Long-term adaptation of the coccolithophore *Emiliania huxleyi* to ocean acidification and global warming

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Lothar Schlüter

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Erster Gutachter: Prof. Dr. Thorsten B.H. Reusch

Zweiter Gutachter: Prof. Dr. Ulf Riebesell

Dritter Gutachter: Prof. Dr. Hinrich Schulenburg

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Zusammenfassung

Die Menschheit verändert den Kohlenstoffkreislauf hochgradig durch die Verbrennung fossiler Kohlenwasserstoffe, welche direkt und indirekt am Klimawandel beiträgt. Der Überschuss an anthropogenem CO_2 Ausstoß treibt nicht nur die Erderwärmung an, sondern verursacht außerdem Ozeanversauerung, wenn CO_2 im Meerwasser gelöst wird. Wie marines Phytoplankton auf diese Veränderungen reagiert ist von besonderem Interesse, da es in etwa die Hälfte der globalen Kohlenstofffixierung leistet. Besonders kalzifizierende Organismen sind besonders sensibel gegen Ozeanversauerung. Coccolithophoriden als kalzifizierendes Phytoplankton sind deshalb besonders im Fokus. Aufgrund ihrer kurzen Reproduktionszeit und ihrer hohen Populationsdichten sind sie besonders dafür geeignet, die Auswirkungen von adaptiver Evolution unter globalem Wandeln zu untersuchen.

Mein Studienalge, die Coccolithophoride *Emiliania huxleyi*, ist eine der häufigsten marinen Mikroalgen, und gleichzeitig einfach im Labor kultivierbar. In Experimenten konnten die physiologischen Abhängigkeiten von CO_2 und Temperatur auf *E. huxleyi* gezeigt werden. Diese deuten darauf hin, dass das Wachstum und Kalzifizierung im Ozean der Zukunft reduziert sein werden. In einem CO_2-Adaptations-Experiment hatte *E. huxleyi* das Potenzial sich an Ozeanversauerung anzupassen. Allerdings ist unklar, ob und wie die Anpassung an erhöhtes CO_2 und Erwärmung miteinander interagieren. Im ersten Teil meiner Arbeit habe ich deswegen die Langzeitauswirkungen von CO_2-Selektion (1100 µatm pCO_2, 2200 µatm pCO_2) sowie die Interaktion mit Temperaturselektion (26.3°C) untersucht, welche nach 1600 asexuellen Generationen CO_2-Selektion hinzugefügt wurde.

Aufbauend auf ein erstes einjähriges Experiment habe ich untersucht, ob sich Adaptation an Ozeanversauerung allein über einen Zeitraum von vier Jahren fortführt. Die Fortsetzung des CO_2-Adaptations-Experiment hat gezeigt, dass langfristige Adaptation komplex ist und sich phänotypische Antworten im Laufe der Zeit umschlagen können. Nach insgesamt 2100 asexuellen Generationen unter CO_2-selektion steigerte sich die Fitness (in Form der Wachstumsrate) unter mittlerem CO_2 (1100 µatm pCO_2) langsam. Der Fitness-Vorteil von 5% nach 500 Generationen unter hoch CO_2 (2200 µatm pCO_2) blieb unverändert. Die Kalzifizierung wurde in den ersten 500 Generationen teilweise wiederhergestellt. Danach schlug die phänotypische Antwort jedoch um, und die Kalzifizierung wurde als Selektionsantwort reduziert. Diese Antwort war nicht grundlegend, da der Kalkgehalt der Zellen wiederhergestellt wurde, nachdem sie zurück unter heutige Ozeanwerte (400 µatm pCO_2) transferiert wurden. Einige phänotypische Veränderungen der Zellen standen wahrscheinlich im Zusammenhang mit der Selektion für höhere Wachstumsraten, so wie eine reduzierte Zellgröße und ein niedriger organischer Kohlenstoffgehalt (POC).

Unabhängig des CO_2-Partialdruckes konnte sich *E. huxleyi* an erhöhte Temperaturen anpassen. Der Fitnesszuwachs betrug bis zu 16% in Populationen welche an hoch CO_2 und Temperatur angepasst waren gegenüber den nicht angepassten Kontrollen unter Selektionsbedingungen. Das Verhältnis von partikulären anorganischen (PIC) und organischen Kohlenstoff (PIC:POC) wurde auf das ursprüngliche Verhältnis wiederhergestellt, auch unter erhöhtem CO_2. Die Zellen wurden mit der Zeit kleiner, begleitet von einer Reduktion des organischen Kohlenstoffgehalts. Die Produktionsraten konnten aufgrund der Adaptation der Wachstumsrate wiederhergestellt werden, welche dem heutigen Niveau entsprechen. Verglichen mit ihren nicht angepassten Kontrollen steigerte sich die Produktion um 101% (PIC) und 55% (POC) unter hoch CO_2/Temperatur-Bedingungen.

In meinem dritten Kapitel wende ich mich der Frage zu, inwieweit Temperaturadaptation Adaptation an Ozeanversauerung beeinflusst. Die Adaptation an erhöhte Temperatur steigerte den Effekt der bestehenden CO_2-Adaptation in der Wachstumsrate. Die adaptive Reduzierung der Zellgröße unter erhöhtem CO_2 wurde nach der Temperaturadaptation umgedreht, so dass die adaptierten Zellen nun unter erhöhtem CO_2 größer waren. Unter erhöhter Temperatur war kein physiologischer Effekt von CO_2 auf den Kalkgehalt der Zellen vorhanden. Dementsprechend konnte ich dort keine evolutiven Veränderungen nachweisen. Die Adaptation an erhöhte Temperatur hat insgesamt die negativen Auswirkungen von Ozeanversauerung verringert. Um den vollen Fitnesszuwachs unter hoch CO_2/Temperatur-Bedingungen zu erhalten, war Adaptation zu CO_2 und Temperatur notwendig. Als Konsequenz daraus ergibt sich, dass CO_2- und Temperaturadaptation sich addieren. Es mag auch einen kleinen nicht signifikanten synergistischen Effekt geben, da der kombinierte Effekt leicht größer war, als die reine Addition der Einzeleffekte.

Zusammengefasst könnte die Erderwärmung die Auswirkungen der Ozeanversauerung für *E. huxleyi* reduzieren. Meine Ergebnisse zeigen auch, dass Mikroalgen phänotypische Plastizität entwickeln können als Antwort auf den Klimawandel. Die Auswirkungen auf biochemisch wichtige Merkmale wie Kalzifizierung könnten dadurch unvorhersehbar beeinflusst werden. Prognosen bezüglich des Ozeans der Zukunft erhalten dadurch einen weiteren Unsicherheitsfaktor. Trotzdem haben marine Einzeller, so wie Phytoplankton, gute Chancen sich an den Klimawandel anzupassen im Gegensatz zu Organismen mit längerer Generationszeit.

Summary

Humans are profoundly altering the carbon cycle by fossil fuel burning, which contributes directly and indirectly to global change. Excess anthropogenic CO_2 emissions not only drive the greenhouse effects and atmospheric warming but also cause ocean acidification when CO_2 dissolves in ocean waters. How marine phytoplankton reacts to these changes is of particular interest as it contributes almost half of the global carbon fixation. Especially calcifying marine organisms are sensitive to ocean acidification. This puts coccolithophores as calcite producing phytoplankton in the focus of research on global change impacts. Because of their short reproduction time and high population densities coccolithophores are the ideal candidate to investigate adaptive evolution to global change.

My study species, the coccolithophore *Emiliania huxleyi* is one of the most abundant phytoplankton in the ocean, and at the same time, can be cultured easily in the laboratory. Coccolithophore growth and calcification display temperature dependent CO_2-optimum curves, which indicate a reduction in growth rate and calcification under future ocean conditions of warming and ocean acidification. While the potential of *E. huxleyi* to adapt to ocean acidification has already been shown, how warming and acidification adaptation interact remained uncertain. In my first chapter, I tested for the long-term effects of CO_2 adaptation (1100 µatm pCO_2, 2200 µatm pCO_2) and the interactions with temperature adaptation (26.3°C) introduced after 1600 generations of CO_2 adaptation.

Building upon first experiments over one year duration, I next assessed whether adaptation to ocean acidification alone would continue over a time interval of 4 yrs. The elongation of the selection to CO_2 revealed that the long-term adaptation is complex and phenotypic responses may revert over time. After 2100 asexual generations of selection to CO_2 the fitness (growth rate) increased slightly over time under medium CO_2 conditions (1100 µatm pCO_2). Under high CO_2 (2200 µatm pCO_2) the fitness advantage of 5% at 500 generations remained unchanged. The phenotypic trait of calcification was partly restored within 500 generations. Thereafter, calcification was reduced in response to selection. The reduction of calcification was not constitutively, as the calcite per cell quotas were restored when assessed with present-day CO_2 conditions (400 µatm pCO_2). Some phenotypic traits were likely associated with selection for higher growth rate, such as a reduced cell size and lower particulate organic carbon (POC) content per cell.

Temperature adaptation occurred independently of ocean acidification levels. The fitness increase in growth rate due was up to 16% in populations adapted to high temperature and high CO_2 compared to not adapted cells under selection conditions. The ratio of particular inorganic (PIC) and organic carbon (PIC:POC) recovered to their initial ratio after temperature adaptation, even under elevated CO_2. Cells evolved to a smaller size accompanied by a reduction in POC-content. Production rates were restored to values under present-day ocean conditions, owing to adaptive evolution in growth

rate. They were 101% (PIC) and 55% (POC) higher under warming compared to the non-adapted controls.

In my third chapter, I addressed how temperature selection changed adaptation to ocean acidification. Temperature adaptation increased the effect on persisting CO_2 adaptation in growth rate. The adaptive reduction of cell size to CO_2 selection was reversed after temperature adaptation, leading to larger CO_2 adapted cells at high temperature. The immediate physiological effect on PIC per cell was diminished compared to the lower temperature treatment, and so were the adaptive effects. Temperature adaptation reduced the negative effects of ocean acidification. Both adaptations were necessary to receive the full fitness effect under high-temperature-high-CO_2-conditions. As consequence both adaptive effects are additive, with a slight and not significant tendency to synergistic, as the combined effect is slightly larger than the addition of both single effects.

Taken together, global warming may reduce the adverse effects of ocean acidification on *E. huxleyi* populations. My results show further, that marine phytoplankton may evolve changes in the plastic response under future ocean conditions. This could affect biogeochemical important traits, such as calcification, in an unpredictable way. Nevertheless, marine microbes like unicellular phytoplankton have quite good chances to adapt to ocean acidification and global warming, in contrast to many organisms with longer generation times.

Introduction

Evolution to global change

Since the industrial revolution starting in the 18th century mankind increased its impact on the environment. In Earth history there has never been a single species with more influence on the appearance of the planet as humans, provoking the postulation of the anthropocene as new geological period (Crutzen 2002). The expansion of civilization causes a reduction of undisturbed ecosystems and a higher demand for energy and resources. The burning of fossil fuels in particular led to an ongoing increase in carbon dioxide (CO_2) in the atmosphere as major greenhouse gas (Mitchell 1989). Atmospheric CO_2 affects the Earth's climate, oceans and ecosystem on a global scale. This far, work on the effects of climate change has focused on range shifts (Parmesan et al. 1999, Parmesan & Yohe 2003, Perry et al. 2005), or on short-term physiological effects of, for example, warming and acidification (Sett et al. 2014). However, predictions about how future changes in climate affect marine life are not strait forward. Short-term experiments in ecosystems under future ocean conditions neglect long-term evolutionary processes that will change the traits of populations to be different than those today while the climate is changing. This raises the question if and how individual species can adapt to future ocean conditions and how the potential of single species adaptation will affect the community composition.

This thesis uses the term adaptation always in the context of evolutionary adaptation, while the term "physiological adaptation" as acclimation/acclimatization (Garland & Kelly 2006). (Evolutionary) Adaptation is a population-level process. The mean fitness of populations changes when a certain genotype contributes more offspring to the next generation than others. In the absence of recombination (i.e. asexual reproducing populations), the abundance of this genotype increases within the population and may displace other genotypes after generations. We also say that this superior genotype is positively selected. Hence, evolution is a process where the mean population fitness increases as a consequence of natural selection.

"Physiological adaptation" refers to processes were individual organisms adjust to a range of environments (Garland & Kelly 2006). Such phenotypic plasticity is defined as acclimation (laboratory-based) or acclimatization when it occurs in nature (Angilletta 2009). Phenotypic plasticity describes how one genotype gives rise to different phenotypes as function of the environment (Scheiner 1993). Phenotypic plasticity can buffer environmental changes and increase the environmental range in which a species can exist. It is important to distinguish between phenotypic plasticity (how much the phenotype changes as a function of environmental change = classical plasticity) and phenotypic buffering (how broad is the environmental range a genotype can maintain its fitness) (Reusch 2014). Under a stressful environment an organism with better phenotypic

buffering would be favored by selection, because it can maintain its fitness. The opposite is the case with enhanced opportunities (e.g. more nutrients available). Here the organism with the higher plasticity would be favored, because it could more easily take advantage of the circumstances (Schaum et al. 2013, Reusch 2014). However, phenotypic plasticity and buffering both underlie evolutionary forces, and are therefore determined by the evolutionary history just as the mutation rate (Schaum & Collins 2014).

An increase in phenotypic plasticity therefore means that the phenotype changes more, not that the environmental range is increased, which is called tolerance. Plasticity often is associated with a cost. The cost is not a reduced fitness due to expression of a certain phenotype, but a general reduced fitness in maintaining the cellular machinery necessary to be plastic (Scheiner 1993). This leads to a trade-off between maintaining the phenotypic plasticity and the maximum possible fitness under constant environmental conditions. As a result we expect organisms living in a stable environment as less plastic than those from instable environments (Scheiner 1993).

Depending on the exact position on the genome where the mutation occurs, mutations can be neutral (have no phenotypic effect), beneficial (increase in fitness) or deleterious. Deleterious mutations are quickly selected against and will therefore only play a negligible role within sexually or asexually propagating populations. Most mutations not causing any phenotypic variation are considered to be neutral, meaning the mutation has no biological meaning. For example the gene sequence of small proteins like insulin, cytochrome c and hemoglobins from various groups of animals showed that the extent of sequence divergence between species increases as the divergence time increases (Nei et al. 2010). However, the function of the gene remains the same, as long as the amino acid sequence of the active sites of the protein remains the same (Nei et al. 2010). The proportion of beneficial mutations is difficult to estimate and depends on the model system (Keightley & Lynch 2003). Before a beneficial mutation can proceed to fixation in a population (permanently established in the population), it has to overcome genetic drift, here the random loss of mutants that always occur by definition at 1/N, where N is the population size. When the mutation is established the frequency of this mutation in the population will increase depending on the selective advantage and the population size (Desai et al. 2007). The time it takes a mutant to get from establishment to being half of the population is approximately $\frac{1}{s}\ln[Ns]$, where N is the population size and s the selective advantage. Initially neutral mutations can become beneficial under different environmental conditions, as any fitness effects are always context (=environment) dependent. It is now well established that evolutionary adaptation can happen on very short timescales. Examples are the rise of antibiotic resistances in bacteria since the medical utilization (Davies & Davies 2010).

Selection to multiple selection factor regimes and how do factors interact has hardly been investigated. Interactions between factors are generally described as synergistic, antagonistic or additive. When factors do note interact, the combined effect is the addition of single effects. When factors act synergistic, the combined effect is stronger than the simple addition of single effects. In case the combined effect is smaller as the addition the factors act antagonistic. I expect to find interactions between the two factors temperature and pH, as enzymatic functioning depends on both these factors (Dixon 1953). However, an important difference between both factors is that the intracellular pH is usually under tight control, whereas the temperature is not.

The three most important approaches to study the adaptive potential are: I. measuring standing genetic variation in climate-sensitive traits, II. inferring past adaptation from comparisons across space and time and III. conducting evolution experiments in real time (Sunday et al. 2014). Only approach III is a direct test of adaptive evolution.

I. A high standing genetic variation could possibly include genotypes that are positively selected under new environmental conditions. In controlled reciprocal exposure experiments where the individual fitness is measured genotypes/phenotypes can be determined which perform better than the population mean and would possibly be positively selected (Shaw & Etterson 2012).

II. The comparison of phenotypes across environmental gradients can give us information about evolution that happened in the past. Effectively this approach tests for local adaptation (e.g. to temperature, pCO_2). With this approach time is substituted with space and other environmental drivers (e.g. nutrients, salinity) would covary in space as they would do in time (Sunday et al. 2014). Such an approach can only tell the adaptive potential, and another shortcoming is that we do not know the time it took for the genotypic cline to build up as function of the environmental gradient.

III. The only direct test of (adaptive) evolution are replicated experimental evolution experiments (Lenski et al. 1991, Sniegowski et al. 1997, Dunham et al. 2002). In marine systems, using experimental evolution adaptation could be confirmed after time spans of less than a year (Lohbeck et al. 2012, Schaum & Collins 2014), which makes experimental evolution a valuable tool to investigate the response of phytoplankton to global change (Reusch & Boyd 2013). So far the number of evolutionary experiments with marine phytoplankton is small (Reusch & Boyd 2013), but increasing (Lohbeck et al. 2012, Benner et al. 2013, Schaum & Collins 2014, Scheinin et al. 2015).

The experimental set-up has large influence on the adaptive process. Most evolutionary experiments are initiated by sudden exposure to the new environment, although a continuous environmental change is more realistic. In a selection experiment with *Chlamydomonas* gradual environmental change led to a higher end-fitness compared to an abrupt change scenario. Here, more mutations

with smaller fitness effect could become fixed and add up in their fitness effects, instead of few with large effect in the abrupt scenario (Collins & de Meaux 2009). Further, a variable selection environment leads to more plasticity (Schaum & Collins 2014). Depending on the founding population used, derived from a single clone or a mixture of clones, fitness increase must derive through novel mutations or can be due to genotypic sorting, respectively (Lohbeck et al. 2012).

Adaptation to ocean acidification has already been demonstrated in *E. huxleyi* (Lohbeck et al. 2012) and *Gephyrocapsa oceanica* (Jin et al. 2013). In both experiments the growth rate of adapted populations exceeded the growth rate of non-adapted populations when tested at elevated CO_2 conditions. While in the *G. oceanica* experiment a non-calcifying strain was used, in *E. huxleyi* the calcification was partly restored and was probably associated with evolution of the pH regulation system (Lohbeck et al. 2014). In this same *E. huxleyi* selection line it could be shown that the adaptation derived from divergent genetic mutation, as the experimental replicates showed different phenotypes when challenged with low salinity (Lohbeck et al. 2013). There is indication that higher levels of CO_2 may lead to a degeneration of the carbon-concentrating mechanism (CCM) in *Chlamydomonas* (Collins & Bell 2004), which could also apply for marine phytoplankton. In *E. huxleyi* local temperature adaptation could be found (Zhang et al. 2014), which is not surprising as it corresponds to the global pattern of temperature adaptation in marine phytoplankton (Thomas et al. 2012).

Phytoplankton as new models for rapid evolution: their role in global climate and potential winners and losers

Although marine phytoplankton account for less than 1% of the Earth's photosynthetic biomass it is responsible for about half of the global net primary production (Field et al. 1998). In terms of global geological earth system evolution, marine phytoplankton has played very decisive roles. In Earth's history it was marine phytoplankton, cyanobacteria to be precise, that elevated the atmospheric oxygen 2.4 billion years ago (Falkowski 2012). The photosynthesis uses inter alia CO_2 as a substrate to produce oxygen and organic matter. This makes photoautotrophic plankton the key driver in the ocean's biological carbon pump (Falkowski 2012). The carbon that is fixed by phytoplankton eventually sinks to the deep sea as fecal pellets or dead bodies of phytoplankton. About 15% of the net primary production gets exported to the deep sea this way and about 0.1% gets buried into the sediment and under the right conditions to be transformed into fossil fuel (Falkowski 2012). Currently we use per day about as much fossil fuel as is produced in a year (Houghton 2007).

Additional to the ecological importance of phytoplankton in Earth's history and the carbon cycle, experiments on marine phytoplankton species in the context of possible evolution is particularly interesting and relevant, as we gain insight from very small beaker experiments that may add to understanding the fate of biogeochemical cycles.

The major functional/taxonomic phytoplankton groups are diatoms, dinoflagellates, cyanobacteria and coccolithophores (Litchman et al. 2007). Each of those groups has a particular combination of traits. Diatoms have a shell made of silicate, which restrict them in their abundance to areas with high silicate input. Some cyanobacteria are able to fix N_2 and therefore are supposed to be most successful in areas with high phosphorus and no nitrate or ammonium. Some dinoflagellate species can also use organic matter as energy source and are therefore mixotrophic. With their flagella they are motile. Unique for dinoflagellates is a cellulose cell wall. Coccolithophores have calcite plates as shells and are therefore especially sensitive to ocean acidification and therefore global change. Hence, the potential of coccolithophores to adapt to global change is of specific relevance for future ocean predictions.

Coccolithophores first appeared in the late Triassic about 200 Mya (million years ago), which makes them a comparatively young group of phytoplankton (Rost & Riebesell 2004). They have had a major impact on biogeochemical cycling over geological eras. The White Cliffs of Dover are actually composed primarily of coccoliths, being a remarkable landmark that highlights the geological impact of coccolithophores during the Jurassic. The biogeochemical relevance of coccolithophores results from the coupling of photosynthesis and calcification, the production of organic matter and calcite at the same time. Since coccolithophores contain organic and inorganic carbon, inorganic carbon is ingested by zooplankton and ends up in fecal pellets. This ballasting of organic matter with inorganic calcite enhances the transport into the deep sea (Armstrong et al. 2001). While photosynthesis binds CO_2 in organic matter, part of which is eventually exported into the deep sea (biological carbon pump), the formation of calcite produces CO_2 and increases the atmospheric pCO_2 (carbonate counter pump). For each mol of calcite formed one mol of CO_2 is released. In numbers, the organic carbon export into the deep sea was calculated to be 11.2 – 15 Pg C yr^{-1} and for calcite 0.82 – 1.20 Pg C yr^{-1} (Jin et al. 2006).

The calcite plates of coccolithophores are termed coccoliths (Paasche 1968). They ballast organic material and therefore enhance the transport of organic matter into the deep sea (Armstrong et al. 2001). Biogenic calcite has a density estimated 2.5 times denser than non-calcified *E.huxleyi* cells (Engel et al. 2009), and organic matter that is ballasted with coccoliths will be exported into the deep sea much faster than non-ballasted organic matter (Bach et al. 2012). The biological function of coccoliths is still under discussion. Prominent explanations are protection against grazing and viruses, ballasting for vertical migration and intracellular light manipulation (Raven & Crawfurd 2012).

Production and morphological integrity of coccoliths is compromised by ocean acidification. In physiological experiments coccolithophores showed reduced growth and calcification under elevated CO_2 (Riebesell & Tortell 2011). However, the sensitivity to ocean acidification is species and strain

specific (Langer et al. 2009, Sett et al. 2014), highlighting the importance of standing genetic variation to phytoplankton adaptive evolution. A potential decline in coccolithophore abundance and production may have crucial consequences for the global carbon cycle (Rost & Riebesell 2004, Riebesell et al. 2009).

Not all species are equally affected by increased CO_2 and/or temperature. Calcifying marine organisms are stressed by ocean acidification (Riebesell et al. 2000, Hoegh-Guldberg et al. 2007, Doney et al. 2009). On the other hand, enrichment in CO_2 leads to a better accessibility of carbon for photosynthesis. For example diatoms could profit from CO_2 fertilization (Wu et al. 2010, Rajanandhini et al. 2014). Of particular interest in terms of ocean acidification are coccolithophores, as they are unicellular calcifying marine phytoplankton and can both profit from excess CO_2 and suffer from acidification, dependent on where the CO_2 optimum of each species is (Sett et al. 2014). In a spring bloom (north Atlantic) experiment coccolithophores were the most abundant genus under greenhouse conditions (elevated CO_2 (690 µatm pCO_2) and temperature (16°C)) (Feng et al. 2009). However, calcification was reduced under those conditions. Additionally the abundance of diatoms was reduced leading to a crucial reduction in biomineralization to organic material production rate (Feng et al. 2009). The ballasting effect of biominerals on organic carbon could be drastically reduced (Armstrong et al. 2001, Feng et al. 2009).

The coccolithophore *Emiliania huxleyi* as a model organism

Emiliania huxleyi is the world's most common coccolithophore and calcifying species with a nearly global distribution, except for polar waters (Westbroek et al. 1989). One remarkable natural phenomenon is blooms of *E. huxleyi*. The coccoliths scatter the light and therefore blooms can be seen from out of space as a "milky cloud" in the ocean (Westbroek et al. 1989). *E. huxleyi* blooms usually occur in eutrophic areas after a diatom bloom, when water is highly stratified, the irradiance is high and silicate is already used up by diatoms (Tyrrell & Merico 2004). Further, the nitrogen to phosphorus (N:P) ratio seems to be relevant, as in most blooms N:P was rather high compared to Redfield (Redfield 1958, Townsend et al. 1994). In mesocosm experiments the success of *E. huxleyi* blooms could be enhanced by adding excess nitrogen over phosphorus (Egge & Heimdal 1994). *E. huxleyi* further contributes to the production of dimethylsulphide (DMS), which is involved in cloud nucleation and may affect the regional climate (Westbroek et al. 1993, Puerta et al. 2005). Aside from the important ecological role for biogeochemical cycles and climate *E. huxleyi* is easy to cultivate in its asexual diploid phase, which made it a model organism for phytoplankton researchers.

E. huxleyi has a complex life cycle consisting of a calcified non-motile diploid and a non-calcified motile haploid life phase (von Dassow et al. 2009). Under laboratory conditions transition of diploid cultures to haploid could be induced by exposure to giant phycodnaviruses (*Emiliania huxleyi* viruses,

EhVs) (Frada et al. 2008). However, the underlying mechanism is still unclear (Frada et al. 2008). The haploid phase is resistant against those viruses leading to the assumption that the haploid phase is a escape strategy to those specific viruses (Frada et al. 2008). Both phases can reproduce asexually, which is actually the case under standard cultivation conditions. However, in nature sexual reproduction is likely to be common, even during blooming (Iglesias-Rodriguez et al. 2006). Evidence are for example many different genotypes even in bloom situations (Krueger-Hadfield et al. 2014). For the calcified phase six morphotypes are known that can be differentiated according to size and coccolith morphology (Young et al. 2003).

E. huxleyi belongs to the group of heterococcoliths were calcification is an intercellular process, in opposition to the group of holococcoliths were coccoliths are formed at the cell surface (Young et al. 1999). In *E. huxleyi* calcification is a highly regulated process and takes place in a Golgi-derived intracellular vesicle, the coccolith vesicle (CV) (van der Wal et al. 1983). The regulation of Ca^{2+} in the CV is complex, since Ca^{2+} is involved in many cellular signal transduction processes and therefore the free cytosolic Ca^{2+} is maintained low (Mackinder et al. 2010). H^+-ATPases maintain an H^+ electrochemical gradient to accumulate Ca^{2+} via Ca^{2+}/H^+ exchangers from the cytosol to the CV. Once the necessary oversaturation is achieved the H^+-ATPases activity is shut down, leading to an alkalinization of the CV lumen and a precipitation of calcite (Mackinder et al. 2010). The carbon source for calcification is believed to be HCO_3^-. The production of calcite out of Ca^{2+} and HCO_3^- produces a proton with a stoichiometry of 1 and hence leads to acidification of the CV lumen if not neutralized or removed. Further, HCO_3^- is part of the intracellular $HCO_3^-/CO_3^{2-}/CO_2$ buffer system, which regulates the cytosolic pH (Brownlee et al. 1995). Under ocean acidification *E. huxleyi* has problems maintaining cytosolic pH (Suffrian et al. 2011). It has been shown, that voltage-gated proton channels serve to quickly release excess protons produced in calcification from the cytosol. The plasma membrane potential is disrupted be seawater acidification and hence the regulation of pH. Hence, growth rate and calcification in *E. huxleyi* under ocean acidification are reduced.

Increasing atmospheric CO_2 imposes two counteracting effects upon *E. huxleyi*. On the one hand, CO_2 and bicarbonate ions can become limiting substrates for photosynthesis (Buitenhuis et al. 1999), on the other hand excessive CO_2 in the atmosphere lowers the pH in seawater, which causes problems in the process of calcification (Bach et al. 2011). At very low concentrations of CO_2, *E. huxleyi* suffers due to lack of photosynthesis substrate (Buitenhuis et al. 1999). At high CO_2 concentrations it suffers of the reduced pH that comes along with the CO_2 increase (Bach et al. 2013). However, the optimum pCO_2 is strain and temperature specific (Langer et al. 2009, Sett et al. 2014). For example the pCO_2 optimum for growth changes from 622 to 1295 µatm for temperature increasing from 15°C to 20°C (Sett et al. 2014). Notice, that the pCO_2 optimum for growth does not equal the pCO_2 optimum for

calcification (Sett et al. 2014). For temperature there are optimum curves as well, given constant carbonate system condition. Depending on the geographical origin (Norway, Azores) the optimum growth is at a different temperature indicating local adaptation (Zhang et al. 2014).

The selection regime: warming and acidification

Global change is induced by mankind through increasing CO_2 emissions. In the ocean that leads to different effects. While ocean acidification is a direct and inevitable result of CO_2 dissolving in seawater, temperature increase is indirect and subject to a certain model uncertainty.

The Earth atmosphere is heated by the sun's short-wave radiation. Without the atmosphere the temperature of the Earth's surface would be around -18°C (Mitchell 1989). This increase in surface temperature which is above the effective radiation temperature is known as the greenhouse effect, and the involved gasses as greenhouse gasses (Mitchell 1989). Approximately 84% of the total heating of the Earth system has gone into the oceans in the past 40 years (Levitus et al. 2005). The upper 75 m of the ocean warmed by 0.11 °C per decade (IPCC 2014), leading to a sea level rise due to the thermal expansion of sea water and more important in this context, to numerous range shifts of animals and plants that 'track' their climate zone (Parmesan & Yohe 2003).

CO_2 is one of the most important greenhouse gasses. Climate reconstructions derived from ice-cores showed a significant relationship between CO_2 concentration in the atmosphere and the mean global temperature. During the last ice age the CO_2 concentration was below 200 ppm CO_2 (Monnin et al. 2001), while the pre-industrial level was about 280 ppm CO_2 (Meinshausen et al. 2011). Today's CO_2 concentration is about 400 ppm CO_2 as a result of anthropogenic CO_2 emissions (March 2015, Mauna Loa, Hawaii)(IPCC 2014). Earth system models project a further increase in CO_2 levels up to 1000 ppm CO_2 until the end of the century in the case of unabated CO_2 emissions (IPCC 2014), with the corresponding global temperature increase of 2-4°C (IPCC 2014).

The atmosphere is in constant equilibrium with the surface ocean for gas exchange. About 30 % of the annual anthropogenic CO_2 emission is absorbed by the ocean (Bakker et al. 2014). However, unlike O_2 or N_2 CO_2 reacts with water (H_2O) according to following equilibrium:

$$CO_2^* + H_2O \leftrightharpoons HCO_3^- + H^+ \leftrightharpoons CO_3^{2-} + H^+$$

Oceanic uptake attenuates excess anthropogenic CO_2 in the atmosphere. However, this uptake entails profound changes in seawater carbonate chemistry. The increasing pCO_2 in the atmosphere leads to an increasing dissociation of CO_2 to HCO_3^- and an increase in H^+-ions. The equilibrium shifts to more CO_2 and a decrease of CO_3^{2-} due to the decrease in pH. This lowering in pH led to the term

'ocean acidification' for the subsequent changes in carbonate chemistry, caused by anthropogenic CO_2 emissions.

Warming has far-reaching implications, since all metabolic processes are temperature dependent. Observations over the past decades revealed a decrease in phytoplankton abundance (Boyce et al. 2010), productivity (Behrenfeld et al. 2006) and phytoplankton size followed by a change in community composition (Morán et al. 2010). These observations are assumed to be direct or indirect associated to temperature increase. Ocean models predict a further decrease in ocean productivity, mainly because of the stronger stratification and accompanied nutrient limitation (Bopp et al. 2001, Steinacher et al. 2009). Not considered in those models is the direct thermal response of individual phytoplankton species. The thermal response in growth can be plotted as temperature-growth-curve. Most of these curves show two particular features, unimodality and negative skewness (i.e., a sharper decline in fitness above the optimum temperature than below). This makes phytoplankton living close to the temperature optimum more sensitive to warming than to cooling. Physiological models therefore predict tropical phytoplankton to be most vulnerable to global warming (Thomas et al. 2012). Dependent on temperature, and the nutrition obviously, the global distribution pattern is about to change (Thomas et al. 2012). However, disregarding the physiological implications, evolutionary adaptation is a possibility to attenuate effects of global warming. Eco-evolutionary models calculate higher optimal temperatures for tropical phytoplankton than they actually are (Thomas et al. 2012), suggesting that there may be constraints between thermal adaptation and other factors (e.g. CO_2, nutrients,…) (Thomas et al. 2012).

