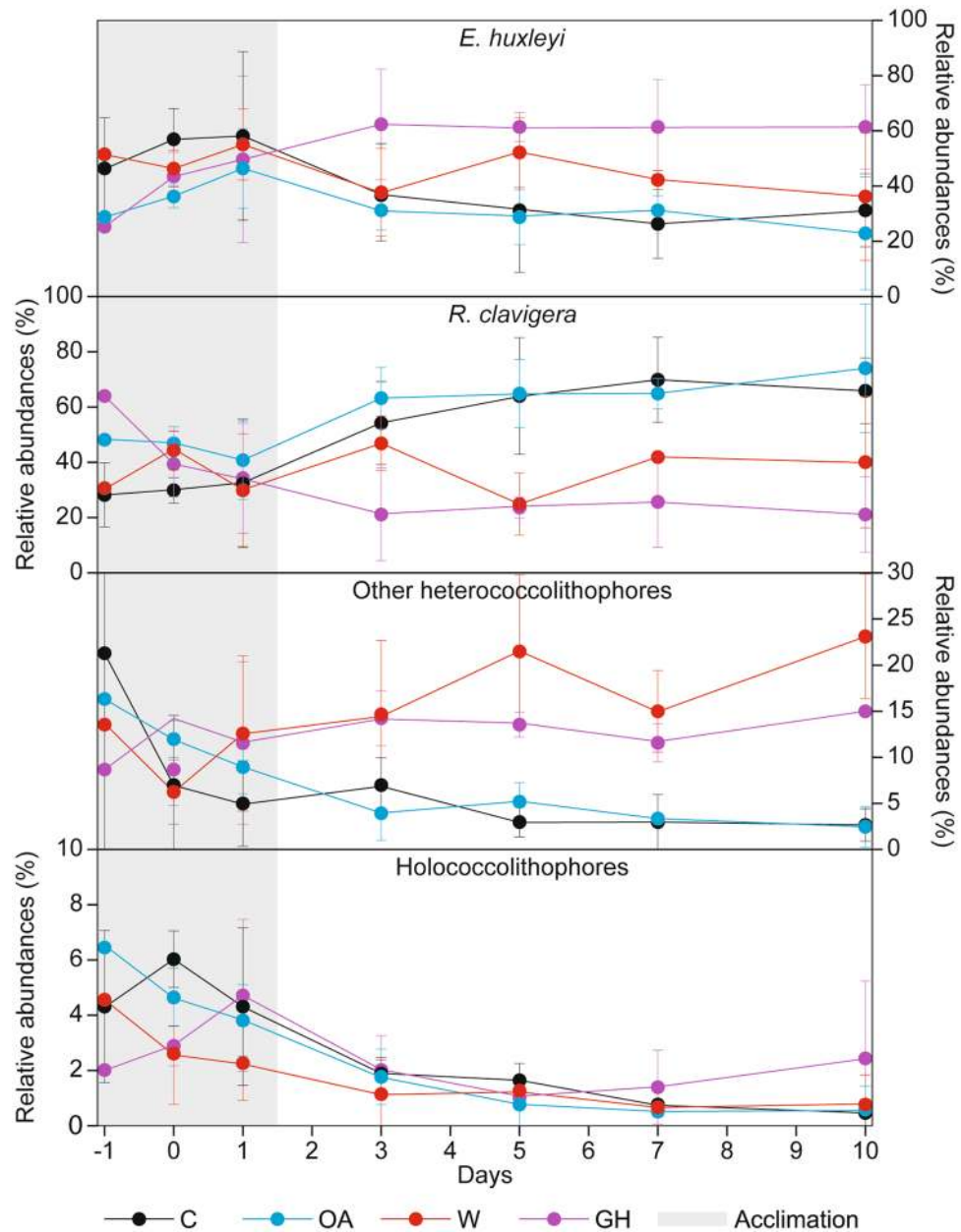
The CaCO<sub>3</sub> concentrations were very similar in the four treatments at the beginning of the experiment (average =  $11.53 \pm 2.58$  µg/L on day 0). Interestingly, the concentrations decreased until day 5, when they reached  $6.10 \pm 1.83$  µg/L on average among all treatments. The values increased again between days 5 and 10 (average =  $13.90 \pm 4.20$  µg/L in the C and OA mesocosms; average =  $10.67 \pm 4.88$  µg/L in the OW and GH mesocosms).



**Figure 2.** Variability of the main physico-chemical parameters during the experiment: temperature (a), salinity (b), CaCO<sub>3</sub> (c), pH in total scale (pH<sub>T</sub>) (d), dissolved inorganic carbon (CT) (e), CO<sub>3</sub><sup>2-</sup> (f), NO<sub>3</sub><sup>-</sup> (g), NH<sub>4</sub><sup>+</sup> (h), and PO<sub>4</sub><sup>3-</sup> (i).

Dissolved PO<sub>4</sub><sup>3-</sup> was very scarce in all mesocosms and did not show a consistent temporal pattern; meanwhile, the concentrations of dissolved NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> decreased gradually over time.

The temperature was maintained stable (average = 27.7 ± 0.5 °C in the OW and greenhouse (GH) mesocosms; average = 25.0 ± 0.3 °C in the control (C) and OA mesocosms) throughout the experiment.