However, how temperature and acidification act together as selection factors has hardly been investigated. There is evidence from ecophysiological experiments that elevated temperature could offset the response of *E. huxleyi* to increasing CO_2 (Benner et al. 2013). However, there is no experiment that distinguishes between the single effects of temperature and CO_2, and the combination of both so far.

Considerations in experimental evolution

According to Kassen (2002) the appropriate evolutionary experiment is as follows.

"A base population, which may be genetically uniform or diverse and either sexual or asexual, is inoculated into an environment, which may be heterogeneous or homogeneous, and natural selection is allowed to proceed for as many generations as one's patience allows. Samples are then extracted from each of the selected lines and their fitness assayed across a range of environments, preferably the same environments used during selection. The results are then cast in a genotype-by-environment matrix, where each of the selected lines are the different levels of genotype."

In the assay experiments the evolutionary adaptation can be revealed by two different approaches: The performance of the selected lines across all environments can be compared against theirs founders or an unselected control line. In this case adaptation to lab condition is part of the tested adaptation (Kassen 2002). To determine specific adaptation to a certain environment the selected lines have to be tested in their selection environment against lines that evolved under control (=ancestral) conditions. If there is adaptation, the selection line should show higher Darwinian fitness in its selection environment (Kassen 2002). Latter approach is suitable for experiments to global change, as the selection lines would evolve at ambient and future ocean conditions. When compared in an assay experiment, the difference between the selected populations at the corresponding conditions (future ocean) and control lines (ambient ocean) is the adaptive effect. The correlated response (the difference of ambient selected lines to future ocean selected lines at ambient conditions) reveals the costs of adaptation to a certain environment (Fig.1).

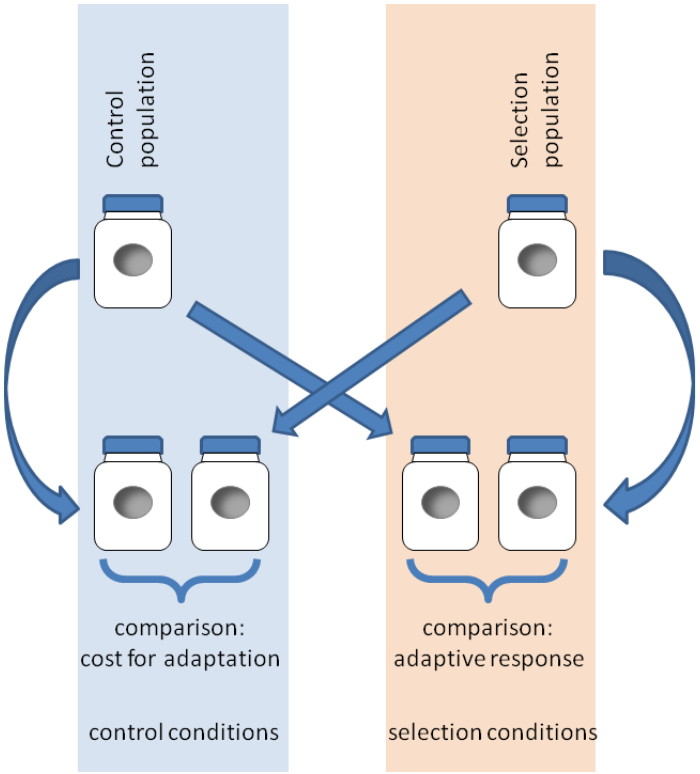

Fig 1. General scheme of a reciprocal experiment. After several generations of selection under selection/control conditions, populations are duplicated and the duplicates transferred into the contrary assay conditions. There the populations are compared in growth rate (the parameter selected for), and other phenotypical parameters. The comparison at selection conditions reveals the adaptive response, while the comparison at control conditions is the cost of adaptation.

Phytoplankton can reproduce both sexually and asexually. Under asexual reproduction the generation time (i.e. time between cell divisions) is relatively short, and in lab culture about 500 generations per year can be accomplished easily. Completely asexual selection regimes of diploid organisms, like *E. huxleyi*, can suffer from mutational load, which is a fitness reduction of a

population owing to accumulated deleterious mutations in the gene pool (does not apply for haploid organisms) (Kimura & Maruyama 1966). Populations with recombination are supposed to tolerate a higher mutational load compared to asexual populations (Rice 2002). Deleterious mutations are faster purged from the population, and beneficial mutations can be combined due to recombination (Rice 2002). However, sex is also accompanied with costs, such as mating and gamete production (Rice 2002). Sexual reproduction increases the rate of adaptation (Kaltz & Bell 2002). Hence, the adaptation rate in our asexual selection regime may underestimate the adaptive rate under natural conditions. However, the selective advantage of sex may only be present as long as adaptation is incomplete (Kaltz & Bell 2002).

Thesis outline

The following three chapters contain the main results of my thesis in the format of scientific publications, one of which (chapter II) is already published (Schlüter et al. 2014). In the end will be a synthesis that discusses the results of all three chapters and sets them into perspective.

Publication 1 is about the longest evolutionary experiment conducted in any marine species to date. This work is continuing the evolutionary experiment described in Lohbeck et al. (2012), which showed increased growth and calcite per cell in *E. huxleyi* after 500 asexual generations of elevated CO_2 selection. The selection was monitored over 2100 generations and revealed that adaptation is certainly not a straight process. While the adaptation in growth rate was still increasing depending on CO_2 level, calcification was adaptively decreased after 500 generations. However, reduction in calcification was an effect of adaptive plasticity.

Publication 2 is whether or not adaptation to temperature is influenced by previous 1600 generations of CO_2 adaptation, compared to treatments which experienced no ocean acidification. *E. huxleyi* could adapt to temperature increase, apparent through higher growth rates which were up to 16% higher in populations adapted for one year to warming when assayed at their upper thermal tolerance limit. The effect of temperature adaptation was roughly three times larger than adaptation to CO_2 at 15°C. There were no constraints of previous adaptation to CO_2 on temperature adaptation. The combined effect of temperature and CO_2 alone was larger than the single temperature effect. A major temperature effect was the decline in cell size over time, which further decreased in replicates subjected to temperature adaptation. After temperature adaptation the cellular PIC:POC-ratio (inorganic to organic carbon-ratio) changed from lower PIC:POC at high CO_2 compared to ambient CO_2 to the opposite.

Publication 3 addresses the complementary question of chapter 2, namely the consequence of co-varying temperature adaptation on the existing CO_2 adaptation. Thus, in the assay experiment only the CO_2 regime was reciprocally varied, while the temperature stayed as under the selection conditions. After 480 generations of combined selection, temperature adaptation caused an increase in the positive adaptive effect to elevated CO_2. The adaptive response to CO_2 selection and temperature selection was additive. Further we found changes in correlated traits after combined selection. The adaptive reaction in cell size to CO_2 reversed from an adaptive decrease at 15°C to an adaptive increase at 26°C. The physiological response in PIC per cell to acidification is diminished at high temperature and no adaptive response was visible. The changes in POC per cell were CO_2-level dependent, with a loss of adaptive response at medium and similar reduction to CO_2 at high level. Adaptive effects on population production were based on adaptation in growth rate and resulted in partial restoration.

Publication I

Author contribution

This chapter is in preparation for publication in a scientific journal under multiple authorship. My contribution to this work is described below.

Title:

Long-term dynamics of adaptive evolution in a globally important coccolithophore to ocean acidification

Authors:
Lothar Schlüter, Kai T. Lohbeck, Joachim P. Gröger, Ulf Riebesell, and Thorsten B. H. Reusch

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Author contributions:
LS and TR conceived the study, LS performed the experiments and conducted the analyses, JG conducted the time series analyses, LS and TR wrote manuscript and all authors edited the manuscript.

Long-term dynamics of adaptive evolution in a globally important coccolithophore to ocean acidification

L. Schlüter,[1] K. T. Lohbeck,[1]† J. P. Gröger,[3] U. Riebesell,[2] T.B.H. Reusch[1]*

[1]Evolutionary Ecology of Marine Fishes, GEOMAR Helmholtz-Centre for Ocean Research Kiel, Düsternbrooker Weg 20, 24105 Kiel, Germany.

[2]Biological Oceanography, GEOMAR Helmholtz-Centre for Ocean Research Kiel Düsternbrooker Weg 20, 24105 Kiel, Germany.

[3]Living Marine Resources Research Unit, Thünen-Institute of Sea Fisheries, Palmaille 9, 22767 Hamburg, Germany.

* corresponding author, e-mail treusch@geomar.de

† present address: University of Gothenburg, Department of Marine Sciences, Sweden, e-mail kai.lohbeck@gu.se.

Abstract

Owing to their large population sizes and short generation times marine phytoplankton may adapt to global change, for example to ocean warming or acidification. Long-term adaptation to novel environments is a dynamic process and phenotypic change can take place thousands of generations after exposure to novel conditions. We conducted a long-term evolution experiment (4 yrs = 2,100 generations) in the abundant and widespread coccolithophore species *Emiliania huxleyi*. We find that long-term adaptation to ocean acidification is complex and initial phenotypic responses may revert for ecologically important traits. While fitness increased slightly over time in response to medium CO_2 conditions (1100 µatm pCO_2), a fitness advantage of 5% remained unchanged over 4 yrs of adaptation in response to high ocean acidification levels (2200 µatm pCO_2). The biogeochemically important trait of calcification was partially restored within the first 500 generations but later reduced in response to selection, enhancing physiological declines of calcification in response to ocean acidification. Interestingly, calcification was not constitutively reduced but particulate inorganic carbon (PIC) cell quotas returned to control treatment levels when transferred back to present-day CO_2 conditions (400 µatm pCO_2). Some trait changes were likely associated with selection for higher cell division rates under laboratory conditions, such as reduced cell size and lower particulate organic carbon content per cell. Our results show that phytoplankton may evolve phenotypic plasticity that can affect biogeochemically important traits, such as calcification, in an unforeseen way under future ocean conditions.

Introduction

About half of the global primary production is contributed by marine phytoplankton (Field, Behrensfled, Randerson, & Falkowski, 1998). They not only form the base of marine food webs, but play a major role in global biogeochemical cycles by turning inorganic carbon and minerals into particulate organic matter that may eventually be transported into the deep ocean via sedimentation, a process known as the biological carbon pump (Falkowski, Fenchel, & Delong, 2008). Coccolithophores, unicellular eukaryotic algae belonging to the haptophytes, are important contributors to the ballasting of organic particles (Armstrong, Lee, & Hedges, 2001). Their cells are covered by tiny calcite platelets, the coccoliths, which have a much higher density than seawater. As many marine calcifying organisms, coccolithophores suffer from ocean acidification (OA, (Kroeker, Kordas, Crim, & Singh, 2010), the dissolution of excess anthropogenic CO_2 in ocean waters (Caldeira & Wickett, 2003), by decreases in calcification and growth rates (Meyer & Riebesell, 2015; Riebesell & Zondervan, 2000). Recent evolution experiments demonstrated, in line with evolutionary theory and results in model microbes, adaptation to OA and warming in the globally important coccolithophore *Emiliania huxleyi* (Lohbeck, Riebesell, & Reusch, 2012; Schlüter et al., 2014). Above studies ran for approximately 1 y (≈500 asexual generations). From evolutionary model species such as *Escherichia coli* and yeast it is well known that adaptive evolution is a dynamic process even in the simplest experiments, designed with a single clone (or genotype) as starting 'population'. For example adaptation to novel conditions via selective sweeps may be delayed by clonal interference (Desai, Fisher, & Murray, 2007; Lang et al., 2013), adaptive improvements may be sudden and step-wise when mutations are rare (Elena, Cooper, & Lenski, 1996) and consecutive beneficial mutations may depend on one another, introducing lineage-specific historical contingency (Blount, Borland, & Lenski, 2008). Hence, further phenotypic changes can occur over several thousand generations. For example, in *E. coli* an adaption to citrate utilization arose 31,500 generations after initial exposure to glucose minimal medium that was citrate enriched (Blount et al., 2008). A recent 15-y time series reports evidence for adaptive changes of natural phytoplankton communities to yearly variation in environmental conditions (temperature and irradiance) (Irwin, Finkel, Müller-Karger, & Troccoli Ghinaglia, 2015).

Here, we evolved replicate populations of *E. huxleyi* for 2100 asexual generations (4 yrs) to ambient (400 µatm pCO_2) and two elevated pCO_2 levels, a medium concentration predicted for the worst-case, end-of-the-century level (1100 µatm, (Stocker, 2013)) and a high, proof-of-principle concentration (2200 µatm pCO_2) that is temporarily found in contemporary coastal waters under upwelling (Melzner et al., 2013), and will be maximally reached in the year 2300 (Caldeira & Wickett, 2003). Upon initial findings of a partial restoration of calcification in *E. huxleyi* (Lohbeck et al., 2012),

we were particularly interested in how calcification played out in subsequent years. Another important question was whether or not adaptive evolution would lead to complete restoration of algal performance and fitness under OA compared to non-adapted controls when both were assayed under ocean acidification conditions.

Results

Over 2100 asexual generations, mean exponential growth rates in all treatments increased with time (μ * generation^{-1}: ambient CO_2 6.56 x 10^{-5}, medium CO_2 4.33 x 10^{-5}; high CO_2 7.60 x 10^{-5}, ARMAX model with significant autocorrelation terms, all trends P<0.001), with no detectable difference between treatments (Fig. 1A). In contrast, cell diameter decreased over time in all treatments, again with no difference among the treatment levels (Fig. 1B, ARMAX model all trends P<0.001). Thus, cells had between 12 and 22% less volume at the end of year 4 compared to the starting populations. These background changes owing to the general selection regime in the laboratory need to be taken into account when interpreting the adaptive responses owing to simulated ocean acidification.

To asses evolutionary responses to elevated CO_2 and in order to control for general laboratory adaptation, we measured growth rates of medium and high CO_2 adapted populations in the respective elevated CO_2 environment via reciprocal assay experiments relative to control populations that at the same time had evolved under ambient CO_2 conditions (Collins, 2011). In all assay experiments, populations were acclimated for at least one full batch cycle (7-8 generations) to their novel condition. Assay experiments were conducted at five time points (500, 1000, 1200, 1600 and 2100 asexual generations), and also included a test of the correlated response, the back-exposure of populations evolved under elevated CO_2 to ambient conditions (Fig. S2).

We focus here on the time course of the adaptive response (Fig. 2) which is given by the difference between control populations (always depicted as solid line) relative to populations that were allowed to long-term adapt to OA, both tested under OA conditions. In all assay experiments CO_2-selected population grew faster than non–adapted ones when assayed under both medium and high CO_2 (Fig. 2A,B; repeated measurement rmANOVA, medium CO_2-selection, $F_{1,8}$=43.92, P=0.0002; high CO_2-selection: $F_{1,8}$ = 53.72, P<0.0001). Growth rate adaptation increased over time under medium CO_2-selection (rmANOVA, time x selection: $F_{4,32}$=2.941, P=0.035). Under high CO_2 selection the adaptation effect was continually present and did not increase further, nor was there a significant fluctuation of the adaptive response (rmANOVA, time x selection, $F_{4,32}$=1.991, ns). Except for the first assay experiment after approximately 450 generations, medium and high-CO_2 selected populations grew slower compared to controls under ambient pCO_2 (Fig. S2A), thus revealing a cost to adaptation.

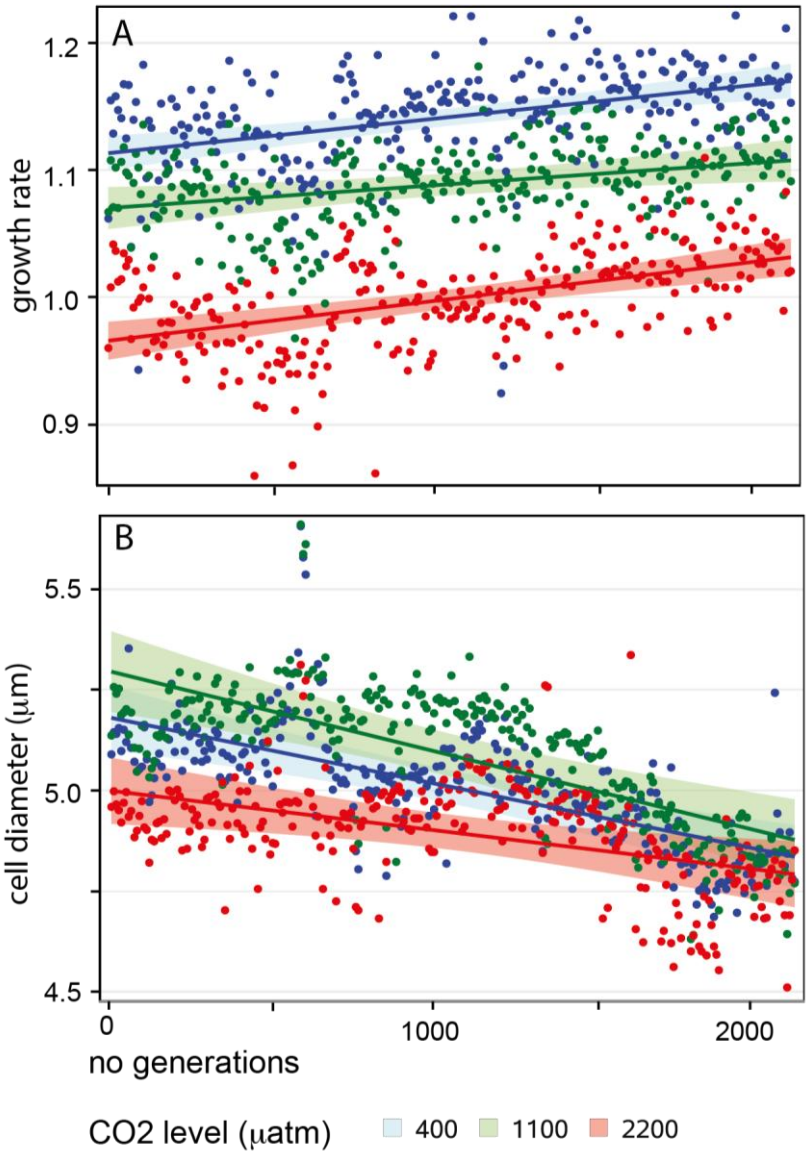

Fig. 1. Time course of exponential growth rates (A) and cell diameter (B) in *Emiliania huxleyi* over 4 yr of selection to three different CO_2 concentrations, simulating ocean acidification. Growth rates and cell sizes were calculated every 5-days upon transfer of batch cultures. All lines reveal highly significant slopes that are not significantly different among CO_2 treatments. As we were not specifically interested in detecting a response level difference but differences in the time trends (i.e. slopes), and as temporal variables are usually autocorrelated, the trend lines were not estimated based on simple regression but using a series of Autoregressive Moving Average Models with exogenous variables (ARMAX models, transfer functions).

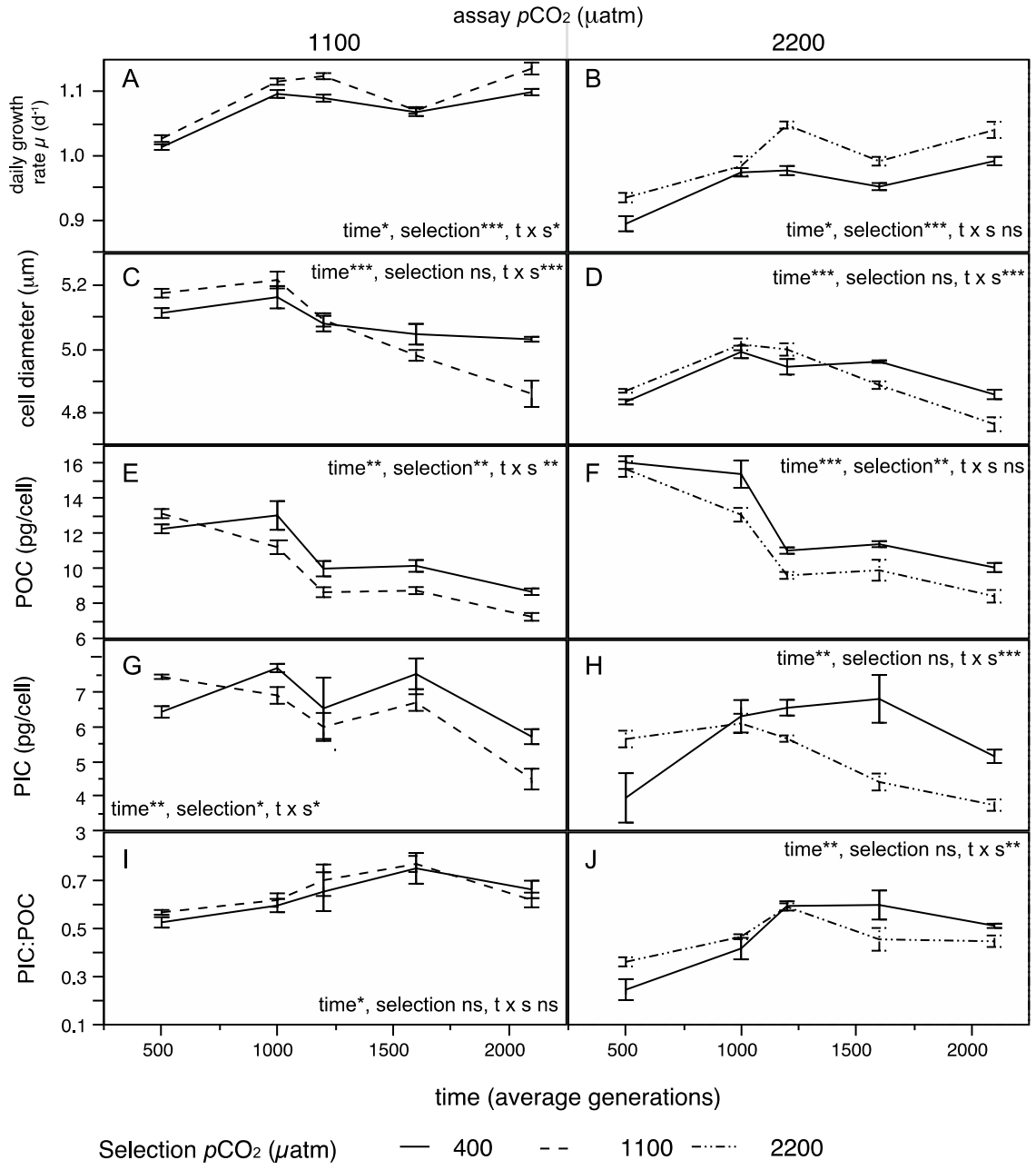

Fig. 2. Evolutionary response of *Emiliania huxleyi* to selection under three CO_2 conditions simulating ocean acidification. Depicted is the adaptive response over 4 years measured during assay experiments at 5 time points (x-axis, average generations of all three CO_2 treatments), always comparing medium- (left) and high-CO_2 adapted (right) vs. non-adapted populations of *E. huxleyi* in three different CO_2 environments, when assayed under elevated CO_2 (mean±SD, *n*=5). (A,B) exponential growth rate (C;D) cell diameter (E,F) particulate organic carbon per cell (POC cell^{-1}) (G;H) particulate inorganic carbon per cell (PIC cell^{-1}) (I,J) ratio of PIC:POC. Significant results of main and interaction effects are depicted with asterisks (*0.05≥*P*>0.01, **0.01≥*P*>0.001, ***P<0.001). Complete repeated measures ANOVA results are given in Table S1. The correlated response, i.e. the performance of all selection treatments under ambient CO_2 is presented in Fig. S2.

Next we focused on ecologically important cell traits that were not directly subjected to selection such as cell sizes and elemental quotas. For cell size the phenotype changed in the course of the 4-yr experiment. While medium and high CO_2 selected cells were initially larger, they became smaller

after 1400 generations relative to control populations (Fig. 2C,D, rmANOVA, time x CO_2-selection, medium: $F_{4,32}$=11.44, P<0.0001; high: $F_{4,32}$=10.89, P<0.0001), although the main effect CO_2 selection was not significant (rmANOVA, medium: $F_{1,8}$=0.762, ns; high: $F_{1,8}$=0.593, ns). Note that cell size changes associated with adaptation to elevated CO_2 are superimposed by a general trend towards smaller cells in all treatments (Fig. S2B; Fig. S2B).

The particulate organic carbon (POC) content of the cells decreased over time in all treatments as a consequence of selection under elevated CO_2. Compared to short-term exposed controls, we observed a decrease of POC-content under both medium and high CO_2 selection (Fig. 2E,F, rmANOVA, medium CO_2 selection: $F_{1,7}$=18.13, P=0.0038; high CO_2 selection: $F_{1,6}$=17.45, P=0.0058), with the interaction with time being non-significant in both cases. Except for a non-significant decrease at 1000-generations, the general decrease in POC is apparent for all three selection treatments under ambient pCO_2 (correlated response, Fig. S2C). Owing to the 12-22% decrease in cell volume across treatments we standardized cell quota by cell volume. The general picture remained the same, especially in the high CO_2 selection treatment (Fig. S3A,B; rmANOVA: medium CO_2 selection $F_{1,7}$=6.47, P=0.0384; high CO_2 selection $F_{1,6}$=12.42, P=0.0124).

We were particularly interested how cell quotas in particulate inorganic carbon (PIC) would change throughout the selection experiment. We expected that given sufficient time, the observed partial restoration of PIC cell quotas after 500 generations (Lohbeck et al., 2012) would be completely restored in both, medium and high-CO_2 treatments. Contrary to expectations, PIC cell quotas markedly decreased after generation 1000 to be lower in populations adapted to high CO_2 than in non-adapted controls when subjected to OA conditions (Fig. 1G,H; rmANOVA, time x CO_2: medium CO_2 selection: $F_{4,28}$=4.85, p=0.0042; high CO_2 selection: $F_{4,24}$=6.79, P=0.0008). In response to medium and high CO_2 selection, CO_2 adapted populations displayed 21% and 22% lower PIC, respectively, compared to the physiological decline of PIC in the control populations under medium and high CO_2 (Fig. 2G,H; all at 2100 generations). This pattern remained after we standardized PIC content on cell volume to compensate for the general decrease in cell size over time (Fig. S3C,D; rmANOVA, time x CO_2: medium CO_2 selection: $F_{4,28}$=3.95, P=0.0115; high CO_2 selection: $F_{4,24}$=5.58, P=0.0025). In terms of PIC:POC ratio we found an interaction of time and CO_2 selection in the high, but not in response to medium CO_2 selection (Fig. 1I,J, rmANOVA, time x CO_2: medium: $F_{4,28}$=0.3378, ns; high: $F_{4,24}$=6.36, P=0.0012).

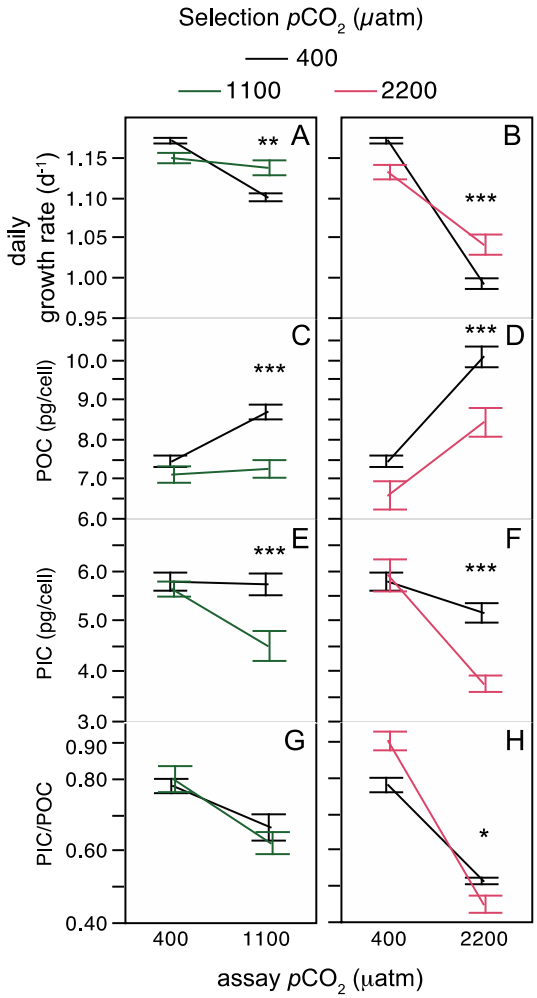

Fig. 3. Reaction norms of *Emiliania huxleyi* populations selected under ambient and high CO_2 conditions as a function of the assay condition. Depicted are (A, B) daily growth rate, (C, D) particulate organic carbon POC; (E, F) particulate inorganic carbon (PIC) and the PIC:POC ratio (G, H) after 2100 generations of evolution (4 yrs). Given are mean values (±1SD, n=5). Significant results of planned contrasts only for the adaptation response (i.e. under elevated CO_2) are indicated by asterisks (*0.05≥*P*>0.01, **0.01≥*P*>0.001, ***P<0.001).

For the last assay experiment we plotted population-wise reaction norms for important parameters under ambient and elevated CO_2 levels to address the evolution of plasticity in traits other than fitness itself that accompanied adaptation (Fig. 2A,B). After 4 yrs, corresponding to ≈2,100 asexual generations, we observed a fitness increase in both selection treatments, resulting in 3.4% and 4.8% fitness increase (calculated according to ref {Lenski, 1991 #2995}) under medium and high CO_2, respectively, compared to control populations. Adaptation to OA was accompanied with 'classical' costs of adaptation in both medium and high CO_2 when back-exposed at ambient CO_2 (Fig. 2A,B; 2x2-ANOVA, interaction selection x assay CO_2, both P<0.001).

The reaction-norm of the POC quota of cells after CO_2 selection revealed a strong 'overshooting' of POC cell quota of non-adapted populations under medium (+20% POC, Fig. 2C) and high CO_2 assay conditions (+19%, Fig. 2D) compared to high-CO_2 adapted ones. Slopes of the reaction norms in

response to assay CO_2, however, were only significantly different among ambient vs. medium CO_2 selected populations (2-factorial ANOVA, interaction selection x assay CO_2, P=0.011; Fig. 2D). Also, medium-CO_2 adapted populations restored their POC quotas under high CO_2 to levels observed in controls under ambient CO_2 (Fig. 2C; planned contrast ns), while there was still 11% more POC per cell for the high-CO_2 selected replicates tested under high CO_2 compared to the ambient control (Fig. 2D, planned contrast P=0.0368).

For calcification (assessed as PIC cell quota) populations selected for 4 yrs to ocean acidification showed markedly different reaction-norms and hence patterns of phenotypic plasticity (interaction selection x assay CO_2, P=0.022 and 0.0033 for medium/high selection, respectively). Despite the loss of calcification under OA assay conditions, CO_2 selected populations increased their PIC quota when transferred back into ambient pCO_2 (+25% for medium CO_2-selection, Fig. 2*E*, +58% for high selection, Fig. 2*F*, planned contrast both P<0.001) which was then indistinguishable from control populations under ambient CO_2. As a result of both, changes in POC and PIC cell quota reaction norms, the PIC:POC ratio determines the specific mass and hence, ballasting effect of an individual coccolithophore (Fig. 2G,H). Here, for the high CO_2 selected populations only, the PIC:POC ratio was significantly reduced after long-term adaptation under high CO_2, while overcompensated upon re-exposure to ambient CO_2 (Fig. 2H).

Discussion

Our experiment is the first to describe extended long-term adaptation for a few thousand generations in any marine microbial species. We find that even one year of evolution reported earlier (Lohbeck et al., 2012) may only cover a transient response in traits correlated with growth rate adaptation when compared to longer time intervals. Such complex long-term dynamics have also been found in selection experiments with model microbes (Blount et al., 2008; Elena & Lenski, 2003; Lang et al., 2013), although these species usually lack an immediate ecological or biogeochemical significance. In contrast, our results have implications for biogenic calcite production and carbon sequestration, given that *E. huxleyi* is the world's most abundant calcifying species (Westbroek, Young, & Linschooten, 1989). We show that long-term evolution exacerbates the immediate physiological decline in PIC quotas, which decreased the ballasting effect of single coccolithophore cells at least under high but not medium CO_2 selection. This in turn may have consequences for particle ballasting and the ocean's biological carbon pump, along with the general abundance of coccolithophores in a phytoplankton community (Armstrong et al., 2001). Using a globally important phytoplankton species that significantly contributes to marine primary production and fuels marine food webs, we are able to merge direct assessments of evolutionary processes with ecological and

biogeochemical significance, an aspect that is rarely considered in experimental evolution (Reusch & Boyd, 2013).

How these changes in the adaptive dynamics came about is currently elusive. It may be that genotypes were already present within the first year of experimental duration that calcified less under high-CO_2 conditions, thus were already 'plastic' with respect to producing calcite plates. recent experiments in a model asexual diploid, yeast, have revealed rampant genetic hitchhiking and clonal competition during the course of simple evolution experiments (Lang et al., 2013).

To assess the contributions of initially rare genotypes one would have to take many sub-replicates during the run time of the experiment, which was prohibited by the large logistical effort to grow *E. huxlyei* in appreciable population sizes in the laboratory.

Growth rate is the parameter we selected for and directly reflects Darwinian fitness in an asexually reproducing population (Lenski, Rose, Simpson, & Tadler, 1991). How other traits respond to CO_2 selection depends upon the genetic architecture (e.g. genetic correlations) and on the contribution of any particular trait to fitness (i.e. its fitness function). Parameters such as cell size are probably closely correlated with cell division and exponential growth rates of the batch cultures (Tang, 1995). The decrease in cell volume observed in all treatments of up to 22% (Fig. 1B) is mainly driven by our selection regime of sequential batch cultures that favor maximal exponential growth rates and thus resulting in smaller cells. When subtracting the decline in cell volume attributable to selection for fast growth, assay experiments revealed that there is still an additional decrease of particulate organic carbon (POC) caused by selection to increased CO_2. Reported physiological responses to ocean acidification are an increase in organic storage compounds including lipids and glucans (Rokitta, John, & Rost, 2012) which may have reverted owing to long-term selection. Detailed physiological assessments along the time course of experimental evolution are clearly warranted that dissect the cellular mechanisms, costs and constraints determining the phenotypic responses observed. Note, however, that the specific time course of adaptation, correlated responses and trait evolution may be contingent upon the particular genotype (clone #62) that was chosen to initiate the experiment (Elena & Lenski, 2001). Even if the genetic starting material is completely identical, historical contingency may produce idiosyncratic outcomes of evolution when "rewinding the evolutionary tape" (Blount et al., 2008).

How coccolith formation and hence the PIC cell quota is linked to fitness is still elusive. The biogenic precipitation of calcium carbonate is an energy demanding process, especially under elevated CO_2 (Mackinder, Wheeler, Schroeder, Riebesell, & Brownlee, 2010). When initiating long-term ocean acidification selection in *E. huxleyi*, we therefore expected a reduction in calcification as one

adaptation mechanism to ocean acidification conditions, assuming that selective pressure to maintain coccolith formation is absent in our microcosm system. After an initial partial restoration of PIC quotas and calcification rates during the first year of experimental evolution (Lohbeck et al., 2012), our data are consistent with initial expectations after about generation 1000. Remarkably, the ability to calcify was not constitutively lost in high-CO_2 adapted populations, but we observed the evolution of a flexible response, i.e. phenotypic plasticity. While we found a complete restoration of PIC per cell under ambient CO_2, elevated CO_2–selection resulted in a further decrease of PIC, lower than in replicates exposed to elevated CO_2 for the first time.

Phenotypic plasticity describes how the same genotype gives rise to different phenotypes as a function of different environments (Scheiner, 1993). One important aspect of studying phenotypic plasticity is to investigate how different genotypes of a particular phytoplankton species respond to higher CO_2 levels and how this may affect primary productivity in future ocean conditions (E. Schaum, Rost, Millar, & Collins, 2013). If the net CO_2 effect is negative, as in *E. huxleyi*, phenotypic buffering may extend the range of tolerances and keep organismal function even under ocean acidification (Reusch, 2014). Here, we found that an energetically costly trait, namely biogenic calcification (Mackinder et al., 2010; Westbroek et al., 1989), is reduced due to long-term selection under high CO_2 conditions, while it is almost unchanged when high CO_2 adapted populations are back exposed to the ancestral condition (ambient CO_2 concentration). Thus, the high-CO_2 adapted replicates have evolved phenotypic plasticity in their novel environment (Ghalambor, McKay, Carroll, & Reznick, 2007) and now reveal two distinct phenotypes, one with reduced and one with 'normal' PIC cell quotas, as a function of the assay CO_2 environment (=correlated response). A selection experiment with increased CO_2 in the freshwater alga *Chlamydomonas* showed that conditionally deleterious mutations accumulated in the high-CO_2 selection lines, leading to lower growth when re-exposed to ambient CO_2 (Collins & Bell, 2004), which may also be the mechanism leading to costs of CO_2 adaptation in the medium and high-CO2 selected replicates past generation 1000. In contrast, the mutations that genotypes carried which we have to invoke in adapting *E. huxleyi* during the first 500 generations of our experiments involved a gain function and visible when comparing control to adapted populations in the novel environment. They probably involved regulatory regions because the phenotypic effects were reversible and only visible in the high-CO_2 assay environment.

The process of calcification requires tight metabolic regulation, such as pH regulation and directed Ca^{2+} and HCO_3^- transport to the coccolith vesicle prior to the precipitation of calcite itself (Mackinder et al., 2011, 2010). The cytosolic pH, in turn, decreases upon acidification (Rokitta et al., 2012; Suffrian, Schulz, Gutowska, Riebesell, & Bleich, 2011). While not only critical for calcification, pH homeostasis is important for many metabolic processes. Initially, adaptation may have improved the

cellular pH regulation under long-term high-CO_2 selection, and calcification may have passively followed, since the energy demand for calcification would decrease (Lohbeck, Riebesell, & Reusch, 2014). The later decrease in PIC cell quota suggests that beyond approx. 1,000 generations, pH homeostasis (determining growth rates) and calcification processes became decoupled.

Care needs to be taken when translating our results from laboratory microcosms face value to the natural system. For example, we always kept our cultures in exponential phase to maintain the desired treatment levels of OA, while in nature, population densities in bloom situations will lead to much higher competition strength. In nature, trade-offs with other important functions of calcification and coccolith production, for example grazer or viral defense (Raven & Crawfurd, 2012), most likely will prohibit the evolution of phenotypic plasticity with respect to coccolith formation observed here. Also, by starting with a single genotype we only allowed for novel mutations as driver of evolutionary adaptation, while genotypic selection will most likely be equally or even more important in nature (Lohbeck et al., 2012; Reusch & Boyd, 2013).

Experimental evolution fills the gap between paleontological studies of evolutionary changes (Kidwell, 2015) and field time-series (Irwin et al., 2015) by directly addressing the evolutionary dynamics on a time scale of months to years. Our data demonstrate that caution is advised even in the simplest experimental set-ups, as responses after one year of adaptation may be transient. Clearly, more complex designs are highly warranted, for example that compare varying versus constant selection regimes (C.-E. Schaum, Rost, & Collins, 2015). Also, experiments including competitors, pathogens and predators are clearly desirable to simulate adaptive evolution under more realistic conditions as one component of phytoplankton community change in a future ocean (Collins, Rost, & Rynearson, 2014; Reusch & Boyd, 2013).

Materials and Methods

The asexual populations in this experiment originate from a single cell, isolated in May 2009 from the coastal waters off Bergen, Norway (clone no. 62, (Lohbeck et al., 2012)). Populations were kept in artificial seawater (ASW, (Kester, Duedall, Connors, & Pytkowicz, 1967)), for details see ref (Lohbeck et al., 2012). To achieve a total alkalinity (TA) of 2380 µmol kg^{-1}, 0.19 g bicarbonate were added. CO_2 levels were manipulated by aerating the ASW for 24 h at 15°C under saturated humidity. Note that the aeration (bubbling) happened separate from growing the cultures in closed bottles allowing no further physical gas exchange. Cultures were not kept axenic, because *Emiliania huxleyi* grows poorly without associated bacteria.

The selection experiment was carried out as a batch culture system with five replicates each for ambient (400 µatm pCO_2, control), medium (1100 µatm pCO_2) and high (2200 µatm pCO_2) under

continuous rotation (0.5 min^{-1}) at 15°C at a photon flux density of 150 µmol $m^{-2}s^{-1}$ and a 16:8 light dark cycle. CO_2 selection was initiated on in May 2010 and lasted for 268 batch cycles (~4 years), corresponding to ~2200 (ambient), ~2100 (medium) and ~1900 (high CO_2) generations of asexual reproduction. The populations were grown with minimal headspace in a total volume of 310 ml. Every 5 days 10^5 cells per replicate were transferred into the next batch. Cell density and size were determined at each transfer using a Coulter Counter Z2 Particle/Size Analyzer. Daily growth rates (µ) were calculated from cell densities according to $\mu = (\ln N_d - \ln N_0)/d$, where $N_{(0)}$ and $N_{(d)}$ are cell numbers at the beginning and end of the batch cycle, and d the duration of the batch cycle in days (d=5). For the assay experiments, conducted after ~500/1000/1200/1600 and 2100 asexual generations, treatments grown at 400 µatm control pCO_2 were acclimated over one full batch cycle to the respective elevated CO_2-levels. Growth rates and additional correlated traits were then measured along with the long-term evolved treatments under medium and high CO_2 assay conditions (adaptive response). Replicates evolving at 1100 and 2200 µatm, respectively, were also back-transferred to ambient CO_2 to assess the correlated response along with control replicates. The reciprocal transfer was incomplete, i.e. the exposure of 1100 and 2200 µmol was omitted. Dissolved inorganic carbon was measured colorimetrically using a SOMMA autoanalyzer or an AIRICA system (Marianda). CO_2 partial pressure values before inoculation were calculated from DIC and TA using the program CO2SYS {Lewis, 1998 #3060}. The draw-down of TA and DIC during the batch cycles were calculated from the total particular carbon measurements. The average dissolved inorganic carbon (DIC) drawdown ±1SD was about 4% with a maximum of 7%. The measured CO_2 partial pressure varied from the desired level (ambient 430±32, medium 1350±207, high 2398±200 µatm). During the selection phase one replicate of the high CO_2 selection line was temporarily contaminated with low numbers of heterotrophic nanoflagellates (<1% in cell number). If excluded, none of the statistical results changed qualitatively, therefore the analyses present the full data set.

The cultures were vacuum filtered (<100mbar) onto combusted glass fiber filters (GF/F) for the quantification of total particulate carbon (TPC) and particulate organic carbon (POC). All filtrations were performed at the same time of the day (≈4-5 hours after start of light phase). POC filters were fumed with 37% HCl for 2h to remove inorganic carbon and dried at 60°C for 12h. The measurements were performed with elemental analyzers. Particulate inorganic carbon (PIC) was calculated by subtracting HCl fumed POC from TPC values.

We estimated changes in terms of cell sizes and growth rates using Autoregressive Moving Average Models with exogenous variables (ARMAX models, transfer functions) including any autocorrelation term, according to

$$\text{Response} = b_0 + b_1 * d_1 * \text{time} + b_2 * d_2 * \text{time} + b_3 * d_1 + \text{ARMA terms} \quad (1)$$

where d_i in {0,1} is a dummy variable (i^{th} d_1 t_i = 1 if observation i at time t belongs to treatment 1 and d_1 t_i = 0 else, d_2 t_i =1- (d_1 t_i) indicates whether (d_2 t_i = 1) or not (d_2 t_i = 0) the observation belongs to treatment 2. Hypotheses of significant slopes and treatment differences were assessed using conventional *t*- and *F*-tests. Note that according to (1) dynamic effects are assumed to be the same in both of the samples. This assumption is justified theoretically due to the unified experimental design, and is empirically corroborated by preliminary empirical tests.

For the statistical analysis of the reciprocal assay experiments the dataset was subdivided, for each level of elevated CO_2, into two parts to avoid singularities. We analyzed the adaptive and correlated response separately because they represented distinct hypotheses and to avoid data interdependencies of the same replicates measured under 2 different assay conditions. For each subset, a repeated measure ANOVA (rmANOVA) including a sphericity test for the within-subject terms was conducted using JMP v. 9.0 (Statsoft Inc.). Subsequent post-hoc contrasts were performed if appropriate. The final assay experiment was subjected to 2-factorial ANOVA with subsequent planned contrasts, separately for each CO_2-treatment. We checked for homogeneity of variances and normal distribution and found no major violation to the test assumptions.

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Author contribution: LS and TR conceived the study, LS performed the experiments and conducted the analyses, JG conducted the time series analyses, LS and TR wrote manuscript and all authors edited the manuscript.

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Data and materials availability: Raw data have been deposited in the World Data Center for Marine Environmental Sciences (WDC-MARE) (accession http://doi.pangaea.de/10.1594/PANGAEA.846062).

References:

Armstrong, R., Lee, C., & Hedges, J. (2001). A new, mechanistic model for organic carbon fluxes in the ocean based on the quantitative association of POC with ballast minerals. *Deep Sea Research …*, *49*, 219–236. Retrieved from http://www.sciencedirect.com/science/article/pii/S0967064501001011

Blount, Z. D., Borland, C. Z., & Lenski, R. E. (2008). Historical contingency and the evolution of a key innovation in an experimental population of Escherichia coli. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(23), 7899–906. doi:10.1073/pnas.0803151105

Caldeira, K., & Wickett, M. E. (2003). Oceanography: Anthropogenic carbon and ocean pH. *Nature*, *425*(6956), 365. Retrieved from http://dx.doi.org/10.1038/425365a

Collins, S. (2011). Many Possible Worlds: Expanding the Ecological Scenarios in Experimental Evolution. *Evolutionary Biology*, *38*(1), 3–14. doi:10.1007/s11692-010-9106-3

Collins, S., & Bell, G. (2004). Phenotypic consequences of 1,000 generations of selection at elevated CO2 in a green alga. *Nature*, *431*(7008), 566–9. doi:10.1038/nature02945

Collins, S., Rost, B., & Rynearson, T. A. (2014). Evolutionary potential of marine phytoplankton under ocean acidification. *Evolutionary Applications*, *7*(1), 140–155.

Desai, M. M., Fisher, D. S., & Murray, A. W. (2007). The speed of evolution and maintenance of variation in asexual populations. *Current Biology : CB*, *17*(5), 385–94. doi:10.1016/j.cub.2007.01.072

Elena, S. F., Cooper, V. S., & Lenski, R. E. (1996). Punctuated Evolution Caused by Selection of Rare Beneficial Mutations. *Science* , *272* (5269), 1802–1804. doi:10.1126/science.272.5269.1802

Elena, S. F., & Lenski, R. E. (2001). EPISTASIS BETWEEN NEW MUTATIONS AND GENETIC BACKGROUND AND A TEST OF GENETIC CANALIZATION. *Evolution*, *55*(9), 1746–1752. doi:10.1111/j.0014-3820.2001.tb00824.x

Elena, S. F., & Lenski, R. E. (2003). Evolution experiments with microorganisms: the dynamics and genetic bases of adaptation. *Nature Reviews. Genetics*, *4*(6), 457–69. doi:10.1038/nrg1088

Falkowski, P. G., Fenchel, T., & Delong, E. F. (2008). The microbial engines that drive Earth's biogeochemical cycles. *Science (New York, N.Y.)*, *320*(5879), 1034–9. doi:10.1126/science.1153213

Field, C. B., Behrensfled, M. J., Randerson, J. T., & Falkowski, P. (1998). Primary Production of the Biosphere: Integrating Terrestrial and Oceanic Components. *Science*, *281*(5374), 237–240. doi:10.1126/science.281.5374.237

Ghalambor, C. K., McKay, J. K., Carroll, S. P., & Reznick, D. N. (2007). Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. *Functional Ecology*, *21*, 394–407. doi:10.1111/j.1365-2435.2007.01283.x

Irwin, A. J., Finkel, Z. V, Müller-Karger, F. E., & Troccoli Ghinaglia, L. (2015). Phytoplankton adapt to changing ocean environments. *Proceedings of the National Academy of Sciences of the United States of America*, (33), 1–5. doi:10.1073/pnas.1414752112

Kester, D., Duedall, I., Connors, D., & Pytkowicz, R. (1967). Preparation of artificial seawater. *Limnol. Oceanogr*, *12*, 176–179. Retrieved from http://www.aslo.org/lo/toc/vol_12/issue_1/0176.html

Kidwell, S. M. (2015). Biology in the Anthropocene: Challenges and insights from young fossil records. *Proceedings of the National Academy of Sciences*, *112*(16), 4922–4929. doi:10.1073/pnas.1403660112

Kroeker, K. J., Kordas, R. L., Crim, R. N., & Singh, G. G. (2010). Meta-analysis reveals negative yet variable effects of ocean acidification on marine organisms. *Ecology Letters*, *13*(11), 1419–1434. doi:10.1111/j.1461-0248.2010.01518.x

Lang, G. I., Rice, D. P., Hickman, M. J., Sodergren, E., Weinstock, G. M., Botstein, D., & Desai, M. M. (2013). Pervasive genetic hitchhiking and clonal interference in forty evolving yeast populations. *Nature*, *500*(7464), 571–4. doi:10.1038/nature12344

Lenski, R. E., Rose, M. R., Simpson, S. C., & Tadler, S. C. (1991). Long-Term Experimental Evolution in Escherichia coli. I. Adaptation and Divergence During 2,000 Generations. *The American Naturalist*. doi:10.1086/285289

Lohbeck, K. T., Riebesell, U., & Reusch, T. B. H. (2012). Adaptive evolution of a key phytoplankton species to ocean acidification. *Nature Geoscience*, *5*(5), 346–351. doi:10.1038/ngeo1441

Lohbeck, K. T., Riebesell, U., & Reusch, T. B. H. (2014). Gene expression changes in the coccolithophore Emiliania huxleyi after 500 generations of selection to ocean acidification. *Proceedings of The Royal Society B*. doi:http://dx.doi.org/10.1098/rspb.2014.0003

Mackinder, L., Wheeler, G., Schroeder, D., Riebesell, U., & Brownlee, C. (2010). Molecular Mechanisms Underlying Calcification in Coccolithophores. *Geomicrobiology Journal*, *27*(6-7), 585–595. doi:10.1080/01490451003703014

Mackinder, L., Wheeler, G., Schroeder, D., von Dassow, P., Riebesell, U., & Brownlee, C. (2011). Expression of biomineralization-related ion transport genes in Emiliania huxleyi. *Environmental Microbiology*, *13*(12), 3250–3265. doi:10.1111/j.1462-2920.2011.02561.x

Melzner, F., Thomsen, J., Koeve, W., Oschlies, A., Gutowska, M., Bange, H., … Körtzinger, A. (2013). Future ocean acidification will be amplified by hypoxia in coastal habitats. *Marine Biology*, *160*(8), 1875–1888. doi:10.1007/s00227-012-1954-1

Meyer, J., & Riebesell, U. (2015). Reviews and Syntheses: Responses of coccolithophores to ocean acidification: a meta-analysis. *Biogeosciences*, *12*(6), 1671–1682. doi:10.5194/bg-12-1671-2015

Raven, J., & Crawfurd, K. (2012). Environmental controls on coccolithophore calcification. *Marine Ecology Progress Series*, *470*, 137–166. doi:10.3354/meps09993

Reusch, T. B. H. (2014). Climate change in the oceans: Evolutionary versus phenotypically plastic responses of marine animals and plants. *Evolutionary Applications*, *7*(Somero 2010), 104–122. doi:10.1111/eva.12109

Reusch, T. B. H., & Boyd, P. W. (2013). Experimental evolution meets marine phytoplankton. *Evolution; International Journal of Organic Evolution*, *67*(7), 1849–59. doi:10.1111/evo.12035

Riebesell, U., & Zondervan, I. (2000). Reduced calcifcation of marine plankton in response to increased atmospheric CO 2. *Nature*, *407*(September), 2–5.

Rokitta, S. D., John, U., & Rost, B. (2012). Ocean acidification affects redox-balance and ion-homeostasis in the life-cycle stages of Emiliania huxleyi. *PloS One*, *7*(12), e52212. doi:10.1371/journal.pone.0052212

Schaum, C.-E., Rost, B., & Collins, S. (2015). Environmental stability affects phenotypic evolution in a globally distributed marine picoplankton. *The ISME Journal*, 1–10. doi:10.1038/ismej.2015.102

Schaum, E., Rost, B., Millar, A. J., & Collins, S. (2013). Variation in plastic responses of a globally distributed picoplankton species to ocean acidification. *Nature Climate Change*, *3*(3), 298–302. doi:10.1038/nclimate1774

Scheiner, S. (1993). Genetics and evolution of phenotypic plasticity. *Annual Review of Ecology and Systematics*, *24*(1993), 35–68. Retrieved from http://www.jstor.org/stable/2097172

Schlüter, L., Lohbeck, K. T., Gutowska, M. a., Gröger, J. P., Riebesell, U., & Reusch, T. B. H. (2014). Adaptation of a globally important coccolithophore to ocean warming and acidification. *Nature Climate Change*, *4*(11), 1024–1030. doi:10.1038/nclimate2379

Stocker, T. F. (2013). The Closing Door of Climate Targets, *280*(339). doi:10.1126/science.1232468

Suffrian, K., Schulz, K. G., Gutowska, M. A., Riebesell, U., & Bleich, M. (2011). Cellular pH measurements in Emiliania huxleyi reveal pronounced membrane proton permeability, (2002), 595–608.

Tang, E. P. Y. (1995). The allometry of algal growth rates. *Journal of Plankton Research*, *17*(0), 1325–1335. Retrieved from /media/kanaloa/biblioteca/articles\tang_95.pdf

Westbroek, P., Young, J. R., & Linschooten, K. (1989). Coccolith production (biomineralization) in the marine alga Emiliania huxleyi. *Journal of Protozoology*, *36*(4), 368–373.

Publication II

Author contribution

This chapter is published in a scientific journal under multiple authorship. My contribution to this work is described below.

Title:

Adaptation of a globally important coccolithophore to ocean warming and acidifications

Authors:
Lothar Schlüter, Kai T. Lohbeck, Magdalena A. Gutowska, Joachim P. Gröger, Ulf Riebesell, and Thorsten B. H. Reusch

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LS, TBHR, UR and KTL designed the experiment, LS performed the experiment. All authors analyzed and interpreted the data. LS wrote an earlier and TBHS the final version of the manuscript.

Adaptation of a globally important coccolithophore to ocean warming and acidification

Lothar Schlüter[1], Kai T. Lohbeck[1], Magdalena A. Gutowska[2,4], Joachim P. Gröger[3], Ulf Riebesell[2], Thorsten B.H. Reusch[1]*

* corresponding author, e-mail treusch@geomar.de

1) Evolutionary Ecology of Marine Fishes, GEOMAR Helmholtz-Centre for Ocean Research Kiel, Düsternbrooker Weg 20, 24105 Kiel, Germany

2) Biological Oceanography, GEOMAR Helmholtz-Centre for Ocean Research Kiel Düsternbrooker Weg 20, 24105 Kiel, Germany

3) Living Marine Resources Research Unit, Thünen-Institute of Sea Fisheries, Palmaille 9, 22767 Hamburg, Germany

4) Present address:

Molecular Microbial Ecology, Monterey Bay Aquarium Research Institute (MBARI), 7700 Sandholdt Road, 95039 Moss Landing, USA

Abstract

While ocean warming and acidification are recognized as two major anthropogenic perturbations of today's oceans we know very little about how marine phytoplankton may respond via evolutionary change. We tested for adaptation to ocean warming in combination with ocean acidification in the globally important phytoplankton species *Emiliania huxleyi*. We found up to 16% higher growth rates in populations adapted for one yr to warming under ambient and elevated CO_2 when assayed at their upper thermal tolerance limit. Particulate inorganic (PIC) and organic (POC) carbon production was restored to values under present-day ocean conditions, owing to adaptive evolution, and were 101% and 55% higher under warming, respectively, than in non-adapted controls. Cells also evolved to a smaller size while they recovered their initial PIC:POC-ratio even under elevated CO_2. The observed changes in coccolithophore growth, calcite and biomass production, cell size and elemental composition demonstrate the importance of evolutionary processes for phytoplankton performance in a future ocean.

Introduction

Marine phytoplankton, a diverse group of photoautotrophic microbes thriving in the world's oceans, generate about half of the global primary production[1,2]. They form the basis of marine food webs and play a major role in the Earth's biogeochemical cycles[2]. Consequently, observed and projected changes in marine primary productivity associated with surface ocean warming[3] are of deep concern for the functioning of the Earth system. Warming may directly impact phytoplankton physiology and productivity[4] and increase growth rates in temperate regions[5]. At the community level, higher temperatures will increase heterotrophy by shifting the balance between photosynthetic and respiratory processes[1,6]. Indirect effects, such as enhanced thermal stratification, will constrain nutrient availability and ultimately limit primary productivity in the sunlit ocean surface layer[7,8]. Ocean warming is progressing along with another major perturbation of today's oceans, the dissolution of excess atmospheric CO_2 in surface waters[9]. This phenomenon, termed ocean acidification[10], improves carbon availability to primary producers, while at the same time it decreases growth and biomineralization in calcifying species[11], including our study species *Emiliania huxleyi*[12,13]. Complex interactions between CO_2 concentration and temperature have recently been reported in physiological experiments with different phytoplankton species[14-16]. For example under high temperatures, the optimal CO_2 level for calcification increases substantially in *E. huxleyi* well beyond ambient CO_2 levels found today[14]. How these interactions play out over longer time scales is largely unknown[17,18].

Recently, the possibility for evolutionary rescue of populations or species to global climate change has become a major area of research[18-22]. The most direct approach to address possible adaptive responses are evolution experiments[23]. Although planktonic microbes lend themselves to such approaches, owing to their short generation time and large population size[18,22,24], there are very few long-term evolution experiments (hundreds of generations) in marine phytoplankton testing responses to global change (but see refs 13, 25), and none tested for thermal adaptation.

Here, we present data from a long-term (≈ 460 asexual generations) evolution experiment that tested for temperature adaptation in factorial combination with increased CO_2 concentration (ocean acidification) in the globally important coccolithophore *E. huxleyi*. This species is a calcifying haptophyte (Prymnesiophyceae) that forms calcium carbonate platelets (coccoliths) and plays an important role in the global carbon cycle[26,27]. *E. huxleyi* is considered to be the single most important calcifying algae in the world's ocean, with blooms that can be seen from outer space[26].

Experimental asexual populations were founded in May 2009 from a single cell isolated from a natural phytoplankton assemblage in the coastal waters off Bergen (Norway). Temperature selection

was initiated in February 2013 by duplicating each of 5 replicate populations that had been maintained in semi-continuous batch cultures at 15.0°C and three defined pCO_2 levels (400 µatm=ambient, 1100 µatm=medium, 2200 µatm=high pCO_2) for about 3 yrs (Supplementary Fig. S1). While the medium level intended to simulate an end-of-the century worst-case scenario, the high concentration corresponds to the highest future level of ocean acidification[9]. To initiate high-temperature selection, populations were subjected to 1°C d^{-1} increments to a final temperature of 26.3°C, a temperature at the upper tolerance limit at which *E. huxleyi* divides at a similar rate as in 15.0°C. Pilot experiments at 26.3°C, 26.5°C and 27.0°C revealed that growth rates decline rapidly >26.3°C (Supplementary Table S1), while the chosen experimental temperature is well within the environmental range for this widely distributed species[28]. Our experiment thus addressed the potential for adaptation to a stressful high temperature at the upper tolerance limit under ambient, medium and high CO_2 conditions. Replicated populations were kept for one yr under temperature selection along with controls that remained at 15.0°C. In order to disentangle the effects of sequential and simultaneous adaptation to two stressors, we also founded new selection lines based on populations that had been kept at 15.0°C and ambient CO_2 and exposed them to high-temperature and CO_2-selection simultaneously, creating a fourth treatment (hereafter 2200_{simult}, Supplementary Fig. S1).

Mean exponential growth rates µ in treatments subjected to high temperature increased rapidly under all high temperature-CO_2 treatment combinations with rates between 0.025 and 0.037 batch $cycle^{-1}$ (Fig. 1a, all slopes highly significant in an autoregressive time series analysis, P<0.0001, Supplementary Table S2). In contrast, 15.0°C treatments maintained their growth rates during 1 yr (Fig. 1b, none of the slopes significant). Pair-wise tests revealed that warming resulted in similar rates of fitness increase in all three CO_2 levels (Supplementary Table S3). The populations that evolved for 1 yr in response to high-temperature and high CO_2-concentration simultaneously (2200_{simult}) had a significantly higher rate of fitness increase than the treatment subjected to high temperature only, and to the populations under medium levels of ocean acidification followed by additional temperature selection (Supplementary Table S3).

At the end of the 1-yr temperature selection phase, we conducted a reciprocal assay experiment[29] in which temperature-adapted asexual populations were compared to the respective non-adapted control populations under high temperature, and *vice versa* (Supplementary Fig. S1). Note that we define "population" here as entirely asexually reproducing individuals that can only generate diversity and adaptive divergence by mitotically derived mutations, since sexual recombination in *E. huxleyi* cannot be induced in the laboratory. We tested for temperature adaptation only within all three CO_2 selection environments that were maintained during the assay experiment, because the

inclusion of ocean acidification as additional factorial treatment levels in the assay phase of the experiment would have resulted in a prohibitively large number of replicates. In the assay experiment, a duplicate of each of the 5 replicate populations of each selection treatment was slowly ($1°C*d^{-1}$) transferred to the higher or lower temperature, respectively, in addition to control assays that remained in their long-term temperature (Fig. S1). In order to verify that the observed phenotypic changes were stable over time we conducted four subsequent batch cycles in addition to an initial (1^{st}) acclimation cycle. No significant effect of batch cycle on growth rates was found (4-way ANOVA, interaction #batch cycle*evol_temp*assay_temp*CO_2 ns), hence all data presented were taken in assay experiment cycle #5 after 30-35 asexual divisions under the respective assay condition.

High-temperature-adapted populations grew significantly better at high temperature than non-temperature adapted populations in all CO_2 conditions (Fig. 2a; Analysis of Variance (ANOVA), log-transformed growth rates, interaction evol_temp*assay_temp: $F_{1,48}$ =56.99, P<0.0001, any interaction with CO_2-level ns). There was also a pronounced correlated response, a growth decline of high-temperature-adapted populations compared to those selected in 15.0°C when assayed under 15.0°C. This suggests that the entire thermal reaction norm shifted to higher temperatures, although confirming this would require further study with more intermediate temperature levels.

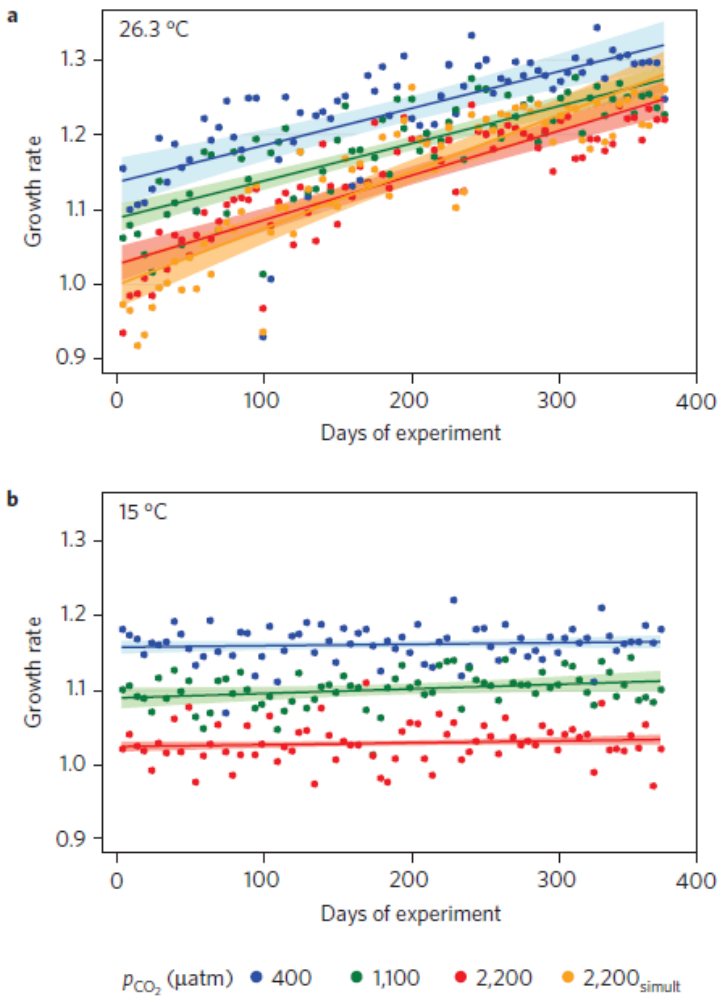

Figure 1. Time course of exponential growth rates in *Emiliania huxleyi* over 1 yr subjected to different combinations of temperature and CO_2 concentration. Two temperatures, 26.3°C (a) and 15.0°C (b), were used in combination with three levels of CO_2 concentration simulating ocean acidification. Growth rates were calculated every 5-days. Fitted lines are based on an Autoregressive Moving Average Model that incorporates significant autocorrelation terms. All lines at 26.3°C reveal highly significant slopes (Supplementary Table S2), whereas none of the slopes is significant at 15.0°C. Pair-wise tests of slopes are given in Supplementary Table S3.