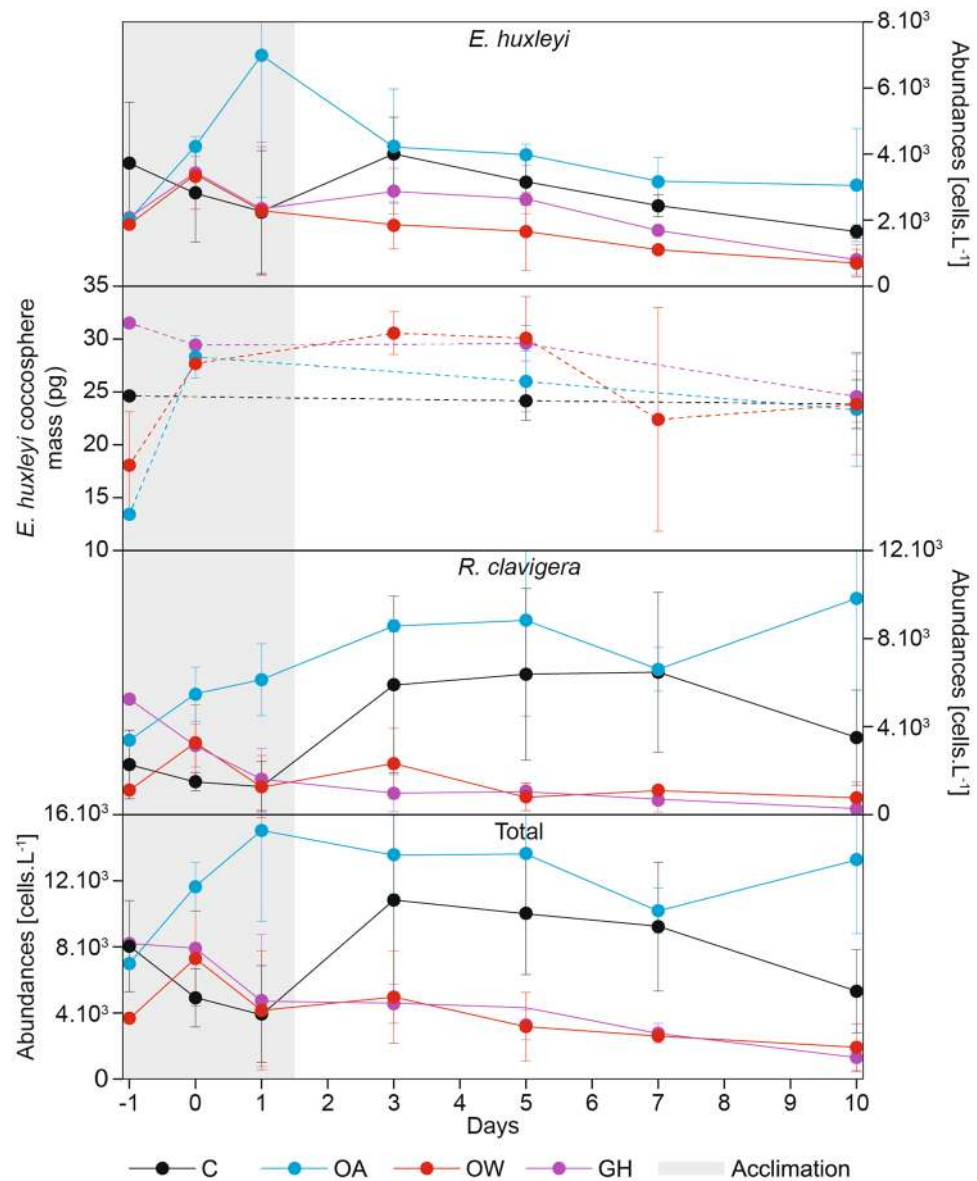
**Figure 3.** Coccolithophore relative abundances: *E. huxleyi* (a), *R. clavigera* (b), all heterococcolithophores except for *E. huxleyi* and *R. clavigera* (c), and holococcolithophores (d). The dots and the vertical bars indicate the average and standard deviation values, respectively.

The seawater pH was maintained at  $8.06 \pm 0.02$  in the C and OW mesocosms throughout experiment (between days -1 and 10). Meanwhile, in the OA and GH mesocosms, the pH dropped until day 1 (following acidification) and remained stable until the end of the experiment (average =  $7.82 \pm 0.02$  between days 2 and 10).

**Coccolithophore production, taxonomy and *Emiliana huxleyi* calcite mass.** The coccolithophore community was mainly composed of HET (94–100%). *Emiliana huxleyi* and *Rhabdosphaera clavigera* were the two major species, representing 23–62% and 21–74% of the total population, respectively. On the other hand, the relative abundances of *Syracosphaera* spp., *Gephyrocapsa muelleriae*, *Umbellosphaera* spp. and HOL oscillated between 1–20%, 0–10%, 0–6% and 0–7%, respectively.

We analysed the temporal evolution of the coccolithophore relative abundances in the four treatments (Fig. 3a–d): *R. clavigera* increased in the C and OA treatments, while it remained relatively stable in the OW treatments and decreased in the GH ones; on the contrary, *E. huxleyi* decreased in the C and OA treatments and increased in the GH ones. Additionally, HOL decreased over time in all treatments.