Effects of temperature selection on traits other than growth rate

Next, we analyzed changes in cell size as an important "master trait" affecting nutrient acquisition, sinking velocity and the trophic role of plankton[30,31]. In addition to established physiological decreases of cell size within species[31] we observed significant reductions in cell diameter associated with temperature adaptation across CO_2 treatments, regardless of whether or not the coccosphere was included into the volume calculation (Fig. 2b and Supplementary Fig. S2a). The size reduction owing to adaptation to warming added to the immediate, physiological decline in cell size upon high temperature exposure in all but the highest CO_2 condition (ANOVA; response cell diameter; assay_temp: $F_{1,48}$=320.5, P<0.0001, evol_temp $F_{1,48}$=27.52, P<0.0001). Size reduction after selection

to warming was stronger for cell sizes excluding the coccosphere, with cells having 15% less volume when assayed under high temperature compared to non-adapted controls (Supplementary Fig. S2a, decalcified cell diameter, evol_temp $F_{1,48}$ =39.76, P<0.0001). Cell size and growth rates were strongly inversely correlated, but this relationship was entirely driven by the experimental treatments (analysis of covariance, covariate cell diameter, categorical factors evol_temp, assay_temp, CO_2_level, and their interactions, ns).

As shown previously, the immediate physiological response to elevated CO_2 is an increase of particulate organic carbon (POC) content (ref 32, Fig. 2c). We can confirm this at medium and high CO_2 levels for populations assayed at 15.0°C. However, high temperature-adapted treatments at all CO_2 levels revealed a pronounced decline in POC cell quotas under high temperatures in the assay when compared to the non-temperature adapted replicates, in particular under medium and high CO_2 conditions (ANOVA; POC cell^{-1}; interaction evol_temp*assay_temp $F_{1,47}$ =28.11, P<0.0001). This response was even stronger when standardizing POC cell quotas to the decreasing decalcified cell volume in warm-adapted populations (Supplementary Fig. S2b). Here, POC cell^{-1} under temperature adaptation was up to 30% lower under medium and high CO_2 when assayed under high temperature (ANOVA, evol_temp*assay_temp*CO_2_level: $F_{2,47}$ =8.37, P=0.0008) as compared to non-temperature adapted populations.

For the particulate inorganic carbon (PIC) content of the cells only immediate temperature- and CO_2 driven declines but no significant evolution-related effects were observed (Fig. 2d; ANOVA, log-transformed PIC * cell^{-1}; assay_temp: $F_{1,47}$=48.59, P<0.0001). For the specific weight and hence, the ballasting effect of single coccolithophore cells, the PIC:POC ratio is an important parameter[33]. Under ambient CO_2 the evolutionary response of PIC:POC to temperature was a decrease, while under long-term medium/high CO_2 the ratio increased relative to non-adapted treatments (Fig. 2e, ANOVA, cubic-root transformed PIC:POC; Evol_temp*CO_2_level: $F_{2,48}$=5.74, P=0.0058) when assayed under warm conditions. Thus, counter-correlated trait changes of cell size, POC and PIC cell quotas cancelled out, with the net results that the PIC:POC ratio was up to 30% higher after temperature adaptation under high CO_2 compared to the temperature response without evolutionary adaptation. Importantly, there was a complete restoration of the initial PIC:POC ratio at 15.0°C (leftmost vs. Rightmost bar in Fig. 2e, ANOVA planned contrast P>0.5, ns).

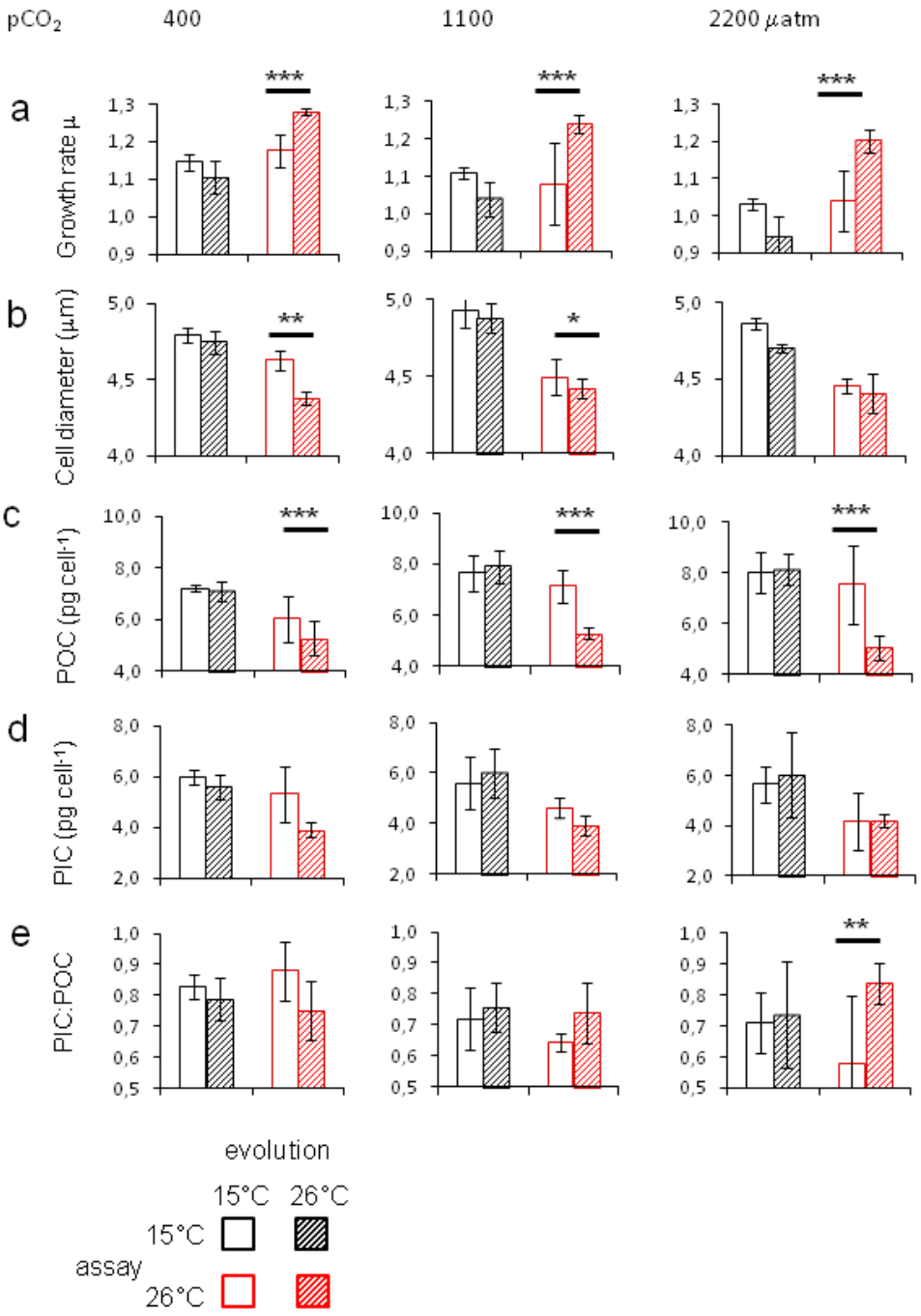

Figure 2. Evolutionary adaptation in *Emiliania huxleyi* after one yr of temperature selection (26.3 vs. 15.0°C) in combination with three CO_2 levels simulating ocean acidification. Mean trait values (±1SD, n=5) of temperature adapted vs. non-adapted (hatched vs. open bars, respectively) populations of *E. huxleyi* are depicted in three different CO_2 environments, when assayed at cold and warm temperatures (black vs. red bars, respectively). (a) exponential growth rate (b) cell diameter (c) particulate organic carbon per cell (POC cell^{-1}) (d) particulate inorganic carbon per cell (PIC cell^{-1}) (e) ratio of PIC:POC. Significant results of planned contrasts after ANOVA are depicted only for temperature adaptation by horizontal lines and asterisks (*0.05≥*P*>0.01, **0.01≥*P*>0.001, ***P<0.001). Complete ANOVA results are given in Supplementary Table S5.

Organic nitrogen content of phytoplankton relative to organic carbon is an important predictor of nutritional quality to higher trophic levels[34]. We find that in temperature-adapted populations, the C:N ratio remained more stable when tested under warming, particularly under the highest CO_2 treatment (Supplementary Fig. S2c, log-transformed C:N ratio, evol_temp*assay_temp*CO_2: $F_{2,47}$ =3.85, P=0.028). This suggests the maintenance of one aspect of nutritional quality under the combination high-temperature/high CO_2 owing to adaptive evolution.

Although cells evolved to a smaller size and contained less carbon, overall production rates in our 5-d experiment were substantially higher in the warm adapted populations compared to controls. Our experiment simulated a coccolithophore bloom by keeping the cells continually in the exponential growth phase, inoculating new batch cycles every five days with exactly 10^5 cells. POC and PIC production (µgC * $5d^{-1}$ * $cell^{-1}$) were 52 and 101% higher, respectively (Fig. 3a,b, ANOVA; interaction evol_temp*assay_temp for PIC and POC both P<0.0001). Five day biomass production under the most stressful future ocean scenario (26.3°C and 2200 µatm pCO_2) was approximately as high as under original conditions prior to initiation of the experiment mirroring the restoration of PIC:POC ratios.

Taken together, both the restored PIC:POC ratio that determines the specific weight of a single cell of *Emiliania huxleyi*, along with the increased biomass production of PIC may enhance the ballasting of coccolith-containing particles for transport to the deep ocean[27,33] as a result of adaptive evolution to warming.

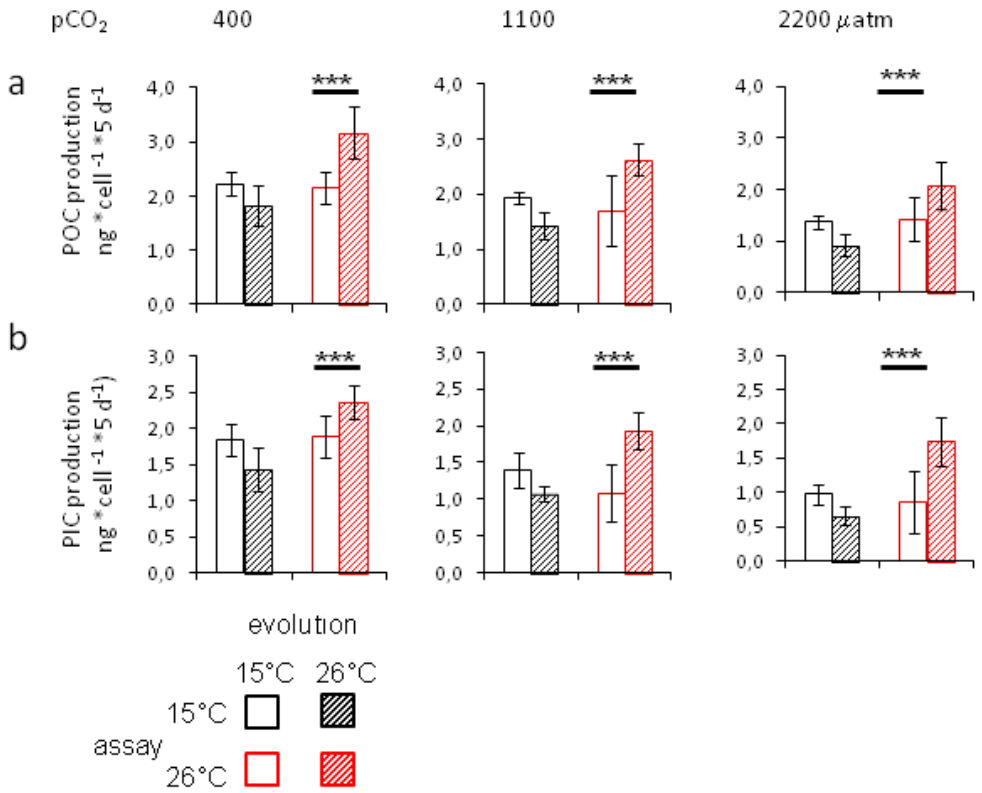

Figure 3. Restoration of biomass production in *E. huxleyi* after 1 yr of temperature selection. Given are mean values (±1SD, n=5) of production per cell over 5 days as a function of assay temperature and CO_2 environment in a fully factorial design. (a) particulate organic carbon (b) particulate inorganic carbon. Further details as in Fig. 2. Complete ANOVA results are given in Supplementary Table S5.

Sequential versus simultaneous adaptation to warming and acidification

Adaptation to warming along with ocean acidification occurred independently of whether or not CO_2 adaptation had happened simultaneously or consecutively to temperature selection. Replicate populations that were subjected to high temperature and CO_2 selection simultaneously were compared to treatments with sequential adaptation (204 batch cycles ≈1500 generations CO_2-selection, then for an additional ≈460 generations of temperature selection, Supplementary Fig. S1). For most response variables, notably growth rate, we found only small and non-significant differences among the sequential and simultaneous treatments (Supplementary Table S4). An exception were PIC cell quotas that were significantly smaller in the simultaneous vs. sequential treatment, which also translated to a lower PIC:POC ratio. Possibly, the duration of high-CO_2 selection for the recovery of this trait was too short to yield the same adaptive outcome.

Adaptation to ocean warming and acidification

A largely open question is how novel selection regimes interact among one another and affect

adaptive dynamics[35]. Experimentally, the scope for adaptation to multiple stressors has previously only been addressed in model microbes, for example adapting to several antibiotics[36], while this remains an open question for global change associated selection. Hence, we compared adaptation to ocean acidification alone from a previous study[13], which was not directly tested in the current experiment, with adaptation to either warming or warming and acidification (this study, details see full material and methods). The magnitude of adaptation in growth rate to warming (under ambient CO_2) was about 3-times larger than adaptation to CO_2 (always at 15.0°C). The adaptation response is composed of the relative fitness difference of the adapted (dark shaded bars) vs. non-adapted populations (light shaded bars) when assayed under the relevant future ocean condition (Fig. 4a). The quotient of the exponential growth rates[37] denotes the relative fitness of the adapted populations (a) over the control populations (c) as $W_{ac} = \mu_a/\mu_c$ (Fig. 4b). We find significantly higher relative fitness in *E. huxleyi* in response to the combined stressors warming and acidification (sequential adaptation) than to acidification alone, while *W* associated with temperature adaptation alone was intermediate (Fig. 4b). Finally, we tested the adaptation response to the simultaneous challenge of warming and acidification using an additional assay treatment in which replicate populations selected under ambient CO_2/low temperature had been immediately exposed to both stressors. Here, *W* of adapted vs. non-adapted populations was largest, and higher compared to both single factor adaptations (1-way ANOVA on randomly combined fitness quotients among adapted/non-adapted populations, subsequent Tukey HSD post hoc test, Fig. 4b).

We thus observed no interference in the adaptation of a phytoplankton species to two major global change related stressors. This finding is of high ecological relevance since adaptation to different stressors may also be antagonistic if there are negative trait correlations[38], here with respect to stress tolerance towards heat and CO_2. The faster fitness gains under simultaneous selection may be due to pervasive sign epistasis of (beneficial) mutations in microbial genomes[39]. Mutations that were fixed under only CO_2 selection may not confer any fitness advantages under selection for warming and 'lock' the evolutionary trajectory at a lower fitness. In contrast, under simultaneous selection, only mutations with a beneficial effect in both environments will rise to fixation.

Previously, a 500-generation evolution experiment revealed adaptation to increased CO_2 concentrations via novel mutations and via genotypic sorting. Both growth and calcification rates were partially restored[13]. Here, we observed even faster adaptation to temperature in *E. huxleyi* that was largely independent of the CO_2 environment. As in many other phytoplankton species, sexual reproduction cannot be induced in *E. huxleyi*, hence our approach must ignore recombination that will generate additional within-population diversity available to selection. In the light of ample within-population diversity in thermal reaction norms among phytoplankton species [5,28,40,41], our

results are conservative because all genetic diversity upon which selection acted must have come from *de novo* mutations. In addition to 1 yr of temperature selection during the thermal adaptation phase, neutral mutations relevant for temperature adaptation could have arisen in all 15 independent replicates (5 at each of three pCO_2 values of 400/1100/2200 µatm) and maintained at a low frequency during the previous cultivation over ≈1500 generations (3 yr) at 15.0°C (refs 42,43), partly explaining the high rate of adaptive evolution. An indirect challenge experiment revealed that the genetic basis for adaptation to ocean acidification must be different among replicate high-CO_2 selected populations[42]. As with the earlier study on adaptation to ocean acidification[42], the phenotypic convergence observed here for thermal adaptation among the replicates was remarkable although the mutational basis is likely to be different owing to the independent cultivation of replicates for a total of ≈2000 asexual generations.

Ocean change and phytoplankton adaptation

Contrary to expectations, there were no apparent antagonistic effects when selecting for ocean acidification and high temperature in the world's most abundant calcifying organism, *Emiliania huxleyi*. Rather, asexual population growth rates fully recovered even under the most stressful future ocean scenario of 26.3°C and 2200 µatm pCO_2 compared to treatments under ambient CO_2 concentration and a benign temperature of 15.0°C. We can only speculate why adaptation to a high temperature at the upper thermal tolerance was up to 6-times faster than to elevated CO_2 concentration. Since most enzymes are temperature-dependent[4] there is possibly a larger mutational target for thermal than for CO_2 adaptation, accelerating the speed of adaptation, provided that the frequency of beneficial mutations is rate limiting. In the same vein, mutations for thermal adaptation may have, on average, a larger beneficial effect, in particular when the sample of mutational effects is larger[44].

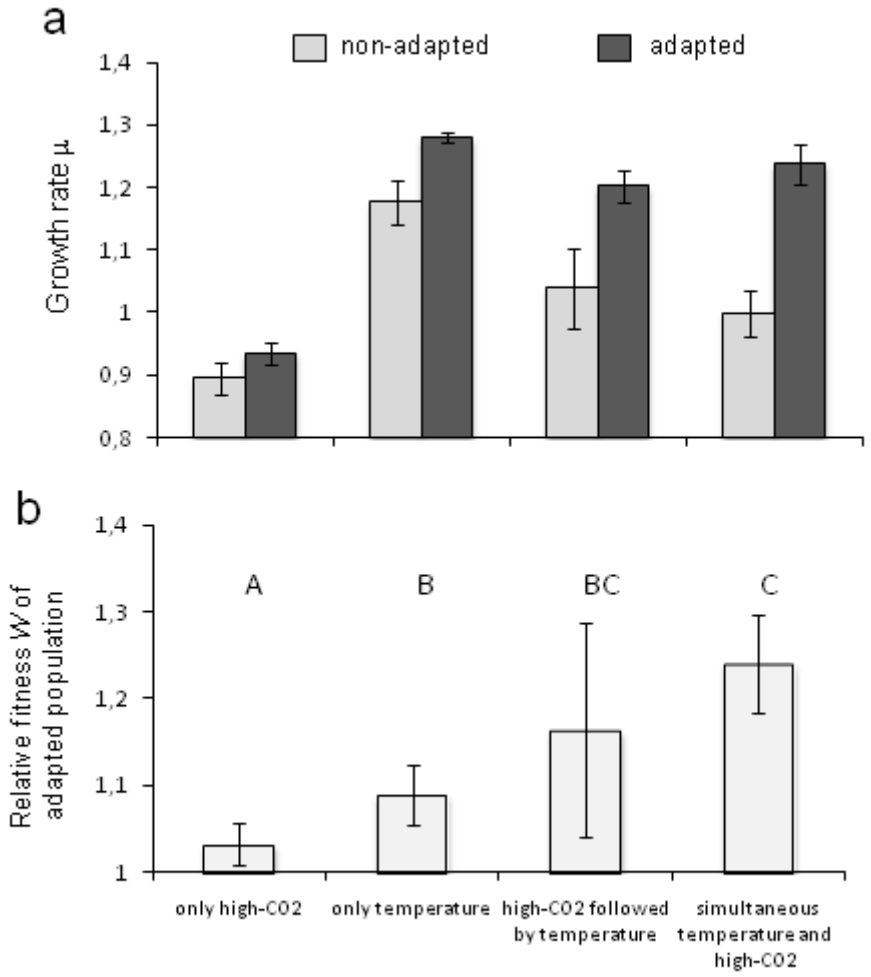

Figure 4. Evolutionary adaptation in *Emiliania huxleyi* to ocean acidification and to temperature alone, and to a combination of both factors. Effect sizes of adaptation to ocean acidification alone (leftmost bar) are from ref. 13. Panel (a) depicts mean growth rates of adapted asexual populations ±1 SD (dark bars) relative to their respective control treatments (light bars). Panel (b) shows the resulting relative fitness increase (±1 SD) of the adapted treatment as quotient of exponential growth rates of the adapted (dark bars) vs. non-adapted (light bars) replicates. Mean values that are significantly different from one another are indicated by different capital letters on top of the relevant column (Tukey HSD post-hoc test (α=0.05) after one-way ANOVA).

We show here that thermal niches in phytoplankton may be more evolutionary flexible than previously thought[1], despite our experiment being ultimately based on asexual offspring of a single wild isolate. Clearly, it would have been desirable to use more than one genotype to address any effects of the specific genetic background of an experimental strain. Many evolution experiments

with classical model organisms identified evolutionary patterns based on a single isolate that were later found to be representative for entire species[23]. Hence, we consider it unlikely that the adaptive capacity via novel mutations of our chosen genotype is not representative for other *E. huxleyi* genotypes. The apparently uninterrupted growth rate increase during a 1-yr time interval observed here leaves it open when adaptation to temperature will reach a fitness plateau, as known from many other evolution experiments[37]. Our results complement several recent large-scale physiological experiments, field surveys and models[1,4,5,45] that have highlighted temperature as the prime factor determining plankton distribution patterns, ocean primary productivity and phytoplankton metabolic function. Controlled laboratory evolution experiments lack the realism of mesocosm experiments employing natural populations or field studies[29], but are currently the only direct test for adaptive change in most marine microbial species where the resurrection of ancient propagules from layered sediments is impossible[46]. That even the asexual offspring of a single isolate will considerably change their performance at high temperature within months to years owing to evolution illustrates that evolutionary processes need to be considered when predicting the effects of a warming ocean on phytoplankton[47].

Material and Methods summary

Experimental populations of *Emiliania huxleyi* were founded in 2009 from a single cell isolated from a natural phytoplankton assemblage in the coastal waters off Bergen (Norway) at an average surface water temperature of 10.0°C. Since then, five independent replicates of each CO_2 treatment were propagated under constant rotation (0.5 min^{-1}) at three levels of CO_2 partial pressure (pCO_2) at 15.0°C and at a photon flux density of 150±15 µmol m^{-2} s^{-1} under a 16:8 light:dark cycle for 1,500 asexual generations. The desired pCO_2 levels (400, 1100 and 2200 µatm) were achieved by aerating the artificial seawater medium for 24 h at the target temperature under saturated humidity. Exactly 10^5 cells were transferred every five days, corresponding to 6-7 asexual generations per batch cycle. The maximal final cell densities did not exceed 2 x 10^5 ml^{-1} such that the DIC drawdown was always <8%. Duplicates of the CO_2 selection lines were created on 8 February 2013 and slowly (1°C*d^{-1}) raised from 15.0°C to 26.3°C, a temperature at the upper thermal tolerance. An additional selection treatment was subjected to high CO_2 and warming simultaneously, originating of duplicates from the original ambient CO_2 /15.0°C selection lines. Temperature selection lasted 1 yr (437-478 asexual generations). In a subsequent assay experiment, replicates were either exposed to their original temperature, or non-temperature adapted populations were exposed to high temperature, and *vice versa*. Prior to all measurements one acclimation batch cycle in addition to 4 more batch cycles were performed to ensure that only persistent phenotypic effects were present in the assay experiment. The cell density and diameter were measured in triplicate at every transfer point using a Beckman

Coulter Z2 Particle and Size Analyzer. The cell counts were always performed three hours after the onset of the light phase. Growth rates were calculated as $\mu = (\ln N_d - \ln N_0)/d$, where $N_{(0,d)}$ are cell concentrations, and d the duration of the batch cycle in days. Changes in growth rates over time were analyzed with an Autoregressive Moving Average Model with exogenous variables to test for significant trends. We measured cell density, cell diameter, total particulate carbon (TPC), particulate organic carbon (POC) and particulate organic nitrogen (PON). In addition to measurements of calcified cell size, we decalcified cells with 10mM EDTA adjusted to pH 8.2 and repeated the size measurements within five minutes of EDTA addition. Culture suspension for the quantification of TPC and POC were vacuum filtered (<100 mbar) onto pre-combusted glass fibre filters. To prevent artefacts due to intrinsic dial cycling, all filtrations were performed at the same time of the day, four hours after lights on. All sampling was completed within approximately one hour. Response variables in the assay experiment were subjected to factorial analysis of variance (ANOVA) followed by planned contrasts if appropriate.

Correspondence and requests for material should be addressed to T.B.H.R.

(treusch@geomar.de).

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L.M., T.B.H.R., U.R., K.T.L. planned the experiment, L. M. conducted the experiment, L.M. collected data, J.P.G. contributed statistical analyses, L.M., K.T.L., M. A. G., U.R. and T.B.H.R. analyzed and interpreted the results, and T.B.H.R. wrote the paper.

Supplementary online material accompanies this manuscript

References

1 Thomas, M. K., Kremer, C. T., Klausmeier, C. A. & Litchman, E. A Global Pattern of Thermal Adaptation in Marine Phytoplankton. *Science* **338**, 1085-1088, doi:10.1126/science.1224836 (2012).

2 Falkowski, P. G., Fenchel, T. & Delong, E. F. The Microbial Engines That Drive Earth's Biogeochemical Cycles. *Science* **320**, 1034-1039, doi:10.1126/science.1153213 (2008).

3 Boyce, D. G., Lewis, M. R. & Worm, B. Global phytoplankton decline over the past century. *Nature* **466**, 591-596 (2010).

4 Toseland, A. *et al.* The impact of temperature on marine phytoplankton resource allocation and metabolism. *Nature Clim. Change* **3**, 979-984, doi:10.1038/nclimate1989

http://www.nature.com/nclimate/journal/v3/n11/abs/nclimate1989.html - supplementary-information (2013).

5 Boyd, P. W. *et al.* Marine Phytoplankton Temperature versus Growth Responses from Polar to Tropical Waters – Outcome of a Scientific Community-Wide Study. *PLOS One* **8**, e63091, doi:10.1371/journal.pone.0063091 (2013).

6 Yvon-Durocher, G. *et al.* Reconciling the temperature dependence of respiration across timescales and ecosystem types. *Nature* **487**, 472-476, doi:http://www.nature.com/nature/journal/v487/n7408/abs/nature11205.html - supplementary-information (2012).

7 Boyd, P. W. Beyond ocean acidification. *Nature Geoscience* **4**, 273-274 (2011).

8 Hofmann, M. & Schellnhuber, H.-J. Oceanic acidification affects marine carbon pump and triggers extended marine oxygen holes. *Proc Natl Acad Sci USA* **106**, 3017-3022, doi:10.1073/pnas.0813384106 (2009).

9 Caldeira, K. & Wickett, M. E. Oceanography: Anthropogenic carbon and ocean pH. *Nature* **425**, 365-365 (2003).

10 Feely, R. A. *et al.* Impact of Anthropogenic CO_2 on the $CaCO_3$ System in the Oceans. *Science* **305**, 362-366, doi:10.1126/science.1097329 (2004).

11 Kroeker, K. J. *et al.* Impacts of ocean acidification on marine organisms: quantifying sensitivities and interaction with warming. *Global Change Biology* **19**, 1884-1896, doi:10.1111/gcb.12179 (2013).

12 Riebesell, U. *et al.* Reduced calcification of marine plankton in response to increased atmospheric CO_2. *Nature* **407**, 364-367 (2000).

13 Lohbeck, K. T., Riebesell, U. & Reusch, T. B. H. Adaptive evolution of a key phytoplankton species to ocean acidification. *Nature Geoscience* **5**, 346-351, doi:http://dx.doi.org/10.1038/NGEO1441 (2012).

14 Sett, S. *et al.* Temperature Modulates Coccolithophorid Sensitivity of Growth, Photosynthesis and Calcification to Increasing Seawater pCO_2. *PLOS One* **9**, e88308, doi:10.1371/journal.pone.0088308 (2014).

15 Feng, Y. *et al.* Interactive effects of increased pCO_2, temperature and irradiance on the marine coccolithophore Emiliania huxleyi (Prymnesiophyceae). *European Journal of Phycology* **43**, 87-98, doi:10.1080/09670260701664674 (2008).

16 Kremp, A. *et al.* Intraspecific variability in the response of bloom-forming marine microalgae to changed climate conditions. *Ecology and Evolution* **2**, 1195-1207, doi:10.1002/ece3.245 (2012).

17 Riebesell, U., Körtzinger, A. & Oschlies, A. Sensitivities of marine carbon fluxes to ocean change. *Proceedings of the National Academy of Sciences* **106**, 20602-20609, doi:10.1073/pnas.0813291106 (2009).

18 Reusch, T. B. H. & Boyd, P. W. Experimental evolution meets marine phytoplankton. *Evolution* **67**, 1849–1859 (2013).

19 Bell, G. & Gonzalez, A. Adaptation and Evolutionary Rescue in Metapopulations Experiencing Environmental Deterioration. *Science* **332**, 1327-1330, doi:10.1126/science.1203105 (2011).

20 Schoener, T. W. The Newest Synthesis: Understanding the Interplay of Evolutionary and Ecological Dynamics. *Science* **331**, 426-429, doi:10.1126/science.1193954 (2011).

21 Hoffmann, A. A. & Sgro, C. M. Climate change and evolutionary adaptation. *Nature* **470**, 479-485 (2011).

22 Kovach-Orr, C. & Fussmann, G. F. Evolutionary and plastic rescue in multitrophic model communities. *Phil Transact R Soc Ser B* **368**, 20120084 (2013).

23 Elena, S. F. & Lenski, R. E. Evolution experiments with microorganisms: the dynamics and genetic bases of adaptation. *Nature Reviews in Genetics* **4**, 457-469 (2003).

24 Collins, S. L. & Bell, G. Phenotypic consequences of 1,000 generations of selection at elevated CO_2 in a green alga. *Nature* **431**, 566-569 (2004).

25 Jin, P., Gao, K. & Beardall, J. Evolutionary responses of a coccolithophorid *Gephyrocapsa* oceanica to ocean acidification *Evolution* **67**, 1869–1878 (2013).

26 Paasche, E. A review of the coccolithophorid *Emiliania huxleyi* (Prymnesiophyceae), with particular reference to growth, coccolith formation, and calcification-photosynthesis interactions. *Phycologia* **40**, 503-529 (2002).

27 Armstrong, R. A., Lee, C., Hedges, J. I., Honjo, S. & Wakeham, S. G. A new, mechanistic model for organic carbon fluxes in the ocean based on the quantitative association of POC with ballast minerals. *Deep Sea Res Part II* **49**, 219-236 (2001).

28 Brand, L. E. Genetic variability and spatial patterns of genetic differentiation in the reproductive rates of the marine coccolithophores *Emiliania huxleyi* and *Gephyrocapsa oceanica*. *Limnol Oceanogr* **27**, 236-245 (1982).

29 Collins, S. Many Possible Worlds: Expanding the Ecological Scenarios in Experimental Evolution. *Evol Biol* **38**, 3-14, doi:10.1007/s11692-010-9106-3 (2011).

30 Daufresne, M., Lengfellner, K. & Sommer, U. Global warming benefits the small in aquatic ecosystems. *Proceedings of the National Academy of Sciences* **106**, 12788-12793, doi:10.1073/pnas.0902080106 (2009).

31 Atkinson, D., Ciotti, B. J. & Montagnes, D. J. S. Protists decrease in size linearly with temperature: ca. 2.5% °C^{-1}. *Proceedings of the Royal Society London Ser B: Biological Sciences* **270**, 2605-2611, doi:10.1098/rspb.2003.2538 (2003).

32 Rokitta, S. D., John, U. & Rost, B. Ocean Acidification Affects Redox-Balance and Ion-Homeostasis in the Life-Cycle Stages of *Emiliania huxleyi*. *PLOS One* **7**, e52212, doi:10.1371/journal.pone.0052212 (2012).

33 Bach, L. T. *et al.* An approach for particle sinking velocity measurements in the 3–400 µm size range and considerations on the effect of temperature on sinking rates. *Marine Biology* **159**, 1853-1864, doi:10.1007/s00227-012-1945-2 (2012).

34 Sterner, R. & Elser, J. in *The Princeton Guide to Ecology* (eds SA Levin *et al.*) 376–385 (Princeton Univeristy Press, 2009).

35 Rizhsky, L. *et al.* When defense pathways collide: the response of *Arabidopsis* to a combination of drought and heat stress. *Plant Physiol.* **134**, 1683-1696, doi:10.1104/pp.103.033431 (2004).

36 Pena-Miller, R. *et al.* When the Most Potent Combination of Antibiotics Selects for the Greatest Bacterial Load: The Smile-Frown Transition. *PLoS Biol* **11**, e1001540, doi:10.1371/journal.pbio.1001540 (2013).

37 Lenski, R., Rose, M., Simpson, S. & Tadler, S. Long-Term Experimental Evolution in Escherichia coli. I. Adaptation and Divergence During 2,000 Generations. *The American Naturalist* **138**, 1315-1341 (1991).

38 Etterson, J. R. & Shaw, R. G. Constraint to adaptive evolution in response to global warming. *Science* **294**, 151-154 (2001).

39 Weinreich, D. M., Watson, R. A. & Chao, L. Perspective: sign epistasis and genetic constraint on evolutionary trajectories. *Evolution* **59**, 1165–1174 (2005).

40 Wood, A. M. & Leatham, T. The species concept in phytoplankton ecology. *J Phycol* **28**, 723-729 (1992).

41 Zhang, Y. *et al.* Between- and within-population variations in thermal reaction norms of the coccolithophore *Emiliania huxleyi*. *Limnol Oceanogr* **59**, 1570–1580 (2014).

42 Lohbeck, K. T., Riebesell, U., Collins, S. & Reusch, T. B. H. Functional genetic divergence in high CO_2 adapted *Emiliania huxleyi* populations. *Evolution* **67**, 1892–1900, doi:DOI: 10.1111/j.1558-5646.2012.01812.x (2013).

43 Lang, G. I. *et al.* Pervasive genetic hitchhiking and clonal interference in forty evolving yeast populations. *Nature* **500**, 571-574, doi:10.1038/nature12344

(2013).

44 Orr, H. A. The genetic theory of adaptation: a brief history. *Nature Reviews in Genetics* **6**, 119-127 (2005).

45 Moran, X. A. G., Lopez-Urrutia, Á., Calvo-DÍAz, A. & Li, W. K. W. Increasing importance of small phytoplankton in a warmer ocean. *Global Change Biology* **16**, 1137-1144, doi:10.1111/j.1365-2486.2009.01960.x (2010).

46 Härnström, K., Ellegaard, M., Andersen, T. J. & Godhe, A. Hundred years of genetic structure in a sediment revived diatom population. *Proc Natl Acad Sci USA* **108**, 4252-4257, doi:10.1073/pnas.1013528108 (2011).

47 Collins, S., Rost, B. & Rynearson, T. A. Evolutionary potential of marine phytoplankton under ocean acidification. *Evolutionary Applications* **7**, 140-155, doi:10.1111/eva.12120 (2014).

Publication III

Author contribution

This chapter is in preparation for publication in a scientific journal under multiple authorship. My contribution to this work is described below.

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Authors:
Lothar Schlüter, Kai T. Lohbeck, Magdalena A. Gutowska, Joachim P. Gröger, Ulf Riebesell, and Thorsten B. H. Reusch

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Author contributions:
LS, TBHR, UR and KTL designed the experiment, LS performed the experiment. All authors analyzed and interpreted the data. LS wrote the manuscript.

Interactions of adaptation to warming and acidification in the coccolithophore *Emiliania huxleyi*

Lothar Schlüter[1], Kai T. Lohbeck[1], Magdalena A. Gutowska[2,3], Ulf Riebesell[2] and Thorsten B.H. Reusch[1]*

1) Evolutionary Ecology of Marine Fishes, GEOMAR Helmholtz-Centre for Ocean Research Kiel, Düsternbrooker Weg 20, 24105 Kiel, Germany

2) Biological Oceanography, GEOMAR Helmholtz-Centre for Ocean Research Kiel Düsternbrooker Weg 20, 24105 Kiel, Germany

3) Present address:

Molecular Microbial Ecology, Monterey Bay Aquarium Research Institute (MBARI), 7700 Sandholdt Road, 95039 Moss Landing, USA

* corresponding author, e-mail treusch@geomar.de

Abstract

Anthropogenic emission of CO_2 causes ocean acidification and global warming. Marine photoautotrophic microbes, which contribute a major part in global carbon fixation, are affected by acidification and warming of the surface ocean. *Emiliania huxleyi* is a globally important marine phytoplankton species and especially sensitive to ocean acidification. It has been shown in long-term selection experiments that *E. huxleyi* can adapt to both ocean acidification and warming. In this study we focus on the effects of sequential adaptation and how previous adaptation to elevated CO_2 is affected by increased temperature as an additional selection pressure. The present study is complementing the work of Schlüter et al. (2014) that quantified temperature adaptation and how this was affected by previous CO_2-adaptation. Replicate asexual populations of E. huxleyi were selected under CO_2 enriched conditions for 1600 generations and then for additional 480 generations under elevated temperature and CO_2. At 1600 generations of CO_2 selection growth rate showed a positive adaptive response to CO_2 at 15°C before the initiation of the experiment. After 480 generations of combined selection, simultaneous temperature exposure caused an increase in the magnitude of adaptive effect to elevated CO_2 compared to populations only long-term subjected to elevated CO_2. As consequence there are no constrains between temperature and CO_2-adaptation. Further we found changes in correlated traits after combined selection. The adaptive reaction in cell size to CO_2 reversed from 15°C to 26°C temperature exposure from a decrease to an increase. The physiological and adaptive response in PIC per cell to acidification (which was present at 15°C) disappeared at high temperature. The changes in POC per cell were CO_2-level dependent, with a loss of adaptive response at medium

and similar reduction to CO_2 at high-level. Adaptive effects on population production were based on adaptation in growth rate and resulted in partly restoration. As a result adaptation to high temperature may reduce the implications of ocean acidification.

Introduction

Experimental evolution is a powerful tool to observe evolutionary processes in 'real time' (Elena & Lenski 2003). Experimental evolution approaches are particularly useful in rapidly reproducing organisms, that are easy cultivable in the lab. These simple requirements are the reason why experimental evolution has mainly been applied to prokaryotic and eukaryotic microbes such as the bacteria *Escherichia coli* and *Pseudomonas* or the yeast *Saccharomyces* (Elena & Lenski 2003, Buckling et al. 2009). Since a few years in marine ecology, experimental evolution approaches have become attractive because they are able to combine evolutionary insight with ecological relevance. Cultivable marine microbes have become model systems in experimental evolution and their adaptive potential is tested in global change scenarios (Reusch & Boyd 2013, Collins et al. 2014). So far most studies are restricted to one selective pressure at a time (but see Schaum et al. 2015, they added fluctuations as second selection pressure and found a stronger response in the fluctuating selection system than in a rather stable one.). A recent study on *Emiliania huxleyi* by Schlüter et al. (2014) revealed that adaptation to a second selection pressure by sequential and simultaneous adaptation is possible.

E. huxleyi is like all calcifying organisms sensitive to ocean acidification. Several strains show reduced growth and calcite production under ocean acidification conditions (Riebesell et al. 2000, Langer et al. 2009). Schlüter et al. (2014) worked with replicate asexual populations that were selected for 1600 generations to elevated CO_2 and added in an additional temperature selection treatment for about 460 generations. However, in their experiment they tested only for temperature adaptation in the assay phase of the study (Collins 2011). A fully crossed design where temperature adaptation and CO_2 adaptation were tested in all combinations was technically not possible due to the large number of cross-exposure replicates. Hence, we address the present separate study how consecutive selection by acidification and temperature play out when adapted vs. non-adapted populations are exposed to different CO_2 regimes, i.e. when formally testing for adaptation to simulated ocean acidification. Accordingly, we conducted a reciprocal experiment, where the selection temperature is the same as the assay temperature, while the adapted cells were challenged with different CO_2 levels (Fig.1).

E. huxleyi is a globally important photoautotrophic marine microbe, fixing carbon via photosynthesis and producing delicate calcite scales that ballast organic material and therefore enhance the transport of organic matter into the deep see (Armstrong et al. 2001). *E. huxleyi* this is of particular interest since ocean acidification is discussed as crucial to calcification in the future (Raven & Crawfurd 2012). Aside from ocean acidification the prognoses for our oceans imply an average temperature increase above 2°C till the end of the century (IPCC 2007). In terms of global change the adaptive potential of such a marine key species is of particular interest. Adaptation via novel

mutations and genotypic selection has been demonstrated on ocean acidification alone, and, based on a single evolving genotype, to temperature, individually and temperature/acidification combined (Lohbeck et al. 2012, Schlüter et al. 2014).

The present study addresses how temperature in full combination with CO_2 selection affects adaptation to ocean acidification in a calcifying marine phytoplankton. We also measure a number of correlated traits that are ecologically important such as cell size and cell quotas for PIC and POC. Further we synthesize our results along with results from Schlüter et al. (2014) to compare magnitudes of adaptation among different treatments and assay conditions, with the ultimate goal to identify synergistic, additive or antagonistic effects of CO_2 and temperature. As we test for adaptive effects to ocean acidification, the selection temperature is the same as the assay temperature.

Material and Methods

This experiment was conducted with the selected populations used by Schlüter et al. (2014). The *E. huxleyi* populations derived from one single clone isolated May 2009 in Bergen, Norway (clone #62, Lohbeck et al. 2012). Thus, any phenotyoic evolution is likely to be due to novel mutations that spread through the population (e.g. Lenski et al. 1991). The CO_2 selection was indicated in May 2010 and lasted approximately 1600 asexual generations prior to temperature selection. For the CO_2 selection three CO_2 levels with five replicates each were launched from one single founder population. Those populations were exposed for 2100 generations of CO_2 adaptation (3 levels: ambient 400 µatm pCO_2, medium 1100 µatm pCO_2 and high 2200 µatm pCO_2) at 15°C and 480 generations of combined CO_2 and temperature selection (2 levels: 15°C and 26.3°C, Fig. 1). The selection levels were chosen in that way, that the cell division rate was about equal at both temperatures. In that why both selection levels would reach about the same number of cell divisions when tested for adaptation. The cultures were unialgal but not axenic, since long term culturing of *E. huxleyi* is not possible without associated bacteria, and a regular use of antibiotics would have been an unintended selection pressure. The cultures were regularly inspected via flow cytometry and microscopy for any contamination, for example for free living bacteria.

The populations were cultured in artificial seawater prepared according to Kester et al. (1967). The seawater was supplemented with 64 µmol kg-1 nitrate and 4 µmol kg-1 phosphate according to (Redfield 1958), trace metals and vitamins consistent to f/8 (Guillard & Ryther 1962) and selenium to a final concentration of 10 nmol Kg^{-1} (Danbara & Shiraiwa 1999). To exclude any limitations of micronutrients 2 ml Kg^{-1} steril filtered North Sea water was added. Total alkalinity (TA) of 2380 µmol kg^{-1} was established by addition of 19.99 g Kg^{-1} bicarbonate. The seawater was sterile filtered

(Whatman Polycap 75AS) after nutrient addition. CO_2 levels were manipulated by aerating the ASW medium at saturated humidity for 24 h at the particular temperature (15°C and 26°C) with air enriched with CO_2 to the desired treatment levels (ambient = 400 µatm pCO_2, medium = 1100 µatm pCO_2 and high = 2200 µatm pCO_2). The carbonate chemistry was regularly controlled via TA and dissolved inorganic carbon (DIC) measurements. The measurements were performed on a Metrohm MATi 01 for Alkalinity and an AIRICA system (Marianda) for DIC. The partial pressure was calculated using the program CO2SYS (Lewis & Wallace 1998). The draw-down in TA and DIC was calculated from the total particular carbon measurements (Lewis & Wallace 1998). Regular DIC and TA measurements at 10 time points revealed that the mean medium CO_2 treatment was 200-300 µatm above the desired treatment level, thus 1400 instead of 1100. Measured CO_2 partial pressures (in µatm±1SD for cold / warm temperature, respectively) were 388±7 and 416±9 (ambient), 1329±177 and 1591±248 (medium) and 2253±30 and 2136±56 (high pCO_2).

Each population was cultured in a 250 ml Schott Duran flasks filled with minimum headspace to avoid gas exchange (total volume about 330 mL). The average light intensity was 150 ± 10 µmol m^{-2} s^{-1} under a 16:8 light dark cycle. The flasks were continuously rotated (0.5 rpm) in two identical Sanyo MLR-351 light cabinets at 15°C and 26.3°C. A small number (<1%) of heterotrophic nanoflagellates were detected in one high CO_2 replicate (at each temperature) by regular microscopic inspections. The statistical results did not change qualitatively, whether the infected cultures were in- or excluded.

Temperature selection was initiated on 8 February 2013 by duplication of each replicate and the slow transfer (1°C day^{-1}) of the duplicates to the high selection temperature (26.3°C), while the control group remained at 15°C. The selection lasted for 58 batch cycles (~1 yr), corresponding to ~500(ambient), ~480 (medium), and ~460 (high CO_2) generations of asexual reproduction. The temperature was chosen for practical reasons. The initial growth rates (before selection) at 26.3°C equaled the growth rates at 15°C. In that way we could avoid drastically different numbers of cell divisions between the selection treatments when testing for adaptation. The high temperature treatment was already above the optimal growth temperature.

At the end of each batch cycle (5 days) exactly 10^5 cells were transferred into fresh medium to start the next cycle. Cell density and cell diameter were determined with a Beckmann Coulter Z2 Particle and Size Analyzer. In addition, we decalcified cells with 10mM EDTA adjusted to pH 8.2 and repeated the size measurements within five minutes of EDTA addition. The cell counts were always performed three hours after the onset of the light phase. Exponential growth rates (µ) were calculated from cell densities according to

$$\mu = (\ln N_d - \ln N_0)/d \quad (1) \qquad (1)$$

where N(0.d) are cell numbers, and d the duration of the batch cycle in days (d=5).

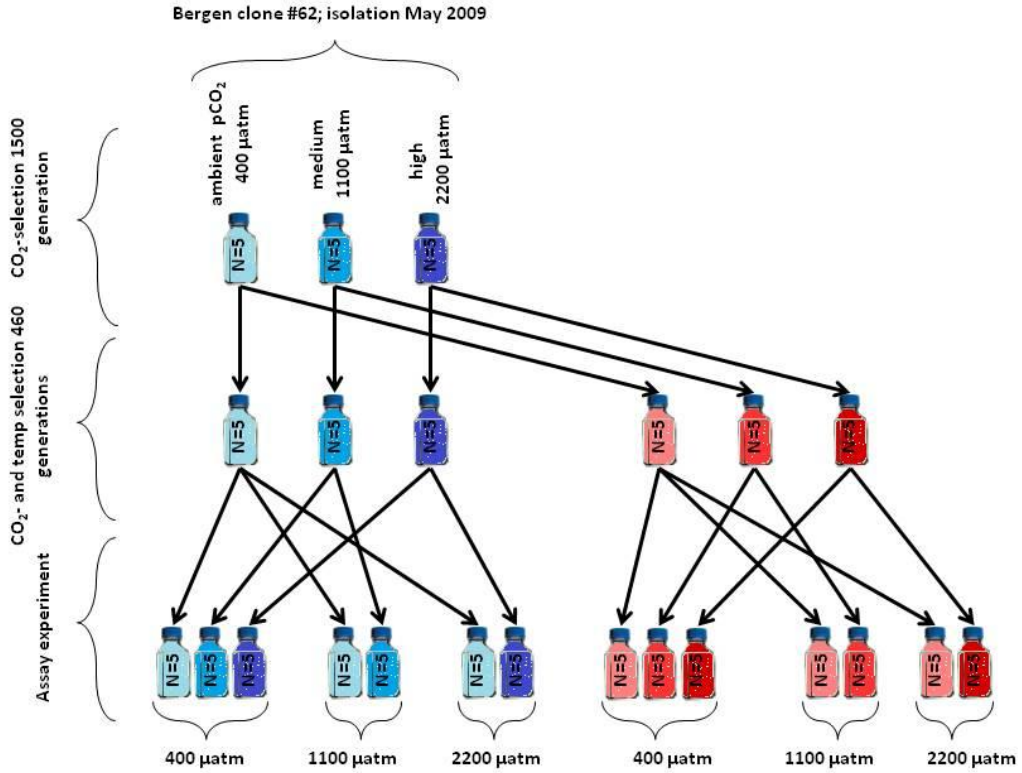

Fig.1 Overview of the selection/assay experiment. All replicates of the selection lines were founded by one single clone (Bergen #62). They were selected at 15°C and ambient, medium and high pCO_2 for approx. 1500 generations of asexual reproduction. After that, they were duplicated and additionally held under 26.3°C for approx. 460 generations. In the assay experiment the CO_2 selected lines were challenged with changed pCO_2. The adaptive response is the difference between the physiological response of non-adapted cell and the adapted cells at a certain pCO_2 concentration.

To measure the adaptive response we performed a reciprocal experiment, where the ambient control populations were tested for their physiological response to elevated CO_2 and the CO_2-selected populations for their response to ambient conditions, both at the temperature of previous section, i.e. 15°C and 26°C (Fig.1). The physiological responses were then compared to the selected responses for each CO_2-level in a 3-way ANOVA, including CO_2-selection treatment (2 levels, control and elevated), temperature (2 levels) and CO_2-assay condition (2 levels, control and elevated). With this approach we exclude the effect of laboratory selection because the salient comparison of adaptation is quantified with respect to a control that was also subjected to laboratory conditions (Kassen 2002). All populations were adapted for 15 days (3 batch cycles, 20-25 cell divisions) to the assay conditions to exclude any acclimatization and phenotypic plasticity effects.

In the fourth and last batch cycle we measured growth rate, cell size, total particular carbon (TPC) and particular organic carbon (POC). 90 and 180 ml culture suspension were vacuum filtered (<100 mbar) onto pre combusted glass fiber filters (GF/F, Whatmann) to determine TPC and POC, respectively. All filtrations were performed at the same time of the day, 4 hours after light exposure, within a timeframe of approximately one hour to avoid artifacts due to daily intrinsic cycling. The filters were handled with carbon free forceps (acetone washed) and petri-dishes (pre-combusted) to avoid carbon contamination. The samples were immediately stored at -20°C until further processing. After thawing, filters for POC-analysis were fumed with 37% HCl to remove all inorganic carbon. All filters were dried at 60°C for 12 h, rolled and put into tin cups (HEKAtech). Carbon content was determined using a Euro EA elemental analyzer (HEKAtech).

The carbon content of the filters was used to calculate the amount of carbon per cell by dividing the filter content by the absolute numbers of cells on the filter (filtered volume in ml x cells per ml). Coccosphere thickness was calculated by subtracting the radius of decalcified from calcified cell. The production was calculated based in the following equation:

$$\text{PIC Production} = \text{PIC cell}^{-1} \times e^{\mu \times \text{days}} \qquad (2)$$

$$\text{POC Production} = \text{POC cell}^{-1} \times e^{\mu \times \text{days}} \qquad (3)$$

As duration period (days) we used the duration of our batch cycle (5 days).

Statistical analysis

The dataset was divided into the two elevated CO_2 levels prior to analysis to avoid singularities. With both datasets we performed a univariate 3-way analysis of variance (ANOVA) with selection CO_2, assay CO_2 and temperature as factors, using JMP v. 9.0 (Statsoft Inc.). For the adaptive and correlated responses we performed planned contrasts, were we compared the control and the CO_2-selected lines in the same assay condition. Further we analyzed relative fitness in a univariate ANOVA were the relevant comparisons were calculated in planned contrasts. We tested each analysis for normal distribution and homogeneity of variances prior to the analysis and found no major violations.

Results

Adaptive response in fitness to elevated CO_2 under different temperature selection regimes

We measured exponential growth rate as fitness proxy, as our experimental set-up will select for those genotypes that display the fastest cell division under exponential growth. Temperature as main factor clearly increased growth rate (ANOVA, temperature: medium: $F_{1,31} = 100.0$ $p < 0.0001$, high: $F_{1,32} = 114.8$, $p < 0.0001$). More importantly, the effect of CO_2-adaptation, the difference between the control in elevated CO_2 and the selected lines, increased by 310 % at medium and 270 % at high CO_2 depending on the assay/selection temperature (Fig. 1, ANOVA, interaction selection CO_2 x assay CO_2 x temp: medium: $F_{7,38} = 24.38$, $p < 0.0001$, high: $F_{7,38} = 34.00$, $p < 0.0001$). Additionally, when testing the correlated response, the CO_2-selected lines grew significant slower under ambient conditions at high temperature. Hence, the interaction effect was not only driven by the adaptive response, but also by a loss of growth rate in high CO_2-selected populations when back-exposed to original CO_2 conditions.

Phenotypic changes associated to adaptation

Next to fitness we examined several other important phenotypic cell traits that were not directly subject to selection. Cell size decreased with temperature (ANOVA, temperature: medium: $F_{1,31} = 412.7$ $p < 0.0001$, high: $F_{1,32} = 351.1$, $p < 0.0001$), while the adaptive changes reversed from smaller adapted cells at 15°C and elevated CO_2 to larger adapted cells at 26°C at the same CO_2- level (ANOVA, interaction selection CO_2 x assay CO_2 x temp: medium: $F_{1,31} = 15.05$, $p = 0.0005$, high: $F_{1,32} = 16.59$, $p = 0.0003$). The general pattern remained the same when looking at the non-calcified cell diameter, i.e. after removing the coccosphere (calcite) of the cells via diluted hydrochloric acid. However, we found differences between the CO_2-levels, as in the medium treatment the interaction was still significant (ANOVA, interaction selection CO_2 x assay CO_2 x temp: medium: $F_{1,31} = 5,3591$, $p = 0,0274$), while at high CO2 it was not (ANOVA, interaction selection CO_2 x assay CO_2 x temp: high: $F_{1,32} = 1.1833$, $p = 0,2848$). In contrast we found CO_2 selection as main factor significant (ANOVA, CO_2 selection: high: $F_{1,32} = 10.4880$, $p = 0.0028$). A closer look into the thickness of the coccosphere, revealed that the adaptive changes switched from a thinner coccosphere of cells selected at elevated CO_2 (both medium and high) at 15°C (compared to the physiological response of ambient CO_2 selected cells at elevated CO_2) to a thicker coccosphere at 26°C (ANOVA, interaction selection CO_2 x temp: medium: $F_{1,31} = 6.4636$, $p = 0.0162$; interaction selection CO_2 x assay CO_2 x temp: high: $F_{1,32} = 22.9812$, $p < 0.0001$).

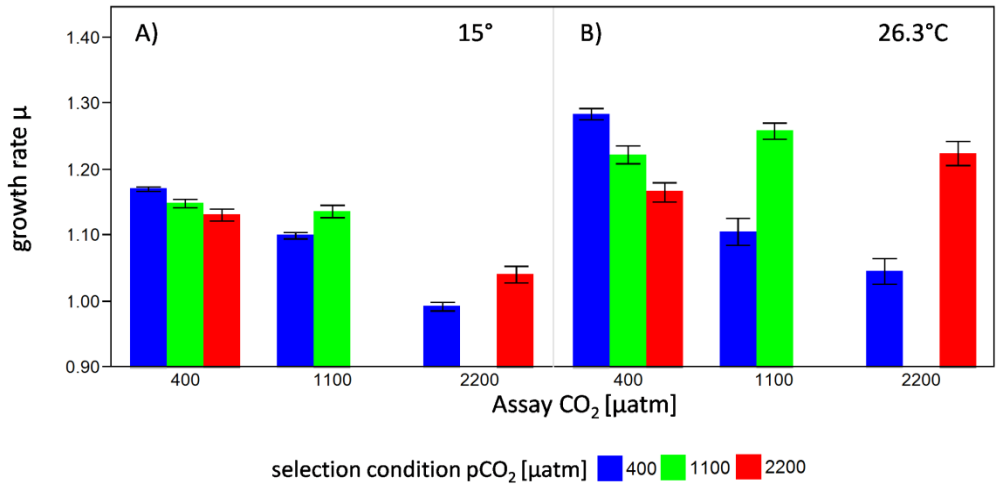

Fig.2 Phenotypic response in growth rate to 1600 generations of CO_2 selection and additional 480 generations of combined selection to temperature and CO_2 in *Emiliania huxleyi*. The test was designed for CO_2 adaptation, hence the selection temperature equals the assay temperature. The difference between the selection backgrounds at each assay condition shows the adaptive effect to CO_2 at given temperature. **A)** exponential growth rate at control temperature. **B)** exponential growth rate at high temperature

Table 1. p-values of corresponding contrasts to the adaptive response to CO_2 at 15 and 26.3°C (ANOVA Contrasts model: medium: $F_{4,31} = 10.66$, $p < 0.0001$, high: $F_{4,32} = 7.211$, $p = 0.0003$).

Assay temperature [°C]	Adaptive response at medium CO_2 contrast: 400->1100 to 1100	Adaptive response at high CO_2 contrast: 400->2200 to 2200
15	p = 0.0169	p = 0.0003
26.3	p = 0.7009	p = 0.6543

Interestingly, a thicker coccosphere did not result in more particulate inorganic carbon (PIC) per cell. At 15°C the PIC content of the cells was adaptively reduced at elevated CO_2. This is in contrast to Lohbeck et al. (2012), where PIC was found to be increased after 500 generations of selection. After high temperature adaptation the adaptive reduction in PIC content at elevated CO_2 was not present anymore (Table1). In general, cells had less PIC at higher temperature (ANOVA, temperature

selection: medium: $F_{1,31}$ = 32.5547, p < 0.0001, high: $F_{1,32}$ = 55.4731, p < 0.0001). This effect, however, was entirely due to the general decrease in cell size, since the temperature effect disappeared when PIC was standardized to total cell volume (ANOVA, temperature: medium: $F_{1,31}$ = 0.4336, p = 0.5151, high: $F_{1,32}$ = 0.9621, p = 0.3340). Standardized to cell volume the adaptive reduction in PIC as response to high CO_2 selection was still present (ANOVA, interaction selection CO_2 x assay CO_2: high: $F_{1,32}$ = 14.61, p = 0.0006). For the particular organic carbon (POC) content of the cells there was a general adaptive decrease to CO_2 (ANOVA, CO_2 selection: medium: $F_{1,31}$ = 7.557, p = 0.0099, high: $F_{1,32}$ = 26.42, p < 0.0001). However, at medium CO_2 the temperature adaptation led to the disappearance of the negative CO_2 effect (ANOVA, interaction selection CO_2 x assay CO_2 x temp: medium: $F_{1,31}$ = 5.82, p = 0.0219).

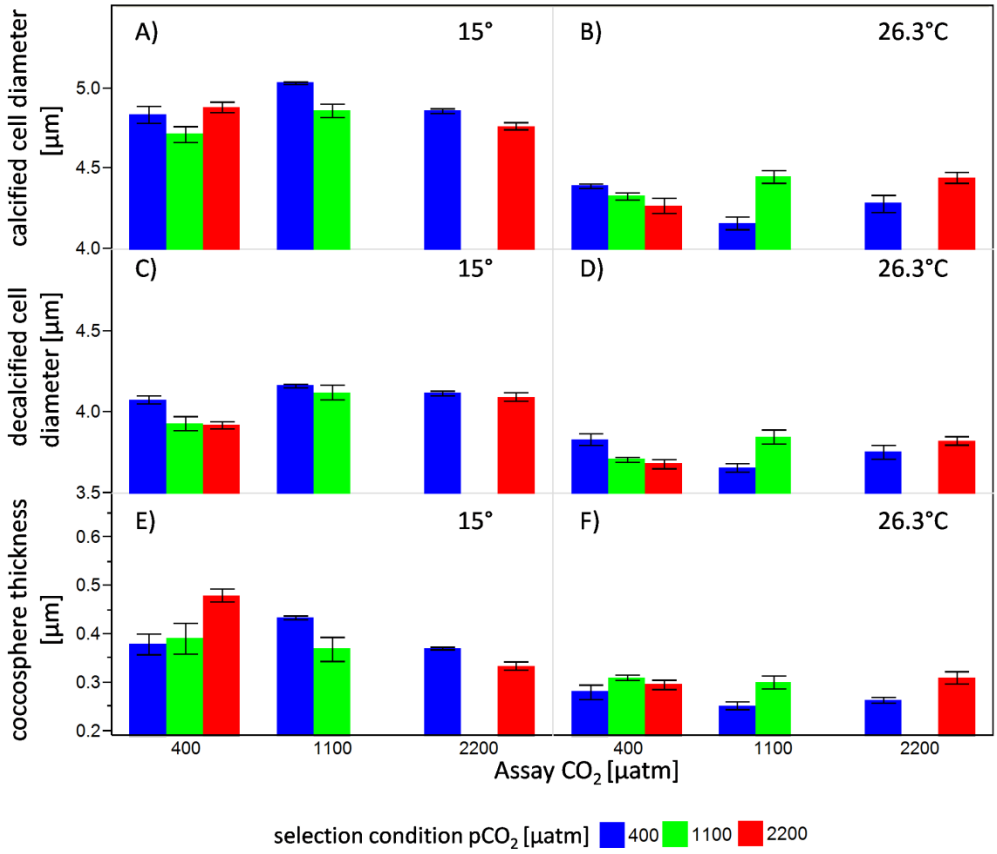

Fig. 3 Correlated traits in cell size to 1600 generations of CO_2 selection and additional 480 generations of combined selection to temperature and CO_2 in *Emiliania huxleyi*. The test was designed for CO_2 adaptation, hence the selection temperature equals the assay temperature. Coccosphere thickness is calculated as half of the difference between calcified and decalcified cell size. Effects caused by adaptation are the differences between the selection backgrounds at each assay condition. **A)** average cell diameter of calcified cells [µm] at control temperature. **B)** average cell diameter of calcified cells [µm] at high temperature. **C)** average cell size of decalcified cell [µm] at control temperature. **D)** average cell size of decalcified cell [µm] at high temperature. **E)** coccosphere thickness ((calcified cell diameter-decalcified cell diameter)/2) [µm] at control temperature. **F)** coccosphere thickness ((calcified cell diameter-decalcified cell diameter)/2) [µm] at high temperature.