On day -1, the coccolithophore cell densities were within the same order of magnitude in all treatments: the total values oscillated between  $3.64 \times 10^3$ – $8.20 \times 10^3$  cells  $L^{-1}$  (Fig. 4d), those of *E. huxleyi* between



**Figure 4.** Coccolithophore absolute abundances: *E. huxleyi* (a), *R. clavigera* (c), and total coccolithophores (d). Average coccosphere calcite mass of *E. huxleyi* (b). The dots and the vertical bars indicate the average and standard deviation values, respectively.

$1.88 \times 10^3$ – $3.72 \times 10^3$  cells  $L^{-1}$  (Fig. 4a) and those of *R. clavigera* between  $1.10 \times 10^3$ – $5.25 \times 10^3$  cells  $L^{-1}$  (Fig. 4c). The average total coccolithophore abundance during the experiment was  $7.14 \times 10^3$  cells  $L^{-1}$ , while the minimum and maximum coccolithophore abundances were 54 cells  $L^{-1}$  (day 1, OW2) and  $1.84 \times 10^4$  cells  $L^{-1}$  (day 10, OA2), respectively. After the acclimation phase, the average absolute cell abundances in the OW and GH treatments were clearly lower than in the C and OA treatments. Notably, in all treatments, the total coccolithophore abundance showed a very low correlation with the total Chlorophyll *a* (Fig. S1 online), suggesting that the contribution of this group of organisms to the total Chlorophyll *a* was marginal. This was to be expected in our mesocosm experiment: eukaryotic picoplankton (defined as cells with size  $< 3 \mu m$ ) is generally the major contributor to plankton biomass and production in the Eastern Mediterranean Sea, especially in summer<sup>59,60</sup>.

A series of ANOVA and Tukey tests highlighted significant differences between the four treatments, in both the total coccolithophore and *R. clavigera* absolute abundances (Tables 1, 2). Two longitudinal data analyses were also conducted. The first (Model 1) demonstrated a significant temporal decrease in the total abundance of coccolithophore cells, as well as in the absolute abundances of *E. huxleyi* and *R. clavigera*, in the OW and GH treatments; moreover, it highlighted a significant increase in *R. clavigera* in the C treatment (Table 3). The second longitudinal data analysis (Model 2) demonstrated an overall positive correlation between the coccolithophore absolute abundances and the dissolved nutrient concentrations; the only exception was found for *R. clavigera*, which was inversely correlated with the  $NO_3^-$  concentrations in the C treatment (Table 4).

Response variable	df	Mean square	F	<i>p</i>
Tot. coccolithophores n°	3	31,276	4.17	<b>0.01</b>
<i>R. clavigera</i> n°	3	68,141	15.39	<b>0.00</b>
<i>E. huxleyi</i> n°	3	9,188	2.00	0.12
<i>E. huxleyi</i> mass	3	29	1.34	0.28

**Table 1.** Results of the ANOVA test. Significant values ( $p < 0.05$ ) are in bold.

Response variable	df	Mean square	HSD	Significant differences found between
Tot. coccolithophore n°	73	7,494	69.71	C-OW; OW-OA
<i>R. clavigera</i> n°	74	4,429	53.59	C-OW; C-GH; OW-OA; GH-OA

**Table 2.** Results of the Tukey test. *HSD* honestly significant difference.

Treatment	Total coccolithophores	<i>E. huxleyi</i>	<i>R. clavigera</i>	<i>E. huxleyi</i> mass
C			↑ ( $p = 4.7 \times 10^{-2}$ )	
W	↓ ( $p = 1.3 \times 10^{-2}$ )	↓ ( $p = 7.0 \times 10^{-3}$ )	↓ ( $p = 5.7 \times 10^{-2}$ )	
OA				
GH	↓ ( $p = 6.5 \times 10^{-5}$ )	↓ ( $p = 6.0 \times 10^{-4}$ )	↓ ( $p = 3.0 \times 10^{-4}$ )	↓ ( $p = 8.0 \times 10^{-3}$ )

**Table 3.** Results of model 1. The arrows pointing upward (downward) indicate significant increases (decreases) in coccolithophore cell abundance and *E. huxleyi* coccosphere calcite mass. The correspondent *p*-values are indicated within parentheses.

Treatment	Total coccolithophores	<i>E. huxleyi</i>	<i>R. clavigera</i>	<i>E. huxleyi</i> mass
C		+NH <sub>4</sub> <sup>+</sup> ( $p = 1.4 \times 10^{-2}$ )	-NO <sub>3</sub> <sup>-</sup> ( $p = 2.6 \times 10^{-2}$ )	
W	+PO <sub>4</sub> <sup>3-</sup> ( $p = 7.0 \times 10^{-2}$ )		+PO <sub>4</sub> <sup>3-</sup> ( $p = 8.6 \times 10^{-2}$ )	
OA		+NO <sub>3</sub> <sup>-</sup> ( $p = 3.0 \times 10^{-2}$ )		+PO <sub>4</sub> <sup>3-</sup> ( $p = 5.0 \times 10^{-3}$ )
GH	+NO <sub>3</sub> <sup>-</sup> ( $p = 7.0 \times 10^{-3}$ ); +NH <sub>4</sub> <sup>+</sup> ( $p = 2.8 \times 10^{-2}$ )	+NO <sub>3</sub> <sup>-</sup> ( $p = 8.7 \times 10^{-2}$ )	+NO <sub>3</sub> <sup>-</sup> ( $p = 5.5 \times 10^{-2}$ ); +NH <sub>4</sub> <sup>+</sup> ( $p = 1.0 \times 10^{-3}$ )	+NH <sub>4</sub> <sup>+</sup> ( $p = 4.9 \times 10^{-2}$ )

**Table 4.** Results of model 2. The positive (negative) signs indicate direct (inverse) significant relationships between the coccolithophore parameters (i.e. coccolithophore cell abundance and *E. huxleyi* coccosphere calcite mass) and nutrient concentrations. The correspondent *p*-values are indicated within parentheses.

The average *E. huxleyi* coccosphere calcite mass during the experiment was 25.44 pg: the values oscillated between a minimum of 10.94 pg (day 7) and a maximum of 32.52 pg (day 5) in W2. *Emiliania huxleyi* coccosphere calcite mass remained relatively stable throughout the experiment in the C treatments, but it showed temporal variations in the others (Fig. 4b): in the OA and OW treatments, the average *E. huxleyi* calcite mass increased between days -1 and 0, and then decreased toward the end of the experiment; meanwhile, in the GH treatments, it decreased quite consistently from the start to the end of the experiment. Although the ANOVA test did not find any significant difference in the average *E. huxleyi* coccosphere calcite mass among the four treatments (Table 1), Model 1 did indicate a significant temporal decrease in mass under GH conditions (Table 3); additionally, Model 2 highlighted a positive relationship between mass and nutrients in both the OA and GH treatments (Table 4).

The percentage of coccospheres with anomalous calcification (i.e. coccospheres with malformed coccoliths; see Supplementary Table S3, Figs. S2 and S3 online) varied in all treatments, but it evolved differently over time. In the C treatment, the percentage of *E. huxleyi* coccospheres with malformed coccoliths remained relatively stable throughout the experiment; meanwhile, that of *R. clavigera* decreased until day 7, and then increased until day 10. In the OA treatment, malformed *E. huxleyi* coccospheres were present between days 3 and 7, and their relative abundance remained high until day 10. No malformed specimens of *R. clavigera* were observed in this treatment during the experiment, except on day 7, when they reached a relative abundance of 65%. In the OW treatment, the percentage of malformed *E. huxleyi* increased and remained high between days 0 and 7 (~20–30%), after which it slightly decreased (25% on day 10). Finally, the percentage of malformed *E. huxleyi* increased continuously between days 0 and 7 in the GH treatment (no data available for day 10). Due to the scarcity of *R. clavigera* coccospheres in the scanning electron microscope (SEM) samples, their temporal patterns

in the OW and GH treatments could not be defined. Overall, we observed considerably higher percentages of coccospheres with malformed coccoliths in the perturbed treatments than in the C: their average percentages (between days 0 and 7) in the C, OA, OW and GH treatments were 8%, 19%, 23% and 36%, respectively.

## Discussion

Sea surface warming, marine heatwaves<sup>8,14,61–65</sup> and OA<sup>11,66</sup> have been anticipated for this century in the Mediterranean Sea. The OA and OW conditions tested in our mesocosm experiment, which reflect those projected for 2,100 under the IPCC RCP8.5 scenario<sup>12</sup>, were found to cause drastic changes in the studied coccolithophore community (Fig. 4d and Tables 1, 2, 3). Mediterranean SSTs have increased since the 1980s. This warming trend has accelerated since the 1990s<sup>67–69</sup> and has been particularly pronounced in the Eastern Basin in the last ~ 10 years compared to the interval 1980–1999<sup>70–73</sup>. Moreover, a new study<sup>74</sup> suggests that the length, severity and spatial extension of surface marine heatwaves increased between 1982 and 2017. The surface waters of the Eastern Mediterranean Sea have already occasionally reached temperatures > 28 °C during heatwaves<sup>75,76</sup>. This temperature seems to represent a biological threshold for many species living in the Mediterranean Sea: it causes the death of infralittoral (e.g. mussels and seagrass)<sup>75,77</sup> and circalittoral (e.g. red coral and red gorgonian)<sup>78,79</sup> species; moreover, 50% of the biological impacts on the growth, survival, fertility, migration and phenology of species pertaining to several marine phyla (including invertebrates, vertebrates, phytoplankton and macrophytes) already occur at summer surface temperatures of 27.5 °C<sup>76</sup>. A numerical model<sup>80</sup> based on the RCP8.5 IPCC scenario<sup>3</sup> indicates that the Mediterranean SST will frequently exceed 28 °C in the next decades. In this study, close correlations were observed between the total coccolithophore cell abundance, the average *E. huxleyi* calcite mass and nutrient concentrations under perturbed conditions (Table 4). Based on these results, we hypothesize that coccolithophore nutrient requirements might have increased under OW and OA. Meanwhile, the extremely low coccolithophore abundance suggest that other phytoplankton groups with a higher total biomass (e.g. picoplankton) should have been the main responsible for the observed decrease in nutrient concentrations.

Eastern Mediterranean surface waters tend to be P- (or N- and P-) limited<sup>17</sup>: the combined effect of heat stress and nutrient limitation may lower the cellular fitness of coccolithophores, affecting both their calcification and growth.