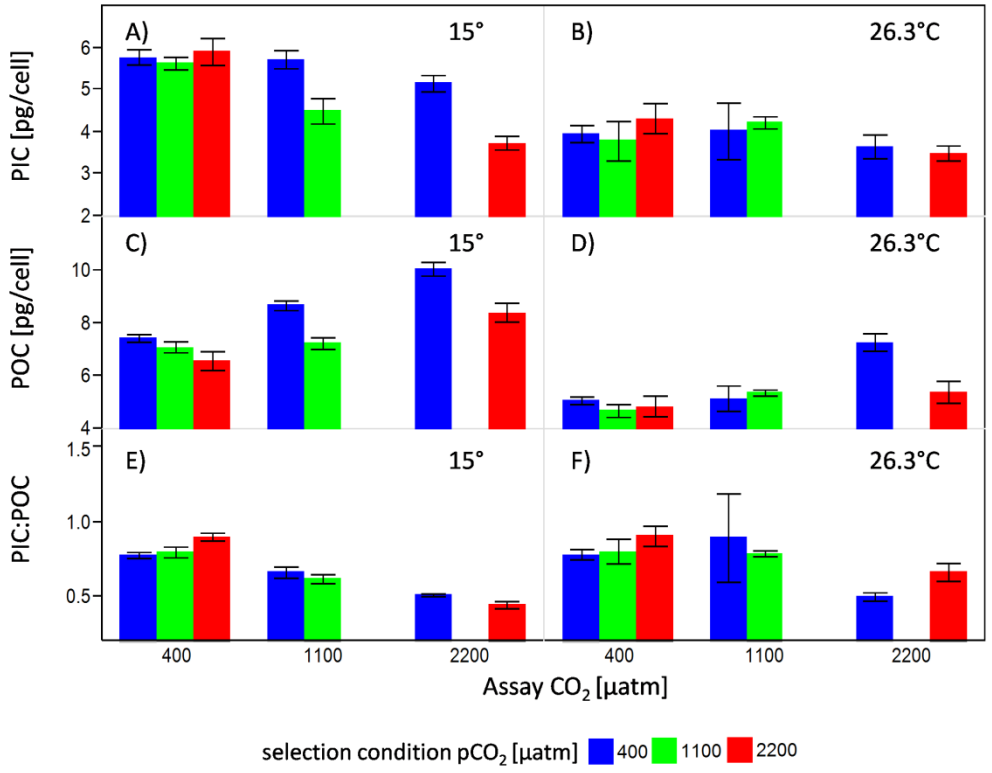

Fig. 4 Physiological and adaptive responses to CO_2 in carbon pools after selection to temperature and CO_2. The test was designed for CO_2 adaptation, hence the selection temperature equals the assay temperature. The physiological responses have the same pCO_2 selection background and different assay pCO_2. The adaptive responses have different selection backgrounds at the same assay pCO_2. **A)** particular inorganic carbon per cell [pg/cell] at control temperature. **B)** particular inorganic carbon per cell [pg/cell] at high temperature. **C)** particular organic carbon per cell [pg/cell] at control temperature. **D)** particular organic carbon per cell [pg/cell] at high temperature. **E)** Ratio of particular inorganic to organic carbon at control temperature. **E)** Ratio of particular inorganic to organic carbon at control temperature.

In addition to the effect of CO_2 adaptation, temperature caused an overall decrease in POC content of the cells (ANOVA, temperature: medium: $F_{1,31} = 208.3$, $p < 0.0001$, high: $F_{1,32} = 121.2$, $p < 0.0001$), regardless of the CO_2 selection /assay regime. Similar to PIC we standardized the POC content of the cells to cell size and the temperature effect disappeared (ANOVA, temperature: medium: $F_{1,31} = 2.432$, $p = 0.129$, high: $F_{1,32} = 1.6531$, $p = 0.2078$), suggesting that the reduction on POC content in the cells was mainly driven by the general decrease in cell size all treat. For the relative distribution among inorganic and organic carbon we found a complete reversal of adaptive effects under high CO_2 selection depending on temperature (ANOVA, interaction selection CO_2 x temp: high: $F_{1,32} = 4.3495$, $p = 0.0451$). In contrast, this did not apply to medium CO_2 selection treatments.

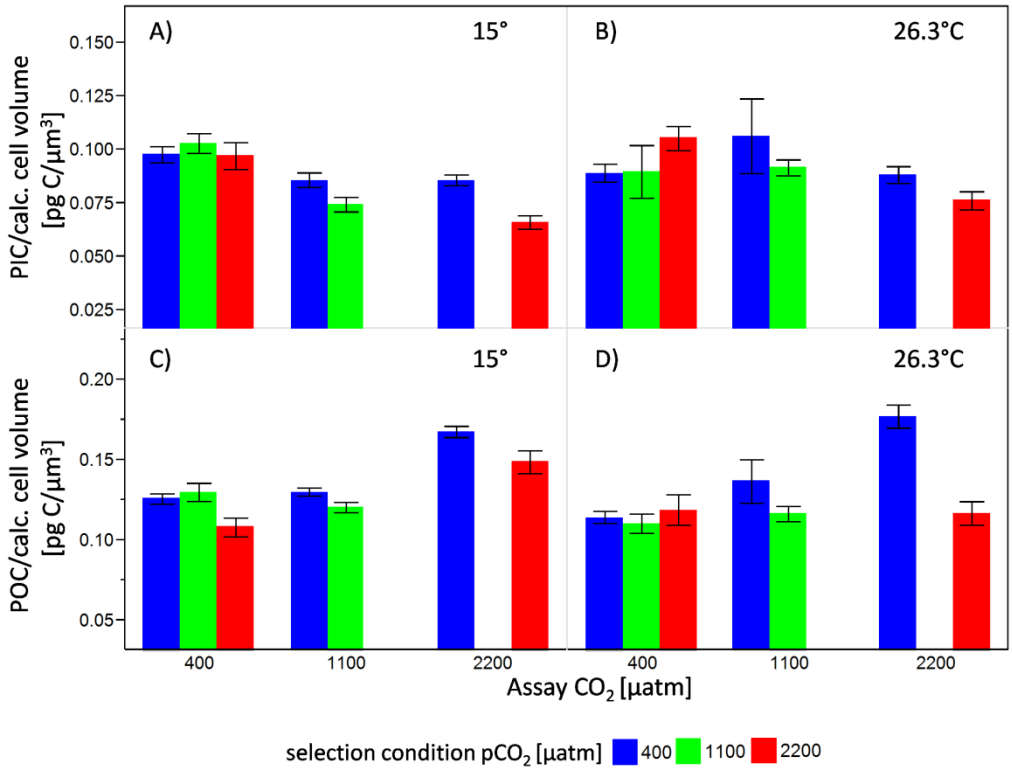

Fig. 5 Physiological and adaptive changes in carbon pools standardized to total cell volume after selection to CO_2 and temperature. The test was designed for CO_2 adaptation, hence the selection temperature equals the assay temperature. The physiological responses have the same pCO_2 selection background and different assay pCO_2. The adaptive responses have different selection backgrounds at the same assay pCO_2. **A)** particular inorganic carbon per calcified cell volume [pg C/µm^3] at control temperature **B)** particular inorganic carbon per calcified cell volume [pg C/µm^3] at high temperature **C)** particular organic carbon per calcified cell volume [pg C/µm^3] at control temperature **D)** particular organic carbon per calcified cell volume [pg C/µm^3] at high temperature.

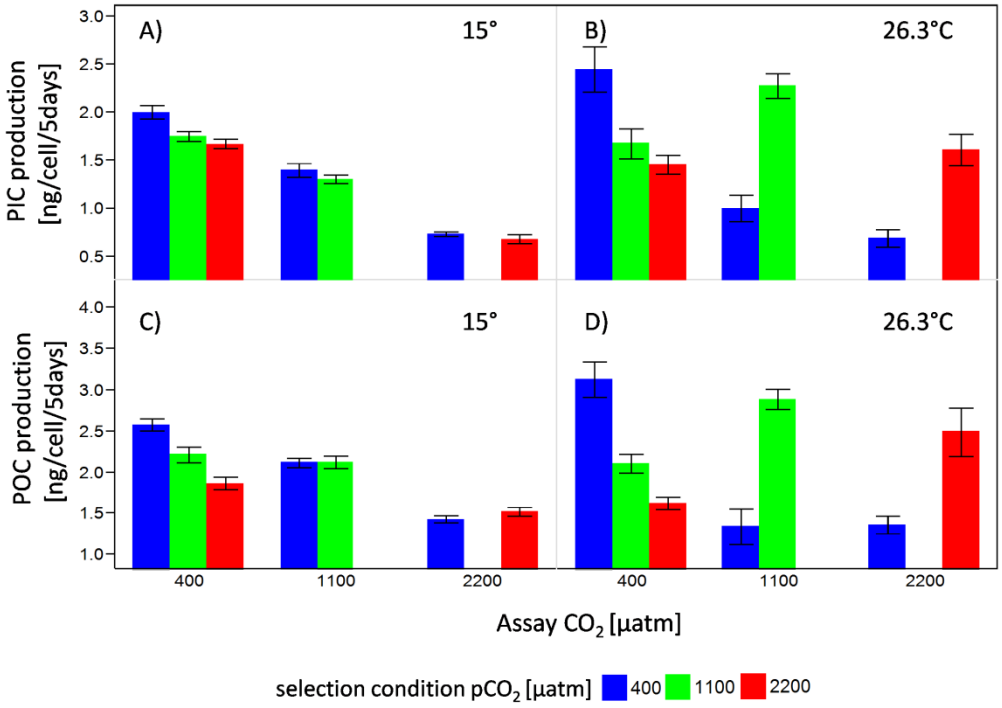

Fig. 6 Physiological and adaptive changes in production after selection to CO_2 and temperature. The test was designed for CO_2 adaptation, hence the selection temperature equals the assay temperature. The physiological responses have the same pCO_2 selection background and different assay pCO_2. The adaptive responses have different selection backgrounds at the same assay pCO_2. **A)** production of particular inorganic carbon after 5 days [ng/cell/5days], starting from a single cell at control temperature. **B)** production of particular inorganic carbon after 5 days [ng/cell/5days], starting from a single cell at control temperature. **C)** production of particular organic carbon after 5 days [ng/cell/5days], starting from a single cell at control temperature. **D)** production of particular organic carbon after 5 days [ng/cell/5days], starting from a single cell at control temperature.

Production rates of inorganic and organic carbon

Production rates were quantified over one batch cycle interval (exactly 5 days). High temperature interacted with adaptation to acidification in terms of population production (5-day interval). Under elevated temperature selection/assay conditions, the increase of adaptive population production of PIC compared to controls was higher, as revealed by a significant three-way interaction (ANOVA, interaction: selection CO_2 x assay CO_2 x temp: medium: $F_{1,31} = 27.0568$, $p < 0.0001$, high: $F_{1,32} = 24.1411$, $p < 0.0001$). We find a similar picture for POC production (ANOVA, interaction: selection CO_2 x assay CO_2 x temp: medium: $F_{1,31} = 32.5504$, $p < 0.0001$, high: $F_{1,32} = 20.1116$, $p < 0.0001$).

Discussion

Here we present the first study on how adaptation to simulated ocean acidification is affected by combinations of previous CO_2-adaptation and further selection to elevated temperature as second selection pressure. This complements an earlier study on how temperature adaptation is influenced by previous CO_2-adaptation (Schlüter et al. (2014). In order to understand how both selection factors interact, it is important to evaluate the impact of each selection factor on the corresponding other.

The CO_2 optimum for growth rate in *E. huxleyi* increases with temperature (Sett et al. 2014). Hence, we assumed that CO_2 would have a smaller selective effect at higher temperature. As consequence we assumed to find a smaller adaptive response. Surprisingly, we found an unexpected increase of the adaptive growth rate response to CO_2 selection (i.e. the difference adapted to non-adapted populations in high CO_2) when selection (=assay) temperature was high.

We used data from Schlüter et al. (2014) to calculate the initial physiological and adaptive response to high CO_2 at 26.3°C before temperature-selection (Table 2). The adaptive effects are described as relative fitness W of the adapted populations (a) over the control populations (c) as $W_{a/c} = \mu a / \mu c$ (Lenski et al. 1991)(Tab.1). The same formula can describe the physiological effects, when (a) is the physiological response in a new environment and (c) the control.

CO_2 adaptation alone has about the same effect size at 15°C and 26.3°C (Fig. 7), which means temperature as physiological factor does not alter the adaptive effect size of CO_2 adaptation (at given conditions). The same applies for CO_2 as physiological factor on temperature adaptation, as the effect size of temperature adaptation is not significantly different at ambient and high CO_2 levels (Fig. 7). When both factors have been selected for, then the adaptive response increases significantly (Fig.7). The adaptive response of to temperature is significantly larger when CO_2 adaptation was present and vice versa. Both adaptive effects, to CO_2 and temperature, are about the same size at double selection conditions (Fig.7). The effect sizes of single adaptive effects add more or less up to the effect size after double selection. Maybe the effect size of double selection is a little more in numbers than both single adaptive effects together, which means both selection factors would act synergetic. However, there is no statistical difference because of the variation and therefore we have to assume that both selective factors act additive. That further means, that we did not find any constrains between both selection factors.

Table 2. Population specifications used to calculate the physiological/adaptive responses. Given are the selection and assay condition of each population, which growth rate μ was used to calculate the effect according to $W_{a/c} = \mu a / \mu c$.

*** values taken from Schlüter et al. (2014).**

	a	**c**
Physiological response to OA at 15°C	Selected: 400µatm 15°C Assay: 2200µatm 15°C	Selected: 400µatm 15°C Assay: 400µatm 15°C
Adaptive response to OA at 15°C	Selected: 2200µatm 15°C Assay: 2200µatm 15°C	Selected: 400µatm 15°C Assay: 2200µatm 15°C
Physiological response to OA before temperature adaptation	Selected: 400µatm 15°C* Assay: 2200µatm 26.3°C*	Selected: 400µatm 15°C* Assay: 400µatm 26.3°C*
Adaptive response to OA before temperature adaptation	Selected: 2200µatm 15°C* Assay: 2200µatm 26.3°C*	Selected: 400µatm 15°C* Assay: 2200µatm 26.3°C*
Physiological response to OA after temperature adaptation	Selected: 400µatm 26.3°C Assay: 2200µatm 26.3°C	Selected: 400µatm 26.3°C Assay: 400µatm 26.3°C
Adaptive response to OA after temperature adaptation	Selected: 2200µatm 26.3°C Assay: 2200µatm 26.3°C	Selected: 400µatm 26.3°C Assay: 2200µatm 26.3°C
Adaptive temperature response at ambient conditons	Selected: 400µatm 26.3°C* Assay: 400µatm 26.3°C*	Selected: 400µatm 15°C Assay: 400µatm 26.3°C
Adaptive temperature response at OA conditons	Selected: 2200µatm 26.3°C* Assay: 2200µatm 26.3°C*	Selected: 2200µatm 15°C* Assay: 2200µatm 26.3°C*
Temperature adaptation without OA adaptation at OA conditions	Selected: 400µatm 26.3°C Assay: 2200µatm 26.3°C	Selected: 400µatm 15°C* Assay: 2200µatm 26.3°C*

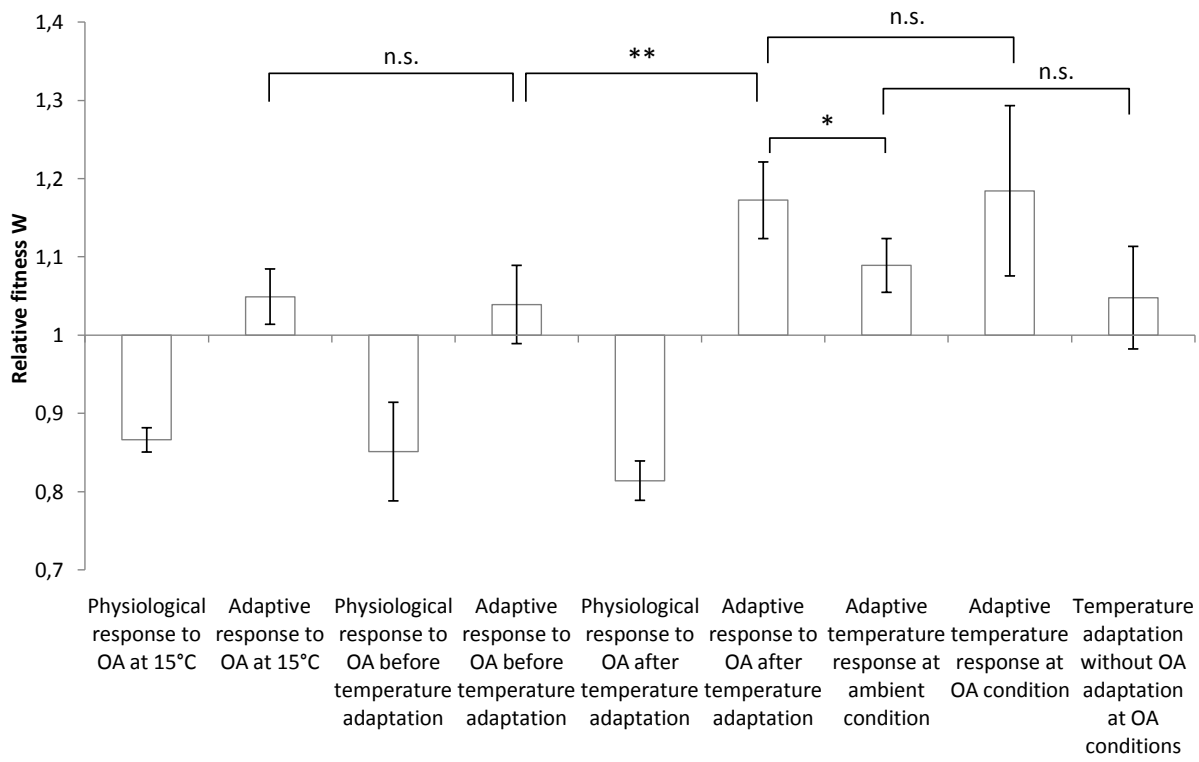

Fig. 7 Effect size as relative fitness increase W denuding on different selection treatments and assay conditions. The quotient of the exponential growth rates denotes the relative fitness of the adapted/challenged populations (a) over the control populations (c) as $W_{a/c}$ = µa /µc. All effect sizes refer to changes from 400 µatm pCO_2 to 2200 µatm pCO_2 and 15°C to 26.3°C (Tab.1). The medium CO_2 treatment had not been accounted for. Effect sizes were tested as contrasts in a univariate ANOVA using JMP 9.0 (Statsoft).

Phenotypical changes associated with adaptation

Cell size itself is temperature dependent, with smaller cell sizes favored at higher temperatures (Atkinson et al. 2003). Moreover, growth rate and cell size are generally negatively correlating (Tang 1995). The logical consequence would have been a decrease in cell size under faster replication rates, which we indeed found as physiological response to temperature. Surprisingly, we found an increase in cell size as result of adaptation despite faster growth. In that way the physiological effect (decrease in cell size) was adaptively reduced (increase in cell size). In theory, an evolutionary stable cell size is determined by the allometric relationships for nutrient uptake kinetics, and by metabolic rates (Verdy et al. 2009). According Verdy et al. (2009) at high nutrient and low (or none) predation availability larger cells are generally favored. The initial reduction in cell size was therefore evolutionary instable. Our experiment was conducted under excess nutrient supply, and hence larger cells could prevail. So far, we cannot tell whether this response is evolutionary stable or just transient. However, the future ocean is predicted to be higher stratified and therefore mainly

nutrient limited (Steinacher et al. 2009). Hence, our observations of cell size are not directly transferable and may be reversed under natural selection conditions.

Further, we found a decline in POC per cell as adaptive effect to high CO_2 conditions. The POC content of the cells was adaptively decreased under elevated CO_2 to a similar level as the ambient control. The physiological response of *E. huxleyi* to ocean acidification is a increase in POC, which corresponds to an increase in storage compounds (Rokitta et al. 2012). The adaptive response presumably readjusted the metabolic pathways to a status similar to the cell status at ambient conditions. We found the same pattern, when standardized to cell volume. We assume that the POC content of the cells was adaptively reduced to a suitable POC/ Volume ratio. This agrees to the literature as POC and cell size are usually correlating (Verity et al. 1992).

The inorganic carbon content per cell is particularly relevant for the ballasting effect of any one coccolithophore cell, as the relatively heavy coccoliths ballast lighter organic particles and therefore enhance the sinking speed (Armstrong et al. 2001, Bach et al. 2012). While at 15°C there was a decrease of PIC per cell with increasing CO_2 (contrary to Lohbeck et al. 2012), physiologically and adaptively, we found no difference for CO_2 at high temperature, neither physiologically nor adaptive. In physiological experiments the negative CO_2 effect remained regardless of temperature (10, 15, 20°C) (Sett et al. 2014). The temperature used in present experiment triggered temperature stress, which hasn't been investigated for calcification so far. Hence, we cannot exclude effects of temperature stress. Further, the cell size seems to play a key factor, as the negative adaptive response to high CO_2 was still present, although there was no physiological effect.

The difference in CO_2 response between both temperatures was the thickness of the coccosphere. Under high temperature selection a thicker coccosphere evolved as a response to CO_2 selection, while at 15°C the coccosphere became thinner due to adaptation. The consequences of those changes in PIC content and coccosphere thickness in terms of function are hard to predict, since the function of coccoliths is still not finally clear (Raven & Crawfurd 2012). Further our experiment was designed to select for exponential growth rate and hence the responses are solely based on the effects of temperature and ocean acidification, but not necessarily relate to natural conditions.

Global biogeochemical implications

Calcification is regarded to have a major impact on global carbon cycling, as it affects ballasting of and sinking velocity organic carbon (Armstrong et al. 2001). The ratio of inorganic to organic carbon in sinking particles is called the rain ratio. The rain ratio results of the production of PIC and POC in the euphotic zone. In absence of other phytoplankton the production ratio of PIC:POC reflects the cellular ratio in E. huxleyi. In the cellular PIC: POC ratio we found a reversal of the adaptive effect at

high CO_2 under high compared to low temperature, which led to a restoration of PIC:POC in the high CO_2 selected populations compared to the ambient controls at 26.3°C. This could potentially reduce the negative effects of acidification.

For the production rates the content of the cells as starting point is only important early during exponential growth, while later, any production will be completely determined by the exponential growth rate, which we already observed during the 5d test interval. (Fig.6). At the observed growth rates the cell concentration of the most intense *E. huxleyi* bloom ever reported in literature (115,000,000 cells/ml (Tyrrell & Merico 2004)) would be reached approximately after 14 - 15 days (assuming a starting cell concentration of 10 cells/ml). The cell concentration in an average bloom (Tyrrell & Merico 2004) would be reached after 10 - 12 days of exponential growth. The production of PIC and POC at high temperature would still be lower at high CO_2 compared to ambient CO_2 conditions, because the (growth) rates are about 6-7 % lower under acidification. However, compared to the production at 15°C the production is massively increased at high temperature, especially when we take the estimated time of an average bloom into account. Assuming sufficient nutrient supply the productivity of *E. huxleyi* in the ocean would increase with higher temperature despite ocean acidification. In reality, the processes in the water column will be much more complex because for example, the predictions for the future ocean are reduced nutrient supply due to enhanced thermal stratification (Steinacher et al. 2009).

Our experiment was highly artificial and meant to be proofs-of-principle. A transfer to nature (and therefore predictions on the future ocean) has to be done very carefully. In contrast to nature our cells were cultured at constant excess nutrient availability. Despite growth rate, we had no other selection factors (like predation, competition to other plankton, sexual reproduction… etc.) included. Hence our data constitute a basis for further research, which need additional studies with increasing experimental complexity.

Conclusions

We here showed in a microcosm batch system the effects of temperature adaptation to previous adaptation to CO_2 and disentangled the single effects of temperature and CO_2 adaptation. The adaptive effects of temperature and CO_2 selection are additive, which also means there were no negative epistatic effects and no constrains visible in our experiment. On a global scale the effect of temperature may be of much more importance than ocean acidification for coccolithophores (Winter et al. 2014), as temperature in our experiment reduced the negative effects of CO_2 and further increased growth rates and therefore productivity. Several large-scale physiological experiments, field surveys and models have already shown that temperature is a prime factor in terms of ocean

primary productivity, plankton metabolic functions and distribution patterns (Thomas et al. 2012, Boyd et al. 2013, Toseland et al. 2013). However, since the experiment selected only on growth rate and neglected ecological factors, the observed correlated traits have to be utilized carefully for predictions of future scenarios. We here propose further evolutionary experiments that should account for factors like predation, light modulation or virus attacks (Raven & Crawfurd 2012).

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References:

Armstrong R, Lee C, Hedges J (2001) A new, mechanistic model for organic carbon fluxes in the ocean based on the quantitative association of POC with ballast minerals. Deep Sea Res … 49:219–236

Atkinson D, Ciotti BJ, Montagnes DJS (2003) Protists decrease in size linearly with temperature: ca. 2.5% degrees C(-1). Proc Biol Sci 270:2605–2611

Bach LT, Riebesell U, Sett S, Febiri S, Rzepka P, Schulz KG (2012) An approach for particle sinking velocity measurements in the 3-400 µm size range and considerations on the effect of temperature on sinking rates. Mar Biol 159:1853–1864

Boyd PW, Rynearson T a, Armstrong E a, Fu F, Hayashi K, Hu Z, Hutchins D a, Kudela RM, Litchman E, Mulholland MR, Passow U, Strzepek RF, Whittaker K a, Yu E, Thomas MK (2013) Marine phytoplankton temperature versus growth responses from polar to tropical waters--outcome of a scientific community-wide study. PLoS One 8:e63091

Buckling A, Craig Maclean R, Brockhurst MA, Colegrave N (2009) The Beagle in a bottle. Nature 457:824–829

Collins S (2011) Many Possible Worlds: Expanding the Ecological Scenarios in Experimental Evolution. Evol Biol 38:3–14

Collins S, Rost B, Rynearson TA (2014) Evolutionary potential of marine phytoplankton under ocean acidification. Evol Appl 7:140–155

Danbara a., Shiraiwa Y (1999) The Requirement of Selenium for the Growth of Marine Coccolithophorids, Emiliania huxleyi, Gephyrocapsa oceanica and Helladosphaera sp. (Prymnesiophyceae). Plant Cell Physiol 40:762–766

Elena SF, Lenski RE (2003) Evolution experiments with microorganisms: the dynamics and genetic bases of adaptation. Nat Rev Genet 4:457–69

Guillard RR, Ryther JH (1962) Studies of marine planktonic diatoms. I. Cyclotella nana Hustedt, and Detonula confervacea (cleve) Gran. Can J Microbiol 8:229–239

IPCC (2007) IPCC Fourth Assessment Report (AR4). IPCC 1:976

Kassen R (2002) The experimental evolution of specialists, generalists, and the\rmaintenance of diversity. J Evol Biol 15:173–190

Kester D, Duedall I, Connors D, Pytkowicz R (1967) Preparation of artificial seawater. Limnol Ocean 12:176–179

Langer G, Nehrke G, Probert I, Ly J, Ziveri P (2009) Strain-specific responses of *Emiliania huxleyi* to changing seawater carbonate chemistry. Biogeosciences 6:2637–2646

Lenski RE, Rose MR, Simpson SC, Tadler SC (1991) Long-Term Experimental Evolution in Escherichia coli. I. Adaptation and Divergence During 2,000 Generations. Am Nat 138:1315

Lewis E, Wallace D (1998) CO2SYS-Program developed for the CO_2 system calculations. Carbon Dioxide Inf. Anal. Center. :105

Lohbeck KT, Riebesell U, Reusch TBH (2012) Adaptive evolution of a key phytoplankton species to ocean acidification. Nat Geosci 5:346–351

Raven J, Crawfurd K (2012) Environmental controls on coccolithophore calcification. Mar Ecol Prog Ser 470:137–166

Redfield A (1958) The biological control of chemical factors in the environment. Am Sci 46:205–221

Reusch TBH, Boyd PW (2013) Experimental evolution meets marine phytoplankton. Evolution 67:1849–59

Riebesell U, Zondervan I, Rost B, Tortell P (2000) Reduced calcification of marine plankton in response to increased atmospheric CO_2. Nature 407:2–5

Rokitta SD, John U, Rost B (2012) Ocean acidification affects redox-balance and ion-homeostasis in the life-cycle stages of Emiliania huxleyi. PLoS One 7:e52212

Schaum C-E, Rost B, Collins S (2015) Environmental stability affects phenotypic evolution in a globally distributed marine picoplankton. ISME J:1–10

Schlüter L, Lohbeck KT, Gutowska M a., Gröger JP, Riebesell U, Reusch TBH (2014) Adaptation of a globally important coccolithophore to ocean warming and acidification. Nat Clim Chang 4:1024–1030

Sett S, Bach LT, Schulz KG, Koch-Klavsen S, Lebrato M, Riebesell U (2014) Temperature modulates coccolithophorid sensitivity of growth, photosynthesis and calcification to increasing seawater pCO_2. PLoS One 9:e88308

Steinacher M, Joos F, Frölicher TL, Bopp L, Cadule P, Doney SC, Gehlen M, Schneider B, Segschneider J (2009) Projected 21st century decrease in marine productivity: a multi-model analysis. Biogeosciences Discuss 6:7933–7981

Tang EPY (1995) The allometry of algal growth rates. J Plankton Res 17:1325–1335

Thomas MK, Kremer CT, Klausmeier C a, Litchman E (2012) A global pattern of thermal adaptation in marine phytoplankton. Science 338:1085–8

Toseland A, Daines SJ, Clark JR, Kirkham A, Strauss J, Uhlig C, Lenton TM, Valentin K, Pearson G a., Moulton V, Mock T (2013) The impact of temperature on marine phytoplankton resource allocation and metabolism. Nat Clim Chang 3:979–984

Tyrrell T, Merico A (2004) Emiliania huxleyi: bloom observation and the conditions that induce them. In: Thierstein HR, Young JR (eds) Coccolithophores: From Molecular Processes to Global Impact. Springer, p 585–604

Verdy A, Follows M, Flierl G (2009) Optimal phytoplankton cell size in an allometric model. Mar Ecol Prog Ser 379:1–12

Verity PG, Robertron CY, Tronzo CR, Andrews MG, Nelson JR, Sieracki ME (1992) Relationships between cell volume and the carbon and nitrogen content of marine photosynthetic nanoplankton. Limnol Oceanogr 37:1434–1446

Winter A, Henderiks J, Beaufort L, Rickaby REM, Brown CW (2014) Poleward expansion of the coccolithophore Emiliania huxleyi. J Plankton Res 36:316–325

Synthesis

This last chapter synthesizes the key findings of the thesis and how they expand our knowledge about adaptive processes in marine phytoplankton under global change. Moreover, I highlight existing gaps and fruitful future research approaches that result from this thesis.

The mutational basis of evolutionary change

The initial experiment was started with one single clone. Hence, all genetic divergence has to be derived from de novo mutations, be they classically genetic (=DNA based) or epi-mutations for example through DNA methylation. I only incorporated one single clone to reveal basic principles in the process of adaptation to global change. From Lohbeck et al. (2012) we already knew, that genotypic sorting has as much effect as appearance of new beneficial mutations. However, a multi-clonal approach wouldn't be able to detect interactions between new mutations. Some mutations could possibly be beneficial to one and deleterious to another selection factor (Weinreich et al. 2005). In a multi-clonal approach the genetic basis would have been different and could have had unwanted epistatic effects based on the different genetic backgrounds (genomes) (Weinreich et al. 2005). In contrast, differences in epistatic effects over treatments in a single-clone approach can only result from new mutations. It is to mention that there could still be epistatic effects based on genome. However, as it is the same for all treatments those effects cannot be seen in a reciprocal experiment. A negative side effect of the single-clone approach is that assumptions about implications on natural systems can hardly be made since natural systems contain more than one genotype and more than one species. In natural systems sex also plays a role in the adaptive process (Kaltz & Bell 2002), which was excluded in my experiments. The effect of sex to adaptation was already summarized in the introduction. Compared to natural populations the adaptive rate was probably underestimated, as sex increases the rate of adaptation (Kaltz & Bell 2002).

Towards more complex selection environments

With 2100 generations of selection to ocean acidification we performed the longest evolutionary experiment in any marine species so far (The experiment was established and run for the first 1000 generations by Kai T. Lohbeck and then by myself). The introduction of temperature as second selection pressure made the experiment highly complex and remarkable within marine science (but see also Schaum et al. 2015). I demonstrated that the

calcifying coccolithophore *E. huxleyi* has the potential to adapt to ocean acidification in combination with selection at the upper thermal limit. I could also identify costs of adaptation, as the growth of the high CO_2 and high temperature adapted populations at control conditions was reduced compared to the control populations.