OA allows relatively high rates of carbon fixation in coccolithophores, but this effect can be influenced by other factors, such as temperature<sup>38,41,81</sup> and the nutrient regime<sup>46,50</sup>. Usually, OW stimulates phytoplankton (including coccolithophores) growth by accelerating its metabolic activities, but only up to a temperature optimum (i.e. a species- or strain-specific threshold)<sup>82–84</sup>. The coccolithophore community tested in our mesocosms was typical of Eastern Mediterranean surface waters<sup>28,85–87</sup>. The distribution of the two most abundant species in all mesocosms, *E. huxleyi* and *R. clavigera*, evolved differently over time (Figs. 3a, b and 4a, c), suggesting a higher tolerance of *R. clavigera* to the extremely low NO<sub>3</sub><sup>-</sup> and PO<sub>4</sub><sup>3-</sup> concentrations reached in all treatments. Coccolithophores, including *E. huxleyi*, are considered good competitors in oligotrophic waters<sup>88,89</sup>; however, their nutrient requirements are species-specific: highly-specialized, K-selected species (e.g. *R. clavigera*), are better equipped for surviving under extreme oligotrophic conditions<sup>90,91</sup>.

The sensitivity of *R. clavigera* to OA and OW has not been tested in laboratory experiments and needs to be inferred from the results of past field studies. This species is preferentially distributed in surface, warm and oligotrophic subtropical waters<sup>92–94</sup>; in fact, it reaches relatively high abundances in the Eastern Mediterranean Sea<sup>93</sup>, especially during summer<sup>95,63</sup> and in concomitance with high CO<sub>3</sub><sup>2-</sup> concentrations (usually ≥ 220 μmol Kg<sup>-1</sup>)<sup>18</sup>. A “substrate-inhibitor concept”, describing the dependence of calcification rates on carbonate chemistry speciation, has been proposed to harmonise the current knowledge about the diverse responses of coccolithophores to OA<sup>30,31,49</sup>. In a recent paper<sup>96</sup> it was suggested that, in an OA scenario, coccolithophore species and strains with higher PIC:POC will be more affected than those with lower PIC:POC; in the future, this could lead to a shift in the coccolithophore communities in favour of low-sensitivity, low-PIC:POC species and strains. In that same paper, *E. huxleyi* was reported to have a typical PIC:POC of 0.67, but no data were provided for *R. clavigera*. Based on previous research<sup>97</sup>, we considered a typical POC value of ~ 18.2 pg C cell<sup>-1</sup> for *R. clavigera*. Moreover, the PIC of *R. clavigera* can be roughly estimated based on the average mass of each rhabdolith (46 pg CaCO<sub>3</sub> or 5.5 pg C)<sup>98</sup> multiplied for their typical number in a coccosphere (~ 20)<sup>99</sup>: ~ 110 pg C cell<sup>-1</sup>. Based on this information, we infer that the PIC:POC of *R. clavigera* (~ 6.04) tends to be much larger than that of *E. huxleyi*. However, the results of our mesocosm experiment suggest that the cell production of *R. clavigera* will not be impacted much more than that of *E. huxleyi* under OA: we did not observe any significant effect of OA alone on neither *R. clavigera*, nor *E. huxleyi* cell abundance (Fig. 4a, c and Tables 2, 3); in particular, the cell abundance of *R. clavigera* remained stable or even increased during the experimental period under such conditions. In accord with our results, a study based on water samples collected along a natural pH gradient in the Eastern Mediterranean Sea demonstrated that both *E. huxleyi* and *R. clavigera* can be adapted to highly acidic conditions and resilient in terms of cell abundance and coccolith morphology<sup>100</sup>.

Interestingly, the abundances of both species, especially those of *R. clavigera*, were lower in the OW and GH treatments (Fig. 4a, c and Tables 2, 3). This suggests a negative effect of the high temperatures tested in those treatments, which likely exceeded the growth optima of the two species and reduced their tolerance to OA (as in the case of the GH treatment). The apparently greater sensitivity of *R. clavigera* could be explained by its eco-physiology and the existence of species-specific temperature optima.

Salinity increased over time in all mesocosms (Fig. 2b). An influence of salinity on the abundance of the two major coccolithophore species is unlikely: both *E. huxleyi* and *R. clavigera* are known for living under a wide range of salinities<sup>28,93,101,102</sup>; hence, we can reasonably expect them to be resilient to small variations (of maximum ~ 0.25 PSU) like those registered during this experiment (Fig. 2b). Notably, this variation is comparable

to the maximum range registered during a previous mesocosm experiment conducted in the Western Mediterranean Sea ( $\sim 0.15$  PSU)<sup>50</sup>.

The preferential distribution of HOL in oligotrophic and stratified waters, like those of the Eastern Mediterranean, is well established<sup>57,103</sup>; accordingly, we would have expected an increase in the relative abundance of the HOL during our experiment, paralleling the decrease in  $\text{NO}_3^-$ . Nevertheless, the relative abundance of HOL was found to decrease rapidly during the experiment in all treatments (Fig. 3d). We can hence suppose that the environmental conditions verified during the experiment (temperatures  $\geq 24.95$  °C and  $\text{NO}_3^-$  concentrations mostly  $< 0.24$   $\mu\text{mol L}^{-1}$ ) exceeded a physiological tipping point for the HOL. Other physico-chemical factors (e.g. turbulence, irradiance, grazing and viral infection) could have also influenced the HOL response, but they were not measured during the experiment.

Changing environmental conditions can regulate the fraction of cellular energy dedicated to calcification in *E. huxleyi*<sup>30,41,101,104–108</sup>. As a matter of fact, a previous study<sup>41</sup> demonstrated that optimum growth, calcification and carbon fixation rates in coccolithophores can occur at different seawater  $\text{CO}_2$  concentrations depending on the environmental temperature. During our experiment, the calcification degree of *E. huxleyi* was found to decrease over time under GH conditions (Fig. 4b; Table 3). Our findings agree with those of a recent model<sup>109</sup>, which projected decreasing levels of coccolithophore growth and calcification throughout the twenty-first century in most tropical and sub-tropical oceanic regions.

A limited amount of morphological data could be obtained in this study (Supplementary Table S3 and Fig. S2 online), due to the low abundance of coccolithophores in the mesocosm samples. Nevertheless, coccolith calcification clearly tended to be disrupted under perturbed conditions, particularly under thermal stress (as seen in the OW and GH treatments).

Primary malformations occur during intracellular coccolith calcification. Malformed coccoliths are relatively rare in coccolithophore specimens from field samples, but are frequently observed in cultured strains, partly due to the high cell densities reached in stock cultures<sup>48,110,111</sup>. In addition, laboratory experiments exposing coccolithophores to various types of physiological stresses (i.e. OA<sup>112,113</sup>, OW<sup>38,104,113–115</sup> and nutrient perturbations<sup>116</sup>, as well as varying trace metal<sup>117</sup>,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  and bisphosphonates<sup>118,119</sup> concentrations) have demonstrated their negative impacts on calcification. Coccolithophores are known for having species-specific physiological requirements for calcification, although most studies have focused on *E. huxleyi*<sup>120,121</sup>. In this experiment, the occurrence of coccospheres composed of anomalously calcified coccoliths suggests a partial disruption of the calcification process. The highest number of such coccospheres was observed in the GH treatment (i.e. under combined OA and OW), followed by the OW and OA treatments.

Overall, our results highlight a clear negative effect of thermal stress on coccolithophore cell abundance and calcification, which was exacerbated under combined OW and OA. To the best of our knowledge, this is the first time an increase in coccolithophore cell abundance was noted in response to OA. Natural living communities have shown only neutral, mixed, or negative responses in terms of cell production<sup>50,123</sup>. For what concerns POC production, culture experiments have demonstrated that it can increase for some species under OA, while coccolithophore PIC generally decreases under such conditions<sup>30,122</sup>.

Most likely, the extreme OW conditions tested during our experiment (temperature  $\geq 28$  °C, the highest ever tested in a mesocosm) were the main responsible for the observed detrimental effects on the coccolithophore population. We infer that the environmental changes projected for this century in the Mediterranean Sea (i.e. OA, OW and increasingly long and frequent marine heatwaves in summer<sup>65</sup>), could have adverse effects on local coccolithophore communities, in terms of both cell abundance and calcification. On one hand, OA might slightly stimulate coccolithophore growth; on the other hand, it might exacerbate the negative effects of OW under sustained elevated temperatures ( $\geq 28$  °C) and ultraoligotrophic conditions. Moreover, coccolithophore species will respond differently depending on their physiological requirements, leading to shifts in species composition. For example, *R. clavigera* may be less resilient than *E. huxleyi*, and hence show a more marked decrease, under extremely high temperatures (e.g.  $\geq 28$  °C). The total coccolithophore  $\text{CaCO}_3$  export in the Mediterranean Sea will be considerably influenced by shifts in the average *E. huxleyi* coccosphere calcite mass and in the proportion of major taxa. Finally, coccolith malformations may become more common under OW and OA, at least until the adaptation of the coccolithophore community to the new environmental conditions. Recent studies suggest that the coccosphere calcification degree and the occurrence of coccolith malformations in *E. huxleyi* coccoliths are not related to photosynthetic rates and cell growth. Nevertheless, any perturbation of the calcification process seem to directly impact the ecological fitness of some coccolithophore species (e.g. *Coccolithus braarudii*)<sup>118–120</sup>.

## Methods

**Experimental setup.** Our land-based mesocosm experiment was carried out for 12 days (1st–12th September 2013) at the Hellenic Centre for Marine Research (HCMR) facilities (CRETACOSM) in Crete, Greece (Fig. 1). The experimental setting included 12 mesocosms of 3 m<sup>3</sup> each (diameter = 1.32 m). Four different treatments were tested: unperturbed ambient conditions (C), ocean acidification (OA), ocean warming (OW) and combined OA + OW (greenhouse, GH). Each treatment was tested on three replicates (Supplementary Tables S1, S2 online).