I showed in my experiments how two selection pressures possibly interact, which hasn't been done before in marine science. Simultaneous selection to CO_2 and temperature increase was studied by Benner et al. (2013). However, in their experiment they did no reciprocal experiment and therefore did not account for lab induced selection (Kassen 2002). Nor did they separate the selection factors, to disentangle their relationship on evolutionary adaptation. Eco-evolutionary models calculate higher optimal temperatures for tropical phytoplankton than they actually are (Thomas et al. 2012). Either these models are wrong, or natural phytoplankton populations cannot tap their full thermal potential. This would suggest that there is a constraint in temperature adaptation. In asexual single-clone selection regimes constraints in adaptive potential are often caused by genetic interaction. Genetic loci (i.e. units of Mendelian inheritance) commonly interact on a functional level, which is called functional epistasis (Weinreich et al. 2005). Hence, the fitness effect of each mutation is dependent on the genetic background it occurs. This has far-reaching consequences for evolution. A special case of epistasis is the so-called sign epistasis. Under sign epistasis, mutations are beneficial under some genetic background and deleterious under others (Weinreich et al. 2005). Further, some mutations that are deleterious on their own, may jointly be beneficial (Weinreich & Chao 2005). Theoretically, a population could fix a mutation which makes potential beneficial mutations in other loci deleterious. The result of such a fixation would be that the population would then be stuck on a local peak on the fitness landscape because no mutational trajectory would be selectively favored (Weinreich et al. 2005). On a fitness landscape every possible genotype is projected in an L+1-dimensional hypercube whose vertices represent genotypes and the edges represent point mutations. L is the number of genotypes, while +1 represents fitness as individual dimension (Weinreich et al. 2005). Due to the interactions of genes and mutations evolutionary trajectories on fitness landscapes are difficult to predict (Weinreich & Chao 2005). I observed positive magnitude epistasis between adaptation to warming and acidification. In the case of magnitude epistasis the magnitude of fitness effect differs in dependency of the genetic background (Weinreich et al. 2005). The adaptive fitness effect of temperature adaptation

was larger at high CO_2 conditions, when the genetic background was also adapted to elevated CO_2. In my experiments the adaptive fitness effects have been additive. However, the combined fitness effect of warming and acidification adaptation was larger than the pure addition of the single effects of warming and acidification. Due to standard deviation this difference is not significant. Either the effects are additive, or the variation in my experiment was too high to detect synergistic effects. My results showed no sign of epistatic constrains between mutations beneficial for temperature and/or CO_2 adaptation. In other words adaptations to warming and ocean acidification are not antagonistic.

How adaptive evolution will mitigate stress effects upon populations and may hence impact ecosystems in the face of global change is relatively little studied. Although our experimental set-up of 330 ml bottles was highly artificial and cannot be directly transferred to the natural open ocean environment, we can use this information and discuss potential implications to improve our understanding of the future ocean.

Evolutionary physiology of adaptation

The adaptive potential to warming is of particular interest, as all metabolic processes are temperature dependent and global mean temperature is expected to increase. Physiological models predict tropical phytoplankton to be most vulnerable to global warming (Thomas et al. 2012). In my experiment I used a strain isolated in Bergen, which is not optimal to investigate the potential effects of warming to tropical coccolithophore populations. The temperature used in the experiment for temperature adaptation was 26.3°C and close to the upper thermal limit of this strain. The strain showed a high adaptive potential to a temperature close to the thermal limit. The thermal adaptive potential cannot directly be transferred to tropical phytoplankton. However, it's an indication that the thermal potential may be underestimated. This calls for further research, especially on the evolution of thermal adaptation in tropical phytoplankton.

However, the temperature increase in the experiment was about 11°C, while the temperature increase to the end of the century is expected to be 2-4°C (IPCC 2014), and therefore the adaptive rate to temperature is probably overestimated. However, as I did not find negative interactions between adaptation to ocean acidification and adaptation to the upper thermal limit, I would expect those results to be consistent at lesser temperature increase. The study of Benner et al. (2013) found also a reduction of the response to ocean acidification with thermal adaptation, with a temperature increase of only 4°C (20-24°C).

Nonetheless, there are indices that under ocean acidification coccolithophores could still remain the dominant species in highly stratified oligotrophic ocean areas. High light conditions could probably still favor coccolithophores, as ocean acidification did not affect the ability of high light tolerance in coccolithophore model organsim *E. huxleyi* (Lohbeck et al. 2013). With temperature increasing the predictions are a higher stratification and a shallower mixed layer depth. Therefore phytoplankton is predicted to experience in average more light than today (IPCC 2014). The reduction in cell size I found throughout the adaptive processes would theoretically be also favorable for oligotrophic conditions (Verdy et al. 2009). However, the reduction in cell size is questionable, as it could only be an artifact of lab selection.

However, considering warming predictions are even more difficult. Temperature adaptation reduced the effects of ocean acidification. In regions where temperature adaptation is more relevant than adaptation to ocean acidification predictions, like the tropics (highly stratified oligotrophic areas), the information of the adaptive potential to the upper thermal limit of different algae taxa are still missing. Hence, coccolithophores could still be outcompeted by an algae with a higher adaptive potential to temperature despite the chances to overcome the effects of ocean acidification.

E. huxleyi has a relatively high pCO_2 optimum for growth (especially at higher temperature) which is still above present values (Sett et al. 2014). Recent studies discovered that *E. huxleyi* is presently benefiting from global change by expanding its range of distribution poleward (Winter et al. 2014). Potential implications may be that at intermediate pCO_2 levels (between ambient and the projections for the end of the century) the abundance of *E. huxleyi* will further increase (Sett et al. 2014).

Implications of study results to the real ocean

Difficult to answer is the question, whether adaptation to ocean acidification or adaptation to temperature increase will play a more important role for the persistence and performance of *E. huxleyi* populations. In my experiment the effect of thermal adaptation on growth rate exceeded the effect of ocean acidification adaptation. However, as mentioned the temperature increase in the experiment was about 3-4 times higher than expected to be for the end of the century (IPCC 2014), and at the upper thermal limit. Populations in blooming regions like the north Atlantic will probably not experience temperatures close to their thermal limit (Tyrrell & Merico 2004, Feng et al. 2009). It is questionable if temperature

adaptation will play a role at all in such regions, so this part of the experiment should rather be seen as proof of principle. The selection pressure due to temperature increase will probably be low, as long as the temperature is colder than the temperature for optimal growth (which is around 22-24°C for *E. huxleyi* strains from Bergen (Zhang et al. 2014)).

For tropical phytoplankton species that live close to their thermal optimum, or even their thermal limit, adaptation to temperature increase will probably be more important than CO_2-adaptation. This results mainly of the unique shape of temperature-growth-curves with a sharper decline in fitness above the optimum temperature than below (Zhang et al. 2014). On the other hand CO_2-optimum-curves look the opposite way (Sett et al. 2014). They have a sharper decline below the optimum than above (Fig. 2). The strength of selection pressure is high when the growth is strongly reduced (Oz et al. 2014). Therefore a sharper decline means a quicker increase in selection pressure. Hence, adaption is more important at sharp declining curves.

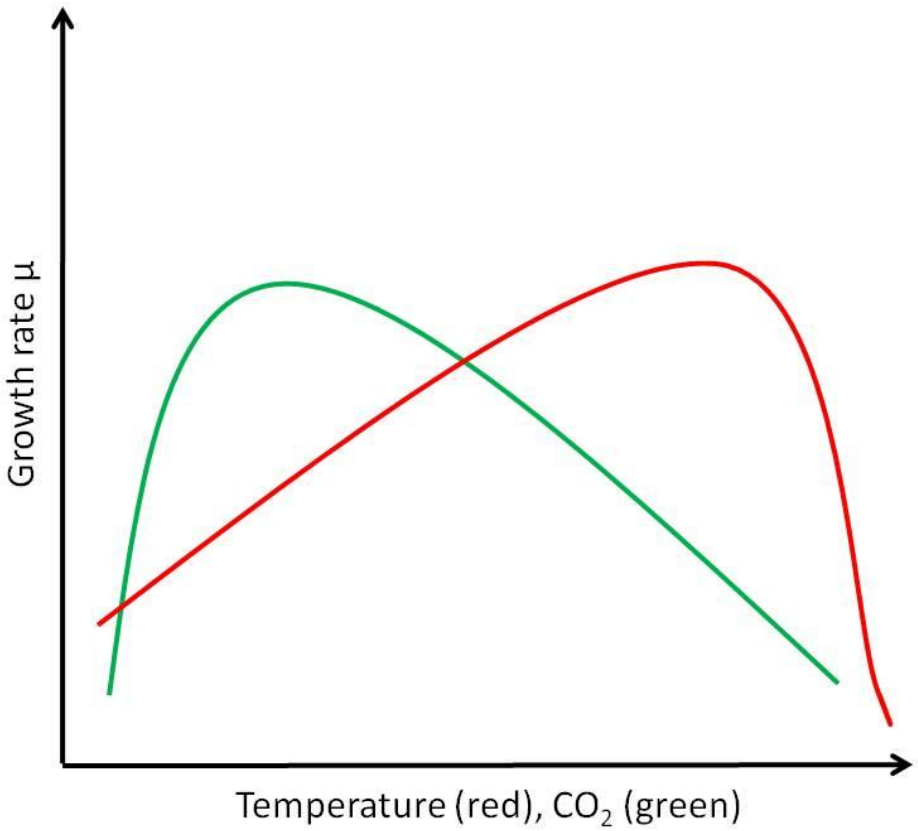

Fig. 2. Schema of temperature- and CO_2-optimum curves. CO_2-optimum curves (green) show a sharp increase at low CO_2 concentrations, while temperature-optimum-curves (temperature-growth-curves) show a sharp decline above their optimal growth temperature.

For temperature adaptation we found a pronounced correlated response suggesting costs to adaptation. High temperature adapted populations grew slower under control conditions than the control. This suggests that the whole thermal reaction norm could be shifted which is currently being tested in a follow-up experiment. A good indication of how those evolved thermal reaction norms could look like is described by Zhang et al. (2014), as they compared low temperature adapted strains isolated off the coast of Norway with high temperature adapted genotypes obtained from the Azores. The temperature norm was not only shifted towards higher temperatures, but the overall maximum growth rate was also lower in warm-adapted Azores genotypes indicating a further cost for high temperature adaptation (Kassen 2002). The same could be true for CO_2 adaptation as we found reduced growth of high CO_2 adapted populations at control conditions.

Mechanisms behind adaptation

My experiment concentrated on the adaptive potential and changes in phenotype. As this thesis doesn't contain genetic analysis or appropriate physiological experiments we can only speculate about the mechanisms underlying adaptation. Lohbeck et al. (2014) discovered adaptive changes in proton channel regulation associated with CO_2 adaptation, which could potentially be involved in pH regulation. In another transcriptomic study, in this case acidification and warming, the authors could not find any adaptive changes in gene regulation associated with pH regulation, which could be due to the missing reciprocal experiment (Benner et al. 2013). Interestingly, they found genes coding for posttranscriptional and cellular processes and signaling were up-regulated (Benner et al. 2013). However, so far only gene regulation has been evaluated, but not specific mutations in protein-coding DNA sequences. This corresponds to my results, as I could identify gene regulation as reason for long-term PIC reduction as byproduct of CO_2 adaptation. In this case it was phenotypic plasticity, which means that calcification pathways in the cells were not damaged, but rather down-regulated. At control conditions the calcite per cell quota could be restored. Further we found cost for adaptation in growth rate under control conditions, which presumably do not underlie regulatory processes, as growth rate could not be restored under control conditions.

For temperature adaptation itself no concrete mechanism could be identified yet. Heat stress will affect a wide range of metabolic processes, as they all are temperature-sensitive. As examples, I will propose two potential mechanisms and further research directions on that. It is to

mention that both proposed mechanisms are solely based on speculation and must not be taken as explanation but rather as proposals for further research.

First we have to consider that we selected for growth rate at a temperature at the upper physiological limit in order to match growth rates with the 15°C treatments. The reduction in growth rate above the optimal growth temperature is most likely due to moderate heat stress. We know from physiology that plants suffer from heat stress above their optimal growth temperature (Salvucci & Crafts-Brandner 2004). Under heat stress the photosynthesis is reduced due to the activation status of the carbon fixing ribulose-1,5-bisphosphate-carboxylase/oxygenase (Rubisco) (Law & Crafts-Brandner 1999, Salvucci & Crafts-Brandner 2004). The optimization of Rubisco activation and therefore energy supply is one possible mechanism that could have increased growth rate. It has further been suggested that adaptation to CO_2 involved pH regulating ion-channels and an improved cytosolic pH regulation (Lohbeck et al. 2014). Photosynthesis is a pH-dependent process (Falkner & Horner 1976) and could benefit from an improved cytosolic pH regulation. Hence, the benefits of an optimization of Rubisco activation may only be disbursed under high CO_2 with improved pH regulation. This could explain the increase in adaptive response to CO_2 under high temperature. Hence, to measure Rubisco activation status could be one way to identify underlying mechanisms.

Secondly, under moderate heat stress all organisms produce heat shock proteins (HSP) while genes active prior to heat stress may be suppressed (Howarth & Ougham 1993). Hence, a totally different set of genes and proteins could be involved in temperature adaptation. Temperature adaptation may also occur in HSP-coding or regulating regions. HSPs interact with other proteins as they function as chaperones and stabilize protein folding (Wang et al. 2004). To evaluate the importance of HSP, a quantitative polymerase chain reaction (qPCR) with HSPs as candidate genes should be performed (samples are existent). Further, the sequencing of HSP genes or their cDNA equivalent could reveal potential mutations within the coding regions of HSP.

A major shortcoming of experimental evolution is the usually unnatural high nutrient supply. In order to keep experiments rather short (<1 year) and feasible, selection regimes are run under constant exponential growth rate, aiming at about one cell division per day (but see Scheinin et al. 2015). To keep the populations in the exponential growth phase the cultures

were under non-limiting nutrients which is unrealistic when compared to the real ocean. I observed a general decline in cell size during adaptation, which is on the one hand an artifact of selection to faster growth, as cell size is strongly inversely correlated to growth rate (Tang 1995). CO_2-selection and temperature (physiologically) further led to a reduction in cell size. Such a reduction of cell size in natural systems could have major implications, since cell size is an important 'master trait' affecting nutrient acquisition, sinking velocity and the trophic position of plankton (Atkinson et al. 2003, Daufresne et al. 2009). However, at high temperature the CO_2 adapted cells became larger after thermal selection. According to the evolutionary stable size-model (ESS) cell size is determined by the allometric relationships for nutrient uptake kinetics, and by metabolic rates (Verdy et al. 2009). Temperature is affecting both, kinetics and metabolic rates. Possibly the physiological reduction in cell size to temperature was evolutionarily instable. However, most of the cell size reduction is probably an artifact of the experimental design. According to Verdy et al. (2009) the maximum cell size increases with nutrient availability (because of the uptake kinetics) and should have induced an increase in cell size over time (Lenski 2004). On the other hand cell size and storage capacity are correlated (Verity et al. 1992, Verdy et al. 2009). Hence, the permanent excess nutrients could have led to a decrease in storage capacity (direct use of resources) and therefore smaller cells. Nutrient uptake rates, and in that way cell size, can be used as ecological classification (Litchman et al. 2007). However, the experimental design was not prepared to investigate the mechanism underlying the cell size reduction. In the design there was no direct selection factor for cell size (like size selective predation). To transfer the experimental results into nature the clarification of cell size under adaptive evolution is a crucial factor that has yet to be investigated.

Final conclusions

In this thesis I showed the adaptive potential of *E. huxleyi* to ocean acidification and global warming. For all calcifying marine organisms it will be a trade-off between benefit and costs for calcification. Temperature and adaptation to it could however reduce the negative effects of ocean acidification on *E. huxleyi*. Temperature is a prime factor of primary production, metabolic and distribution patterns (Thomas et al. 2012, Boyd et al. 2013, Toseland et al. 2013), and had higher physiological and adaptive effects in the present experiment compared to ocean acidification. However, whether adaptation to temperature or to acidification is of higher importance depends on temperature. Hence, at a high latitude

or in upwelling regions adaptation to ocean acidification will be the key factor, while in warm regions adaptation to temperature is more important will be. Marine microbes, like unicellular phytoplankton have quite good chances to adapt to ocean acidification and global warming, because of the short generation time and the large population sizes (Reusch & Boyd 2013). According to the space-time comparison approach (Sunday et al. 2014), the adaptive potential increases with distribution. As *E. huxleyi* is the world's most common coccolithophore with a global distribution (except for polar waters) (Westbroek et al. 1989) and an accordingly local adaptation (Zhang et al. 2014), it has probably the highest adaptive potential within the group of coccolithophores. Adaptive potential in general includes biological and non-biological selection factors. Hence, I expect *E. huxleyi* to remain the most abundant coccolithophore in the future ocean. Model calculations predict changes in ocean currents and stratification accompanied by shifting in global primary production because of nutrient limitation (Bopp et al. 2001, Steinacher et al. 2009). However, the conditions for *E. huxleyi* to bloom should still occur, although the areas where blooms occur may be shifted north (Bopp et al. 2001). The question remains if other coccolithophores will be able to adapt, as their adaptive potential might be lower (less far distributed). Hence, this experiment should be repeated with other coccolithophores to evaluate if *E. huxleyi* is a useful representative for coccolithophores in terms of evolutionary adaptation.

References:

Angilletta MJ (2009) Thermal adaptation: a theoretical and empirical synthesis (MJ Angilletta Jr., Ed.). Oxford University Press

Armstrong R, Lee C, Hedges J (2001) A new, mechanistic model for organic carbon fluxes in the ocean based on the quantitative association of POC with ballast minerals. Deep Sea Res … 49:219–236

Atkinson D, Ciotti BJ, Montagnes DJS (2003) Protists decrease in size linearly with temperature: ca. 2.5% degrees C(-1). Proc Biol Sci 270:2605–2611

Bach LT, Mackinder LCM, Schulz KG, Wheeler G, Schroeder DC, Brownlee C, Riebesell U (2013) Dissecting the impact of CO_2 and pH on the mechanisms of photosynthesis and calcification in the coccolithophore Emiliania huxleyi. New Phytol 199:121–34

Bach LT, Riebesell U, Georg Schulz K (2011) Distinguishing between the effects of ocean acidification and ocean carbonation in the coccolithophore Emiliania huxleyi. Limnol Oceanogr 56:2040–2050

Bach LT, Riebesell U, Sett S, Febiri S, Rzepka P, Schulz KG (2012) An approach for particle sinking velocity measurements in the 3-400 µm size range and considerations on the effect of temperature on sinking rates. Mar Biol 159:1853–1864

Bakker DCE, Pfeil B, Smith K, Hankin S, Olsen a., Alin SR, Cosca C, Harasawa S, Kozyr a., Nojiri Y, O'Brien KM, Schuster U, Telszewski M, Tilbrook B, Wada C, Akl J, Barbero L, Bates NR, Boutin J, Bozec Y, Cai WJ, Castle RD, Chavez FP, Chen L, Chierici M, Currie K, Baar HJW De, Evans W, Feely R a., Fransson a., Gao Z, Hales B, Hardman-Mountford NJ, Hoppema M, Huang WJ, Hunt CW, Huss B, Ichikawa T, Johannessen T, Jones EM, Jones SD, Jutterström S, Kitidis V, Körtzinger a., Landschützer P, Lauvset SK, Lefèvre N, Manke a. B, Mathis JT, Merlivat L, Metzl N, Murata a., Newberger T, Omar a. M, Ono T, Park GH, Paterson K, Pierrot D, Ríos a. F, Sabine CL, Saito S, Salisbury J, S. Sarma VVS, Schlitzer R, Sieger R, Skjelvan I, Steinhoff T, Sullivan KF, Sun H, Sutton a. J, Suzuki T, Sweeney C, Takahashi T, Tjiputra J, Tsurushima N, C. Van Heuven SM a, Vandemark D, Vlahos P, Wallace DWR, Wanninkhof R, Watson a. J (2014) An update to the surface ocean CO_2 atlas (SOCAT version 2). Earth Syst Sci Data 6:69–90

Behrenfeld MJ, O'Malley RT, Siegel D a, McClain CR, Sarmiento JL, Feldman GC, Milligan AJ, Falkowski PG, Letelier RM, Boss ES (2006) Climate-driven trends in contemporary ocean productivity. Nature 444:752–755

Benner I, Diner RE, Lefebvre SC, Li D, Komada T, Carpenter EJ, Stillman JH, B PTRS (2013) Emiliania huxleyi increases calcification but not expression of calcification-related genes in long-term exposure to elevated temperature and pCO. Philos Trans Biol Sci 368:1–17

Bopp L, Monfray P, Aumont O, Dufresne J-L, Treut H Le, Madec G, Terray L, Orr JC (2001) Potential impact of climate change on marine export production. Global Biogeochem Cycles 15:81

Boyce DG, Lewis MR, Worm B (2010) Global phytoplankton decline over the past century. Nature 466:591–596

Boyd PW, Rynearson T a, Armstrong E a, Fu F, Hayashi K, Hu Z, Hutchins D a, Kudela RM, Litchman E, Mulholland MR, Passow U, Strzepek RF, Whittaker K a, Yu E, Thomas MK (2013) Marine phytoplankton temperature versus growth responses from polar to tropical waters--outcome of

a scientific community-wide study. PLoS One 8:e63091

Brownlee C, Davies M, Nimer N, Dong LF, Merrett MJ (1995) Calcification, photosynthesis and intracellular regulation in Emiliania huxleyi. Bull Oceanogr MONACO-NUMERO Spec:19–36

Buitenhuis ET, Baar HJW de, Veldhuis MJW (1999) Photosynthesis and Calcification By Emiliania Huxleyi (Prymnesiophyceae) As a Function of Inorganic Carbon Species. J Phycol 35:949–959

Collins S, Bell G (2004) Phenotypic consequences of 1,000 generations of selection at elevated CO_2 in a green alga. Nature 431:566–9

Collins S, Meaux J de (2009) Adaptation to different rates of environmental change in Chlamydomonas. Evolution 63:2952–65

Crutzen PJ (2002) Geology of mankind. Nature 415:23

Dassow P von, Ogata H, Probert I, Wincker P, Silva C Da, Audic S, Claverie J-M, Vargas C de (2009) Transcriptome analysis of functional differentiation between haploid and diploid cells of Emiliania huxleyi, a globally significant photosynthetic calcifying cell. Genome Biol 10:R114

Daufresne M, Lengfellner K, Sommer U (2009) Global warming benefits the small in aquatic ecosystems. Proc Natl Acad Sci U S A 106:12788–12793

Davies J, Davies D (2010) Origins and evolution of antibiotic resistance. Microbiol Mol Biol Rev 74:417–433

Desai MM, Fisher DS, Murray AW (2007) The speed of evolution and maintenance of variation in asexual populations. Curr Biol 17:385–94

Dixon M (1953) The effect of pH on the affinities of enzymes for substrates and inhibitors. Biochem j 55:161–170

Doney SC, Fabry VJ, Feely R a, Kleypas J a (2009) Ocean acidification: the other CO_2 problem. Ann Rev Mar Sci 1:169–192

Dunham MJ, Badrane H, Ferea T, Adams J, Brown PO, Rosenzweig F, Botstein D (2002) Characteristic genome rearrangements in experimental evolution of Saccharomyces cerevisiae. Proc Natl Acad Sci U S A 99:16144–16149

Egge JK, Heimdal BR (1994) Blooms of phytoplankton including Emiliania huxleyi (Haptophyta): Effects of nutrient supply in different N:P ratios. Sarsia 79:333–348

Engel A, Szlosek J, Abramson L, Liu Z, Lee C (2009) Investigating the effect of ballasting by CaCO3 in Emiliania huxleyi: I. Formation, settling velocities and physical properties of aggregates. Deep Res Part II Top Stud Oceanogr 56:1396–1407

Falkner G, Horner F (1976) pH Changes in the Cytoplasm of the Blue-Green Alga Anacystis nidulans Caused by Light-dependent Proton Flux into the Thylakoid Space. Plant Physiol 58:717–718

Falkowski P (2012) Ocean Science: The power of plankton. Nature 483:S17–S20

Feng Y, Hare CE, Leblanc K, Rose JM, Zhang Y, DiTullio GR, Lee P a., Wilhelm SW, Rowe JM, Sun J, Nemcek N, Gueguen C, Passow U, Benner I, Brown C, Hutchins D a. (2009) Effects of increased pCO_2 and temperature on the north atlantic spring bloom. I. The phytoplankton community and

biogeochemical response. Mar Ecol Prog Ser 388:13–25

Field CB, Behrensfled MJ, Randerson JT, Falkowski P (1998) Primary Production of the Biosphere: Integrating Terrestrial and Oceanic Components. Science (80-) 281:237–240

Frada M, Probert I, Allen MJ, Wilson WH, Vargas C de (2008) The “Cheshire Cat” escape strategy of the coccolithophore Emiliania huxleyi in response to viral infection. Proc Natl Acad Sci U S A 105:15944–9

Garland T, Kelly S a (2006) Phenotypic plasticity and experimental evolution. J Exp Biol 209:2344–2361

Hoegh-Guldberg O, Mumby PJ, Hooten a J, Steneck RS, Greenfield P, Gomez E, Harvell CD, Sale PF, Edwards a J, Caldeira K, Knowlton N, Eakin CM, Iglesias-Prieto R, Muthiga N, Bradbury RH, Dubi A, Hatziolos ME (2007) Coral reefs under rapid climate change and ocean acidification. Science 318:1737–42

Houghton R a. (2007) Balancing the Global Carbon Budget. Annu Rev Earth Planet Sci 35:313–347

Howarth CJ, Ougham HJ (1993) Gene expression under temperature stress [Review]. New Phytol 125:1–26

Iglesias-Rodriguez DM, Schofield OM, Batley J, Medlin LK, Hayes PK (2006) Intraspecific genetic diversity in the marine coccolithophore Emiliania huxleyi (Prymnesiophyceae): The use of microsatellite analysis in marine phytoplankton population studies. J Phycol 42:526–536

IPCC (2014) Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change (RK Pachauri, L Meyer, J-P Van Ypersele, S Brinkman, L Van Kesteren, N Leprince-Ringuet, and F Van Boxmeer, Eds.). IPCC

Jin P, Gao K, Beardall J (2013) Evolutionary Responses Of A Coccolithophorid Gephyrocapsa Oceanica To Ocean Acidification. Evolution (N Y) 67:1869–1878

Jin X, Gruber N, Dunne JP, Sarmiento JL, Armstrong RA (2006) Diagnosing the contribution of phytoplankton functional groups to the production and export of particulate organic carbon, CaCO 3 , and opal from global nutrient and alkalinity distributions. Global Biogeochem Cycles 20:n/a–n/a

Kaltz O, Bell G (2002) The ecology and genetics of fitness in Chlamydomonas. XII. Repeated sexual episodes increase rates of adaptation to novel environments. Evolution 56:1743–1753

Kassen R (2002) The experimental evolution of specialists, generalists, and the\rmaintenance of diversity. J Evol Biol 15:173–190

Keightley PD, Lynch M (2003) Toward a realistic model of mutations affecting fitness. Evolution 57:683–685; discussion 686–689

Kimura M, Maruyama T (1966) The mutational load with epistatic gene interactions in fitness. Genetics 54:1337–1351

Krueger-Hadfield S a., Balestreri C, Schroeder J, Highfield a., Helaouët P, Allum J, Moate R, Lohbeck KT, Miller PI, Riebesell U, Reusch TBH, Rickaby REM, Young J, Hallegraeff G, Brownlee C,

Schroeder DC (2014) Genotyping an Emiliania huxleyi (Prymnesiophyceae) bloom event in the North Sea reveals evidence of asexual reproduction. Biogeosciences Discuss 11:4359–4408

Langer G, Nehrke G, Probert I, Ly J, Ziveri P (2009) Strain-specific responses of *Emiliania huxleyi* to changing seawater carbonate chemistry. Biogeosciences 6:2637–2646

Law RD, Crafts-Brandner SJ (1999) Inhibition and Acclimation of Photosynthesis to Heat Stress Is Closely Correlated with Activation of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase. Plant Physiol 120:173–182

Lenski R (2004) Phenotypic and genomic evolution during a 20,000-generation experiment with the bacterium Escherichia coli. Plant Breed Rev 24:225–265

Lenski RE, Rose MR, Simpson SC, Tadler SC (1991) Long-Term Experimental Evolution in Escherichia coli. I. Adaptation and Divergence During 2,000 Generations. Am Nat 138:1315

Levitus S, Antonov J, Boyer T (2005) Warming of the world ocean, 1955-2003. Geophys Res Lett 32:1–4

Litchman E, Klausmeier C a., Schofield OM, Falkowski PG (2007) The role of functional traits and trade-offs in structuring phytoplankton communities: Scaling from cellular to ecosystem level. Ecol Lett 10:1170–1181

Lohbeck KT, Riebesell U, Collins S, Reusch TBH (2013) Functional genetic divergence in high CO_2 adapted Emiliania huxleyi populations. Evolution 67:1892–900

Lohbeck KT, Riebesell U, Reusch TBH (2012) Adaptive evolution of a key phytoplankton species to ocean acidification. Nat Geosci 5:346–351

Lohbeck KT, Riebesell U, Reusch TBH (2014) Gene expression changes in the coccolithophore Emiliania huxleyi after 500 generations of selection to ocean acidification. Proc R Soc B

Mackinder L, Wheeler G, Schroeder D, Riebesell U, Brownlee C (2010) Molecular Mechanisms Underlying Calcification in Coccolithophores. Geomicrobiol J 27:585–595

Meinshausen M, Smith SJ, Calvin K, Daniel JS, Kainuma MLT, Lamarque J, Matsumoto K, Montzka S a., Raper SCB, Riahi K, Thomson a., Velders GJM, Vuuren DPP van (2011) The RCP greenhouse gas concentrations and their extensions from 1765 to 2300. Clim Change 109:213–241

Mitchell JFB (1989) The “Greenhouse” effect and climate change. Rev Geophys 27:115

Monnin E, Indermühle A, Dällenbach A, Flückiger J, Stauffer B, Stocker TF, Raynaud D, Barnola JM (2001) Atmospheric CO_2 concentrations over the last glacial termination. Science 291:112–114

Morán XAG, López-Urrutia Á, Calvo-Díaz A, LI WKW (2010) Increasing importance of small phytoplankton in a warmer ocean. Glob Chang Biol 16:1137–1144

Nei M, Suzuki Y, Nozawa M (2010) The neutral theory of molecular evolution in the genomic era. Annu Rev Genomics Hum Genet 11:265–289

Oz T, Guvenek A, Yildiz S, Karaboga E, Tamer YT, Mumcuyan N, Ozan VB, Senturk GH, Cokol M, Yeh P, Toprak E (2014) Strength of Selection Pressure Is an Important Parameter Contributing to the Complexity of Antibiotic Resistance Evolution. Mol Biol Evol 31:2387–2401

Paasche E (1968) Biology and physiology of coccolithophorids. Annu Rev Microbiol 22:71–86

Parmesan C, Ryrholm N, Stefanescu C, Hill JK, Thomas CD, Descimon H, Huntley B, Kaila L, Kullberg J, Tammaru T, Tennent WJ, Thomas JA, Warren M (1999) Poleward shifts in geographical ranges of butterfly species associated with regional warming. Nature 399:579–583

Parmesan C, Yohe G (2003) A globally coherent fingerprint of climate change impacts across natural systems. Nature 421:37–42

Perry a L, Low PJ, Ellis JR, Reynolds JD (2005) Climate change and distribution shifts in marine fishes. Science (80-) 308:1912–1915

Puerta MVS, Bachvaroff TR, Delwiche CF (2005) The complete plastid genome sequence of the haptophyte Emiliania huxleyi: A comparison to other plastid genomes. DNA Res 12:151–156

Rajanandhini K, Santhanam P, Jeyanthi S, Shenbaga Devi A, Dinesh Kumar S, Balaji Prasath B (2014) Impact of CO_2 Fertilization on Growth and Biomass in Marine Diatom Nitzschia SP. Int J Earth Sci Eng 7:1049–1054

Raven J, Crawfurd K (2012) Environmental controls on coccolithophore calcification. Mar Ecol Prog Ser 470:137–166

Redfield A (1958) The biological control of chemical factors in the environment. Am Sci 46:205–221

Reusch TBH (2014) Climate change in the oceans: Evolutionary versus phenotypically plastic responses of marine animals and plants. Evol Appl 7:104–122

Reusch TBH, Boyd PW (2013) Experimental evolution meets marine phytoplankton. Evolution 67:1849–59

Rice WR (2002) Experimental tests of the adaptive significance of sexual recombination. Nat Rev Genet 3:241–251

Riebesell U, Körtzinger A, Oschlies A (2009) Sensitivities of marine carbon fluxes to ocean change. Proc Natl Acad Sci U S A 106:20602–9

Riebesell U, Tortell PD (2011) Effects of ocean acidification on pelagic organisms and ecosystems BT - Ocean Acidification. In: Ocean Acidification. OUP Oxford, p 99–121

Riebesell U, Zondervan I, Rost B, Tortell P (2000) Reduced calcification of marine plankton in response to increased atmospheric CO_2. Nature 407:2–5

Rost B, Riebesell U (2004) Coccolithophores and the biological pump: Responses to environmental changes. In: Coccolithophores. Springer, p 99–125