The seawater used for this experiment was collected aboard the *R/V Philia* using a submersible pump offshore Crete (35° 24.96' N, 25° 14.44' E, site depth = 170 m, sampling depth = 10 m, sampling temperature = 25 °C) between the 30th–31st August 2013. About 36 m<sup>3</sup> of water were transferred into polyethylene containers (1 m<sup>3</sup> each) that were previously filled with tap water (for 1 week), washed with HCl 10% and rinsed with deionized water. The collected seawater was maintained under a constant temperature of 25 °C during transportation and reached the HCMR CRETACOSMOS 2 h after collection. The seawater in each container was split equally by gravity siphoning between 12 polyethylene mesocosm bags, which were then covered with a plexiglass lid (to



protect the mesocosm water from atmospheric deposition) and a mesh screen (to mimic the light conditions at 10 m depth). The bags were deployed in two separate external pools (of 350 m<sup>3</sup> and 150 m<sup>3</sup>, respectively) filled with water. The seawater temperature for the larger pool (containing the C and OA mesocosms) was maintained at 25 °C, while the target temperature for the seawater in the smaller tank (containing the OW and GH mesocosms) was 28 °C. Three mesocosm bags from both pools (OA1, OA2, OA3, GH1, GH2 and GH3) were acidified by dispersing 28.5–31 L of CO<sub>2</sub>-saturated seawater in each bag. Such water had been separated from the original batch before the mesocosm filling, bubbled several minutes with CO<sub>2</sub> and transferred into 10-L Nalgene plastic containers. The acidification was implemented over 3 days (1st–3rd September 2013) using a special-designed diffusing system<sup>47</sup> in order to minimize the biological stress. On day 2, after the completion of the acidification stage, the average pH<sub>T</sub> values of the three OA and of the three GH mesocosms were 7.83 ± 0.01 and 7.79 ± 0.01, respectively; afterwards, the carbonate system was left to evolve independently. Notably, no nutrients were added during the experiment. Every day before sampling, the water in all mesocosm bags was mixed for 2 min using a clean paddle in order to avoid possible “bottle effects”; then, it was vacuum-forced through a plastic tubing into 10- and 20-L containers previously washed with Elix water (resistivity > 5 MΩ cm<sup>-1</sup> at 25 °C, typically 10–15 MΩ cm<sup>-1</sup>).

**Environmental parameters.** The water temperature was measured every 2 min in all mesocosms with HOBO UA-002-64 sensors and once per day with an Aanderaa Conductivity-Temperature sensor 3,919. All of these sensors were connected to a control panel (IKS Aquastar, IKS ComputerSysteme GmbH). The salinity was checked once per day using the Aanderaa Conductivity-Temperature sensor 3,919. The carbonate chemistry was also checked daily: duplicate samples were collected from the mesocosms and directly poisoned with HgCl<sub>2</sub>. Then, their total alkalinity was measured using a VINDTA 3C analyser (Versatile Instrument for the Determination of Total inorganic carbon and titration Alkalinity). Titrations of certified reference seawater (CRM Batch #82, A.G. Dickson, Scripps Institution of Oceanography, USA) yielded on average total alkalinity values within 0.8 μmol kg<sup>-1</sup> of the nominal value (standard deviation = 1.8 μmol kg<sup>-1</sup>; n = 24). The pH of the seawater was potentiometrically determined using a pH meter (Metrohm, 827 pH lab) fitted with a glass electrode (Metrohm, Aquatrode Plus) and calibrated on the total H<sup>+</sup> concentration scale (pH<sub>T</sub>) with a Tri/HCl buffer solution<sup>124</sup> at a salinity of 38.0, provided by A. Borges (University of Liege). The standard deviation of the Tris/HCl buffer pH measured at 25.4 °C during the whole experiment was 0.010. All the other carbonate chemistry parameters, including the dissolved inorganic carbon (CT) and CO<sub>3</sub><sup>2-</sup> concentrations, were calculated using the R package “seacarb”<sup>125</sup>; moreover, the uncertainties in CT and CO<sub>3</sub><sup>2-</sup> were estimated with the “errors” function of “seacarb” and based on the abovementioned standard deviations<sup>126</sup>. The combined uncertainty for CT and CO<sub>3</sub><sup>2-</sup> ranged between 5.1–6.7 and 2.9–4.3 μmol kg<sup>-1</sup>, respectively, being lower in the OA mesocosms and higher in the OW ones.

Water samples were collected daily also for the nutrient measurements: NO<sub>3</sub><sup>-</sup> was analysed following<sup>127</sup>, PO<sub>4</sub><sup>3-</sup> according to the MAGIC25 method<sup>128</sup>, and NH<sub>4</sub><sup>+</sup> following<sup>129</sup>.

**Coccolithophore abundance.** A total of 78 water samples were collected during the experiment to monitor any changes in the abundance and composition of the coccolithophore community. The sample collection occurred daily for the first three days of experiment, and then continued every second day; three replicates per treatment were included, with the exception of day -1 (Supplementary Table S1 online). A vacuum pump system (Eyela, A-1000S) and cellulose acetate-nitrate filters (Millipore, Ø 47 mm, 0.45 μm) were used to filtrate 3–5 L of water per sample; subsequently, the filters were rinsed with buffered Elix water (63 ml NH<sub>3</sub> + 500 ml of Elix water) to dissolve any salt residues and oven-dried at 40 °C for ~8 h. A portion of each filter was radially cut and mounted on a microscope slide using transparent immersion oil. Between 120 and 1,895 fields of view (1 FOV = 0.05 mm<sup>2</sup>) per slide were observed at ×1,000 magnification using a polarizing light microscope (Leica DM6000B). The observed area varied depending on the cell abundance; on average, it corresponded to 68 mL of water per sample. The 95% confidence interval, assuming a Poisson distribution, varied between 21–139 cells L<sup>-1</sup> (for an abundance of 54 cells L<sup>-1</sup>) and 1.66 × 10<sup>4</sup>–2.05 × 10<sup>4</sup> cells L<sup>-1</sup> (for an abundance of 1.84 × 10<sup>4</sup> cells L<sup>-1</sup>). The cell densities and confidence limits were calculated following<sup>130</sup>. The HET were identified down to species level wherever possible, while the HOL species were not differentiated.

**Emiliania huxleyi calcite mass.** Forty-one of the original 78 phytoplankton samples were analysed to determine the average *E. huxleyi* coccosphere calcite mass values. The luminosity level of the light microscope (Leica DM6000B) was adjusted before starting the analysis, as in<sup>131</sup>. For each slide, a minimum of 50 coccosphere pictures were taken at ×1,000 magnification using a SPOT Insight Camera, and then processed by an automated system for coccolith/coccosphere recognition called SYRACO<sup>132,133</sup>. Coccolith calcite is bright when viewed in cross-polarized light; moreover, its brightness increases with its thickness<sup>98</sup>: SYRACO records calcite brightness in grey levels, which can then be converted into calcite mass (in pg<sup>131</sup>). This software is able to differentiate among several coccolithophore species, as well as between coccoliths and coccospheres. In this study, we considered only the coccosphere calcite mass of *E. huxleyi* and calculated the correspondent average value for each sample.

**Coccolithophore morphology.** Fifteen of the original 78 phytoplankton samples were selected to perform a semiquantitative analysis of coccolithophore morphology. Such samples were collected on experimental days 0, 3, 7 and 10 from different treatments (except in the case of GH, for which we analysed only samples collected on days 0, 3 and 7; see Supplementary Table S1 online). A piece of filter was radially cut from each of these filters, attached to a stub, and coated with a Au/Pd alloy. One to 50 coccospheres per sample were observed using a

SEM (Zeiss EVO MA 10) at  $\times 10,000$ – $30,000$  magnification: the number of observed specimens depended on the sample richness. The data collected through this analysis were used to calculate the percentages of malformed *E. huxleyi*, *R. clavigera* and of the total malformed coccospheres (Supplementary Table S3 and Fig. S3 online).

**Statistics.** Different types of statistical tests were conducted to analyse the response of the coccolithophore population by considering all the phytoplankton samples collected between experimental days – 1 and 10 (Table S1), except those from OA3 (see the “Results” section for a detailed explanation).

First, Microsoft Excel was used to perform a series of ANOVA and Tukey tests. The ANOVA tests were conducted to assess any statistical differences in the average coccolithophore abundance (total coccolithophores, *R. clavigera* and *E. huxleyi*) or *E. huxleyi* mass between different treatments. The final aim was to tease apart any significant effect of temperature and pH on the coccolithophore population. If any statistically significant effect was recognized, a post-hoc Tukey test was also performed to discern its occurrence among pairs of treatments.

Second, RStudio (version 3.3.3, package lmer4)<sup>134</sup> was used to run two longitudinal data analysis models (Model 1, Model 2). Longitudinal data analyses are based on measures performed on a response variable (continuous or discrete) repeatedly over time and for multiple subjects. Generally, the objective of this type of analyses is to model the expected value of the response variable as a linear or nonlinear function of a set of explanatory variables. Based on Model 1 and Model 2, we aimed at defining the temporal evolution of the coccolithophore population and its dependence on several environmental parameters. In particular, Model 1 (Eq. 1) was used to assess the statistical significance of the treatment conditions over both the coccolithophore abundance and *E. huxleyi* coccosphere calcite mass:

$$\text{Model 1} = X \sim \text{Days} + (1|\text{Replicate}) \quad (1)$$

where  $X = [\text{Total coccolithophores}], [E. huxleyi], [R. clavigera]$  or *E. huxleyi* coccosphere calcite mass.

Model 2 (Eq. 2) was used to assess the influence of nutrients over both the coccolithophore abundance and *E. huxleyi* coccosphere calcite mass in each treatment:

$$\text{Model 2} = X \sim Y + (1|\text{Replicate}) \quad (2)$$

where  $X = [\text{Total coccolithophores}], [E. huxleyi], [R. clavigera]$  or *E. huxleyi* coccosphere calcite mass, while  $Y = [\text{NO}_3^-], [\text{NH}_4^+]$  or  $[\text{PO}_4^{3-}]$ .

Notably, the results of all the statistical analysis were considered significant for  $p < 0.05$ .

## Data availability

The environmental and coccolithophore datasets used in this work are available at <https://doi.pangaea.de/10.1594/PANGAEA.836005> and the data will be uploaded on the PANGAEA (<https://www.pangaea.de>) platform, respectively.

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## Author contributions

The experiment was designed by P.Z., P.P. and E.K., and performed by P.P. and E.K. B.D., P.Z., M.G. and C.P. contributed to the design of the coccolithophore study; moreover, M.G., B.D. and C.P. performed the analyses, including the statistics, created the graphs and wrote the first draft of the manuscript. All authors contributed substantially to the manuscript revisions.

### Competing interests

The authors declare no competing interests.

### Additional information

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