Salvucci ME, Crafts-Brandner SJ (2004) Inhibition of photosynthesis by heat stress: The activation state of Rubisco as a limiting factor in photosynthesis. Physiol Plant 120:179–186

Schaum E, Collins S (2014) Plasticity predicts evolution in a marine alga. Proc R Soc B 281

Schaum C-E, Rost B, Collins S (2015) Environmental stability affects phenotypic evolution in a globally distributed marine picoplankton. ISME J:1–10

Schaum E, Rost B, Millar AJ, Collins S (2013) Variation in plastic responses of a globally distributed picoplankton species to ocean acidification. Nat Clim Chang 3:298–302

Scheiner S (1993) Genetics and evolution of phenotypic plasticity. Annu Rev Ecol Syst 24:35–68

Scheinin M, Riebesell U, Rynearson TA, Lohbeck KT, Collins S (2015) Experimental evolution gone wild. :1–5

Schlüter L, Lohbeck KT, Gutowska M a., Gröger JP, Riebesell U, Reusch TBH (2014) Adaptation of a globally important coccolithophore to ocean warming and acidification. Nat Clim Chang 4:1024–1030

Sett S, Bach LT, Schulz KG, Koch-Klavsen S, Lebrato M, Riebesell U (2014) Temperature modulates coccolithophorid sensitivity of growth, photosynthesis and calcification to increasing seawater pCO_2. PLoS One 9:e88308

Shaw RG, Etterson JR (2012) Tansley review Rapid climate change and the rate of adaptation : insight from experimental quantitative genetics. New Phytol

Sniegowski PD, Gerrish PJ, Lenski RE (1997) Evolution of high mutation rates in experimental populations of E. coli. Nature 387:703–705

Steinacher M, Joos F, Frölicher TL, Bopp L, Cadule P, Doney SC, Gehlen M, Schneider B, Segschneider J (2009) Projected 21st century decrease in marine productivity: a multi-model analysis. Biogeosciences Discuss 6:7933–7981

Suffrian K, Schulz KG, Gutowska MA, Riebesell U, Bleich M (2011) Cellular pH measurements in Emiliania huxleyi reveal pronounced membrane proton permeability. :595–608

Sunday JM, Calosi P, Dupont S, Munday PL, Stillman JH, Reusch TBH (2014) Evolution in an acidifying ocean. Trends Ecol Evol 29:117–125

Tang EPY (1995) The allometry of algal growth rates. J Plankton Res 17:1325–1335

Thomas MK, Kremer CT, Klausmeier C a, Litchman E (2012) A global pattern of thermal adaptation in marine phytoplankton. Science 338:1085–8

Toseland A, Daines SJ, Clark JR, Kirkham A, Strauss J, Uhlig C, Lenton TM, Valentin K, Pearson G a., Moulton V, Mock T (2013) The impact of temperature on marine phytoplankton resource allocation and metabolism. Nat Clim Chang 3:979–984

Townsend DW, Keller MD, Holligan PM, Ackleson SG, Balch WM (1994) Blooms of the coccolithophore Emiliania huxleyi with respect to hydrography in the Gulf of Maine. Cont Shelf Res 14:979–1000

Tyrrell T, Merico A (2004) Emiliania huxleyi: bloom observation and the conditions that induce them. In: Thierstein HR, Young JR (eds) Coccolithophores: From Molecular Processes to Global Impact. Springer, p 585–604

Verdy A, Follows M, Flierl G (2009) Optimal phytoplankton cell size in an allometric model. Mar Ecol Prog Ser 379:1–12

Verity PG, Robertron CY, Tronzo CR, Andrews MG, Nelson JR, Sieracki ME (1992) Relationships between cell volume and the carbon and nitrogen content of marine photosynthetic nanoplankton. Limnol Oceanogr 37:1434–1446

Wal P van der, Jong EW de, Westbroek P, Bruijn WC de, Mulder-Stapel a a (1983) Polysaccharide

localization, coccolith formation, and Golgi dynamics in the coccolithophorid Hymenomonas carterae. J Ultrastruct Res 85:139–58

Wang W, Vinocur B, Shoseyov O, Altman A (2004) Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. Trends Plant Sci 9:244–252

Weinreich DM, Chao L (2005) Rapid evolutionary escape by large populations from local fitness peaks is likely in nature. Evolution 59:1175–1182

Weinreich DM, Watson R a, Chao L (2005) Perspective: Sign epistasis and genetic constraint on evolutionary trajectories. Evolution 59:1165–74

Westbroek P, Brown CW, Bleijswijk J van, Brownlee C, Brummer GJ, Conte M, Egge J, Fernández E, Jordan R, Knappertsbusch M, Stefels J, Veldhuis M, Wal P van der, Young J (1993) A model system approach to biological climate forcing. The example of Emiliania huxleyi. Glob Planet Change 8:27–46

Westbroek P, Young JR, Linschooten K (1989) Coccolith production (biomineralization) in the marine alga Emiliania huxleyi. J Protozool 36:368–373

Winter A, Henderiks J, Beaufort L, Rickaby REM, Brown CW (2014) Poleward expansion of the coccolithophore Emiliania huxleyi. J Plankton Res 36:316–325

Wu Y, Gao K, Riebesell U (2010) CO_2-induced seawater acidification affects physiological performance of the marine diatom Phaeodactylum tricornutum. Biogeosciences 7:2915–2923

Young J, Davis S, Bown P, Mann S (1999) Coccolith ultrastructure and biomineralisation. J Struct Biol 126:195–215

Young JR, Geisen M, Cros L, Kleijne A, Sprengel C, Probert I, Ostergaard JB (2003) A guide to extant coccolithophore taxonomy. International Nanoplankton Association, London

Zhang Y, Klapper R, Lohbeck KT, Bach LT, Schulz KG, Reusch TBH, Riebesell U (2014) Between- and within-population variations in thermal reaction norms of the coccolithophore Emiliania huxleyi. Limnol Oceanogr 59:1570–1580

Appendix:

Supplementary Material Publication I

Title:

Long-term dynamics of adaptive evolution in a globally important coccolithophore to ocean acidification

Authors:
Lothar Schlüter, Kai T. Lohbeck, Joachim P. Gröger, Ulf Riebesell, and Thorsten B. H. Reusch

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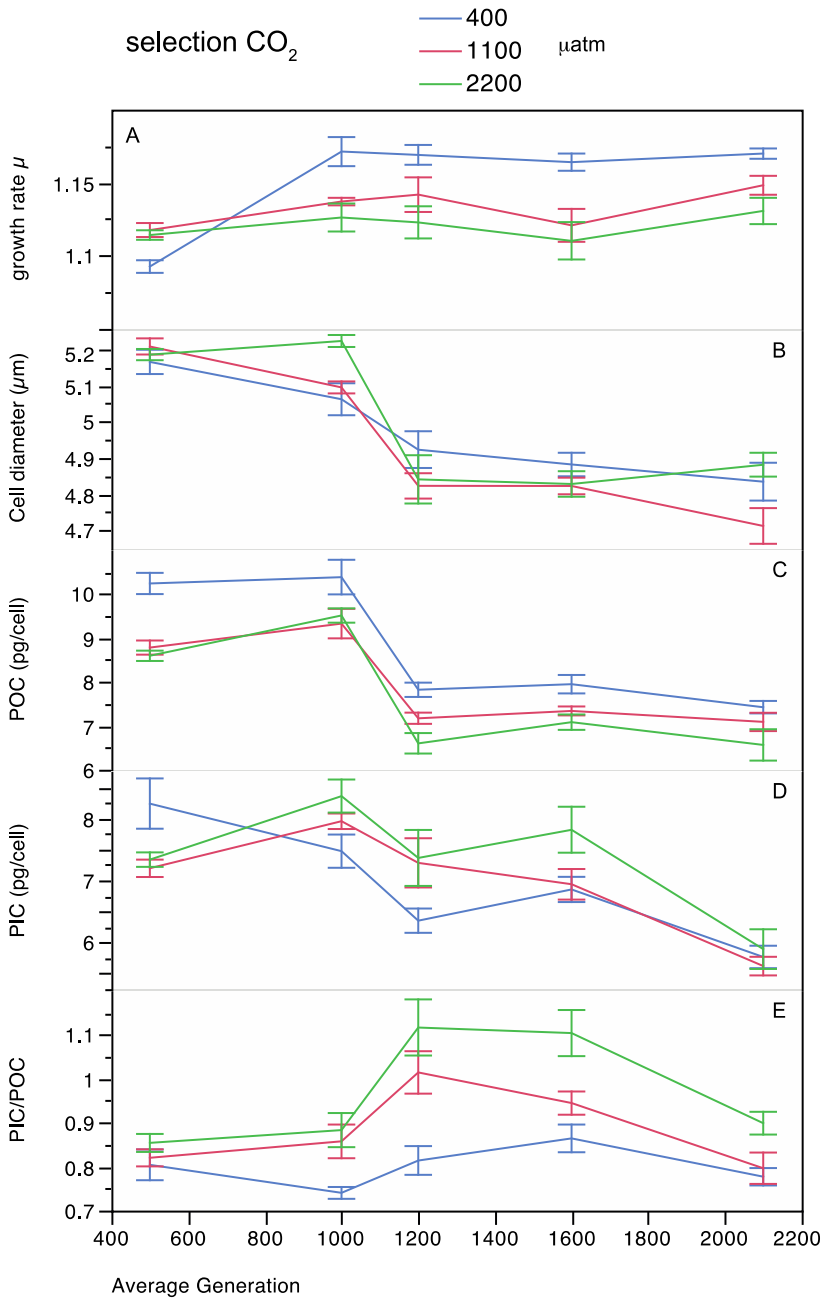
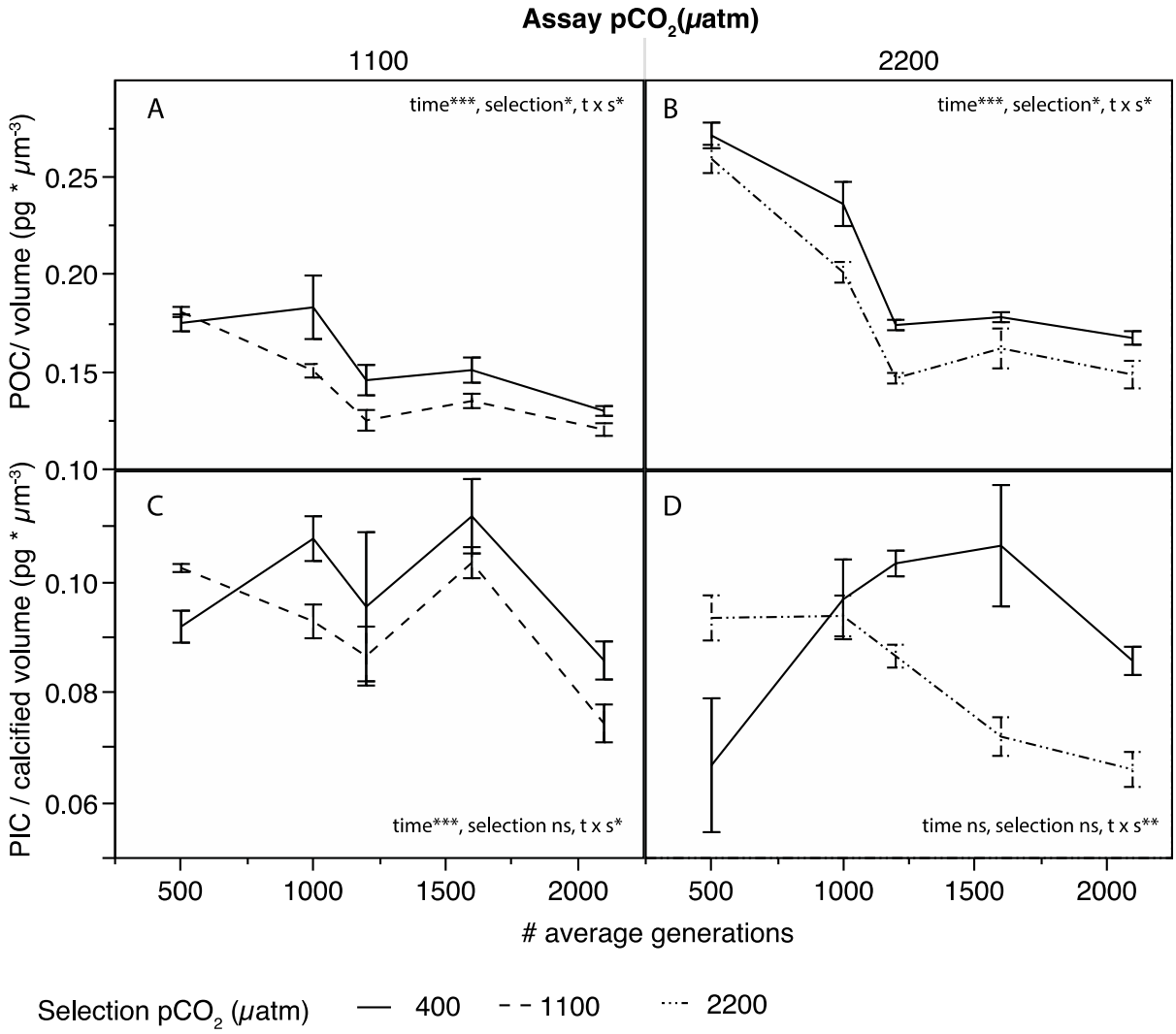


Supplementary Figure S2 (overleaf). Correlated response in replicated *Emiliania huxleyi* populations during 4 years of selection under three CO_2 levels simulating ocean acidification. Depicted is the response (mean±1SE, *n*=5) of medium- and high-CO_2 adapted vs. non-adapted (=control) populations in the ambient CO_2 condition (400 µatm) measured during assay experiments at 5 time points (x-axis, average generations), (A) exponential growth rate (B) cell diameter (C) particulate organic carbon per cell (POC $cell^{-1}$) (D) particulate inorganic carbon per cell (PIC $cell^{-1}$) (E) ratio of PIC:POC).



Supplementary Figure S3. Evolutionary response of *Emiliania huxleyi* to selection under three CO_2 conditions, simulating ocean acidification. Depicted is the adaptive response measured during assay experiments at 5 time points (x-axis, average generations), always comparing medium- (left) and high-CO_2 adapted (right) vs. non-adapted populations of *E. huxleyi* in three different CO_2 environments, when assayed under elevated CO_2 (mean±SD, n=5). (A,B) POC standardized by cell volume (C;D) PIC standardized by cell volume Significant results of main and interaction effects are depicted with asterisks (*0.05≥*P*>0.01, **0.01≥*P*>0.001, ***P<0.001). Complete repeated measures ANOVA results are given in Table S1.

Supplementary Table S1: Statistical analysis of the adaptive response in exponential growth rate and other cell traits of *Emiliania huxleyi* to elevated CO_2 under two different CO_2-levels as repeated measures ANOVA (rmANOVA) with univariate and sphericity tests. *p*CO_2-selection is a between-subjects effect, CO_2 x time and time are within subject effects. For particulate inorganic carbon (PIC) additionally the correlated response was analyzed.

response/trait	Assay level	*p*CO_2 selection	time	CO_2 selection x time
growth rate	medium	$F_{1,8}$=43.92 p=0.0002	$F_{4,32}$=92.46 p<0.0001	$F_{4,32}$=2.941 p=0.0354
	high	$F_{1,8}$ = 53.72 p<0.0001	$F_{4,32}$=47.58 p<0.0001	$F_{4,32}$=1.991 ns
cell size	medium	$F_{1,8}$=0.762 ns	$F_{4,32}$=46.40 p<0.0001	$F_{4,32}$=11.44 p<0.0001
	high	$F_{1,8}$=0.0741 ns	$F_{4,32}$=61.83 p<0.0001	$F_{4,32}$=10.89 p<0.0001
particular organic carbon POC (pg/cell)	medium	$F_{1,7}$=2.590 P=0.0038	$F_{4,28}$=56.55 p<0.0001	$F_{4,28}$=4.68 p<0. 0051
	high	$F_{1,6}$=2.908 p=0.0058	$F_{4,24}$=70.76 p<0.0001	$F_{4,24}$=0.897 ns
particular inorganic carbon PIC (pg/cell)	medium	$F_{1,7}$=6.534 p=0.0378	$F_{4,28}$=17.749 p<0.0001	$F_{4,28}$=4.852 p=0.0042
	high	$F_{1,6}$=3.171 ns	$F_{4,24}$=5.345 p=0.0032	$F_{4,24}$=6.798 p=0.0008
PIC:POC ratio	medium	$F_{1,7}$=0.121 ns	$F_{4,28}$=1.000 p<0.0001	$F_{4,28}$=0,3378 ns
	high	$F_{1,6}$=0.500 ns	$F_{4,24}$=14.72 p<0.0001	$F_{4,24}$=6.356 p=0.0012
POC per cell volume	medium	$F_{1,7}$=6.471 p=0.0384	$F_{4,28}$=26.46 p<0.0001	$F_{4,28}$=3.444 p=0.0207
	high	$F_{1,6}$=12.42 p=0.0124	$F_{4,24}$=88.8577 p<0.0001	$F_{4,24}$=1.932 ns
PIC per cell volume	medium	$F_{1,7}$=4.736 p=0.066	$F_{4,28}$=14.08 p<0.0001	$F_{4,28}$=3.954 p=0.0115
	high	$F_{1,6}$=2.754 ns	$F_{4,24}$=2.349 ns	$F_{4,24}$=5.585 p=0.0025
PIC per cell correlated response	medium	$F_{1,7}$=0.311 ns	$F_{4,28}$=19.46 p<0.0001	$F_{4,28}$=3.797 p=0.0137
	high	$F_{1,6}$=3.091 ns	$F_{4,24}$=14.10 p<0.0001	$F_{4,24}$=2.460 p=0.0727

Supplementary Table S2: Statistical analysis of the reciprocal assay experiment after 4 yrs (average asexual generations = 2,100) to assess the adaptive and correlated response of *Emiliania huxleyi* to elevated CO_2. Evolutionary adaptation to two different CO_2-levels was analyzed as two separate 2x2 factorial ANOVA (see Fig. 2 for graphical depiction of treatment means). Medium OA treatment = 1,100 µatm pCO_2, high = 2,200 µatm pCO_2.

response/trait	OA treatment (sub-experiment)	CO_2 selection	Assay condition	CO_2 selection x assay condition
Daily growth rate µ	medium	$F_{1,16}$=1.37 ns	$F_{1,16}$=41.18 p<0.001	$F_{1,16}$=20.60 P<0.001
	high	$F_{1,16}$=0.3 ns	$F_{1,16}$=242.9 p<0.001	$F_{1,16}$=25.99 P<0.001
particular organic carbon POC (pg/cell)	medium	$F_{1,16}$=21.26 p<0.001	$F_{1,16}$=12.95 P=0.002	$F_{1,16}$=8.212 P=0.011
	high	$F_{1,16}$=18.18 p<0.001	$F_{1,16}$=57.69 p<0.001	$F_{1,16}$=1.802 ns
particular inorganic carbon PIC (pg/cell)	medium	$F_{1,16}$=9.862 p=0.0063	$F_{1,16}$=7.344 P=0.016	$F_{1,16}$=6.073 P=0.025
	high	$F_{1,16}$=8.322 P=0.011	$F_{1,16}$=38.96 p<0.001	$F_{1,16}$=11.88 P=0.0033
PIC:POC ratio	medium	$F_{1,16}$=0.150 ns	$F_{1,16}$=21.67 p<0.001	$F_{1,16}$=1.003 ns
	high	$F_{1,16}$=1.930 ns	$F_{1,16}$=299.2 p<0.001	$F_{1,16}$=20.20 P<0.001

Supplementary Material Publication II

Title:

Adaptation of a globally important coccolithophore to ocean warming and acidifications

Authors:
Lothar Schlüter, Kai T. Lohbeck, Magdalena A. Gutowska, Joachim P. Gröger, Ulf Riebesell, and Thorsten B. H. Reusch

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"Adaptation of a globally important coccolithophore to ocean warming and acidification"

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Full Material & Methods

The *Emiliania huxleyi* populations used in this experiment were ultimately derived from a mono-clonal culture isolated from a natural *E. huxleyi* bloom in Raunefjorden near Bergen, Norway in May 2009 (clone #62, ref 1). Cultures were unialgal but not axenic, since *Emiliania huxleyi* does not grow properly without associated bacteria. Regular inspection via flow cytometry detected no measurable abundance of free-living bacteria in the cultures. Clonal lineages were split into 15 independent asexually propagating populations that were kept separately under defined CO_2 selection conditions for ≈1,500 asexual generations prior to the temperature adaptation experiment. Populations were maintained in artificial seawater (ASW, ref 2) that was sterile filtered (Whatman Polycap 75AS) and supplemented with 64 µmol kg^{-1} nitrate and 4 µmol kg^{-1} phosphate (ratio after Redfield[3]), trace metals and vitamins were added consistent to f/8 (ref 4) and selenium to a final concentration of 10 nmol kg^{-1} (ref 5). Two ml kg-1 sterile filtered North Sea water were added to exclude any limitations by micronutrients. To achieve a total alkalinity (TA) of 2380 µmol kg^{-1}, 19.99 g per kg ASW bicarbonate were added.

Replicate populations were grown in 250 ml Schott Duran flasks, carefully filled with a minimum headspace to a volume of approximately 310 ml to avoid gas exchange. The flasks were stored in the dark at 15°C before inoculation. In addition to the culture flasks two flasks were prepared for dissolved inorganic carbon (DIC) and total alkanility (TA) measurements. DIC samples were taken for all CO_2 and temperature levels every 10 batch cycles and measured colorimetrically[6] using an AIRICA system (Marianda). CO_2 partial pressure values before inoculation were calculated from DIC and TA using the program CO2SYS (ref 7), with the solubility constants after Ref [8]. The draw-down of TA and DIC during the batch cycles were calculated from the total particular carbon measurements[7]. CO_2 levels were manipulated by aerating the ASW medium for 24 h at the respective treatment temperatures (15.0°C and 26.3°C) under saturated humidity[8]. Regular DIC and TA measurements at 10 time points revealed that the mean medium CO_2 treatment was 200-300 µatm above the desired treatment level, thus 1400 instead of 1100. Measured CO_2 partial pressures (in µatm±1SD for cold / warm temperature, respectively) were 388±7 and 416±9 (ambient), 1329±177 and 1591±248 (medium) and 2253±30 and 2136±56 (high pCO_2).

The cultures were continuously rotated (0.5 min^{-1}) in two identical Sanyo MLR-351 light cabinets (one at 15.0°C and one at 26.3°C) at a photon flux density of 150±15 µmol m^{-2} s^{-1} under a 16:8 light:dark cycle. ASW media preparation and all culture work were conducted with sterilized equipment under a clean bench.

Temperature selection was initiated on 8 February 2013 and lasted for 73 batch cycles (1 yr), corresponding to ~478 (ambient), ~459 (medium), and ~440 (high CO_2) generations, ~437

generations in the simultaneous selection of high temperature / high CO_2. Replicated selection lines where split in order to raise the temperature to 26.3°C (1°C d^{-1}) in one group while maintaining CO_2 selection at 15.0°C in the other group. This temperature is close to the upper thermal threshold for growth of *E. huxleyi* populations from Bergen and was chosen in order to achieve approximately similar growth rates in the low and high temperature treatment. The chosen temperature of 26.3°C is at the upper thermal tolerance for *Emiliania huxleyi*. Pilot experiments revealed that already at 27°C, thus 0.7°C warmer, cultures almost stop growing (Supplementary Table S1). The batch cycles were reduced to four days, adjusted to the higher growth rates during temperature transition. During temperature acclimation ASW medium was prepared separately and aerated under intermediate temperature conditions. In a fourth warm-temperature treatment, the effect of simultaneous selection to pCO_2 (400 ->2200 µatm) and temperature was tested. This treatment was based on duplicates of the populations kept for 1500 generations at 400 µatm pCO_2 (2200_{simult}; Supplementary Fig. S1).

During the selection phase 10^5 cells were transferred into fresh medium every five days to initiate the next batch cycle. The cell density and diameter were measured in triplicate at every transfer point using a Beckman Coulter Z2 Particle and Size Analyzer. The cell counts were always performed three hours after the onset of the light phase. Exponential growth rates (µ) were calculated from cell densities according to

$$\mu = (\ln N_d - \ln N_0)/d \quad (1)$$

where $N_{(0.d)}$ are cell numbers, and d the duration of the batch cycle in days (d=5).

For the assay experiment, after ~478 ambient, ~459 medium, ~440 high and ~437 simultaneous asexual generations, the temperature transition was performed exactly as in the initial phase, transferring replicates from 26.3°C back to 15.0°C and vice versa. Populations evolving under 400 µatm pCO_2 and 15.0°C were challenged in an additional assay treatment with both stressors simultaneously. This treatment served as non-adapted control for the 26.3°C / high CO_2 selection populations (Supplementary Fig. S1). Microscopic inspection revealed that one high-temperature selected replicate at 2200 µatm contained small numbers of heterotrophic nanoflagellates (<1% of cell number). If excluded, none of the statistical results changed qualitatively, hence this values was kept in the data set.

In order to exclude phenotypic plasticity in the assay experiment we included an acclimatisation phase of ten days and measured response variables within the reciprocal exposure over 20 additional days (4 batch cycles). Growth rates and other response variables presented in this paper were obtained in the last (5^{th}) batch cycle. We measured cell density, cell diameter, total particular carbon

(TPC), particular organic carbon (POC) and particular organic nitrogen (PON). In addition to measurements of calcified cell size, we decalcified cells with 10mM EDTA adjusted to pH 8.2 and repeated the size measurements within five minutes of EDTA addition. We used 90 and 180 ml culture suspension for the quantification of TPC and POC, respectively, which were vacuum filtered (<100 mbar) onto pre-combusted Whatman glass fibre filters (GF/F). To prevent artefacts due to intrinsic dial cycling, all filtrations were performed at the same time of the day, four hours after lights on. All sampling was completed within approximately one hour. To avoid carbon contamination all filters were handled with carbon free (combusted petri-dishes, acetone cleaned forceps) devices and immediately stored at -20°C until further processing. After thawing, filters for POC-analysis were fumed with 37% HCl to remove all inorganic carbon. All filters were dried at 60°C for 12 h, rolled and put into tin cups (HEKAtech). Carbon and Nitrogen content were determined using a Euro EA elemental analyser (HEKAtech).

Statistical analysis

We performed a trend analysis with the mean values of the 7 selection treatments because we were specifically interested in differences in the time trends (i.e. slopes) rather than response level differences. Classical analysis tool of an ANOVA cannot be used because the response variables (growth rate) were auto-correlated. We thus evaluated the data in the context of a Linear Dynamic model. Given this, we estimated a series of Autoregressive Moving Average Models with exogenous variables (ARMAX models. transfer functions). The exogenous variable here is an extra input variable, reflecting the time in terms of weeks to indicate a potential linear trend arising from global warming. In this framework, two samples can be compared in a unified setting by introducing binary indicators (dummy variables) d_i in {0.1}: With $d_1\ t_i = 1$ if observation i at time t belongs to sample 1 and $d_1\ t_i = 0$ else. d_2 ti =1- ($d_1\ t_i$) indicates whether (d_2 ti = 1) or not (d_2 ti = 0) the observation belongs to sample 2. With these indicators several hypotheses can be tested in following regression equation

$$\text{Response} = b_0 + b_1 * d_1 * \text{time} + b_2 * d_2 * \text{time} + b_3 * d_1 + \text{ARMA terms} \quad (2)$$

using conventional *t*- and *F*-tests. For instance, H0: $b_1 = b_2 = b_3 = 0$ denotes no effects (i.e. compliance with the controls). H0: $b_3 = 0$ indicates same response levels in both of the samples and H0: $b_2 = b_3$ says that global warming has the same effect in both samples. Note that according to (equ 2) dynamic effects are assumed to be the same in both of the samples. This assumption is justified theoretically due to the unified experimental design and is empirically corroborated by preliminary empirical tests. There was no evidence for asymptotic behaviour of growth rates over time during the experimental duration. This was tested via a transformation of the model to the log-scale under the ARIMA setup using log-transformed data on both sides (response, time) which would reveal a better fit when asymptotic. No improvement became evident when compared with the findings

based on the original data series, i.e. the quality-of-fit criteria were worse in case of the power model, implying no indication of asymptotic behavior.

For the statistical analysis of the reciprocal assay experiment the dataset was subdivided into two parts to answer the general temperature adaptation and to answer differences between sequential and simultaneous adaptation. A univariate 4-way analysis of variance (ANOVA) was performed using JMP v. 9.0 (Statsoft Inc.) with selection temperature, assay temperature, assay pCO_2 and #batch cycle as factors to check for stability of the trait over time. No significant 4-way interaction could be detected. Hence, we focus on data from the fourth batch cycle. Single and interactive effects of selection /assay temperature and CO_2 environment were analyzed by a 3-way factorial analysis of variance with subsequent planned contrasts if the evolution treatment was significant alone or in an interaction. We inspected residuals visually for normal distribution and tested for homogeneity of variances using Cochran's and Levene's test at α=0.05 (ref 9). If appropriate, variables were transformed to achieve variance homogeneity. For growth rate, this was not successful. Here, we tested for general differences among treatment means using Welch 1-way-ANOVA allowing for unequal variance. We nevertheless proceeded with conventional ANOVA using above factorial structure that permits the calculation of factor interactions, since parametric ANOVA is robust against the violation of assumptions if designs are balanced[10]. However, for growth rate, the essential contrast between temperature-adapted and non-temperature adapted populations was confirmed with a Wilcoxon Rank test. Detailed statistical results are provided in Table S5.

Relative fitness increases owing to adaptation were computed as quotient of the exponential growth rates of the adapted treatment[11], relative to the non-adapted one $W_{ac} = \mu_a/\mu_c$. For the statistical test using one-way ANOVA (cf. Fig 4b), random pairs within the following adapted /non-adapted treatments were formed, always assayed in the evolution environment: CO_2 selection (always 2200 µatm) vs. control (from ref 1); warming selection vs. control; sequential CO_2 and warming selection vs. control; and simultaneous CO_2 /warming selection vs. control.

References full material and methods

[1] Lohbeck, K. T., Riebesell, U. & Reusch, T. B. H. Adaptive evolution of a key phytoplankton species to ocean acidification. *Nature Geoscience* **5**, 346-351 (2012).

[2] Kester. D. R.. Duedall. I. W.. Connors. D. N. & Pytkowicz. R. M. Preparation of artificial seawater. *Limnology and Oceanography* **12**, 176-179 (1967).

[3] Redfield. A. C.. Ketchum. B. H. & Richards. F. A. in *The Sea Vol.2* (ed M.N. Hill), pp 26-77 (Wiley 1963).

[4] Guillard. R. R. L. Studies of marine planktonic diatoms: I. *Cyclotella nana* Hustedt. and *Detonula confervacea* (Cleve) Gran. *Canadian Journal of Microbiology* **8**, 229-239 (1962).

[5] Danbara A. & Shiraiwa Y. The requirement of selenium for the growth of marine coccolithophorids. *Emiliania huxleyi*. *Gephyrocapsa oceanica* and *Helladosphaera sp* (Prymnesiophyceae). *Plant and Cell Physiology* **40**, 762-766 (1999).

[6] Dickson. A. G.. Sabine. C. L. & Christian. J. R. *Guide to best practices for ocean CO_2 measurements* Vol. 3. 191pp. (PICES Special Publication, 2007).

[7] Lewis. E. & Wallace. D. W. R. *Program Developed for CO_2 System Calculations*. Vol. ORNL/CDIAC-105. Carbon Dioxide Information Analysis Center (Oak Ridge National Laboratory. U.S. Department of Energy, Oak Ridge, Tennessee, 1998).

[8] Roy. R. N. *et al.* The dissociation constants of carbonic acid in seawater at salinities 5 to 45 and temperatures 0 to 45° C. *Marine Chemistry* **44**, 249-267 (1993).

[9] Winer. J. T. *Statistical principles in experimental design*. (McGraw-Hill, Kogashua, 2nd edition, 1971).

[10] Underwood. A. J. *Experiments in ecology* (Cambridge University Press, 1997).

[11] Lenski, R., Rose, M., Simpson, S. & Tadler, S. Long-Term Experimental Evolution in Escherichia coli. I. Adaptation and Divergence During 2,000 Generations. The American Naturalist 138, 1315-1341 (1991).

Fig. S1. Design and treatment structure of the selection phases and the assay experiment. Bottle colors depict the two different experimental temperatures (15.0°C blue, 26.3°C red).

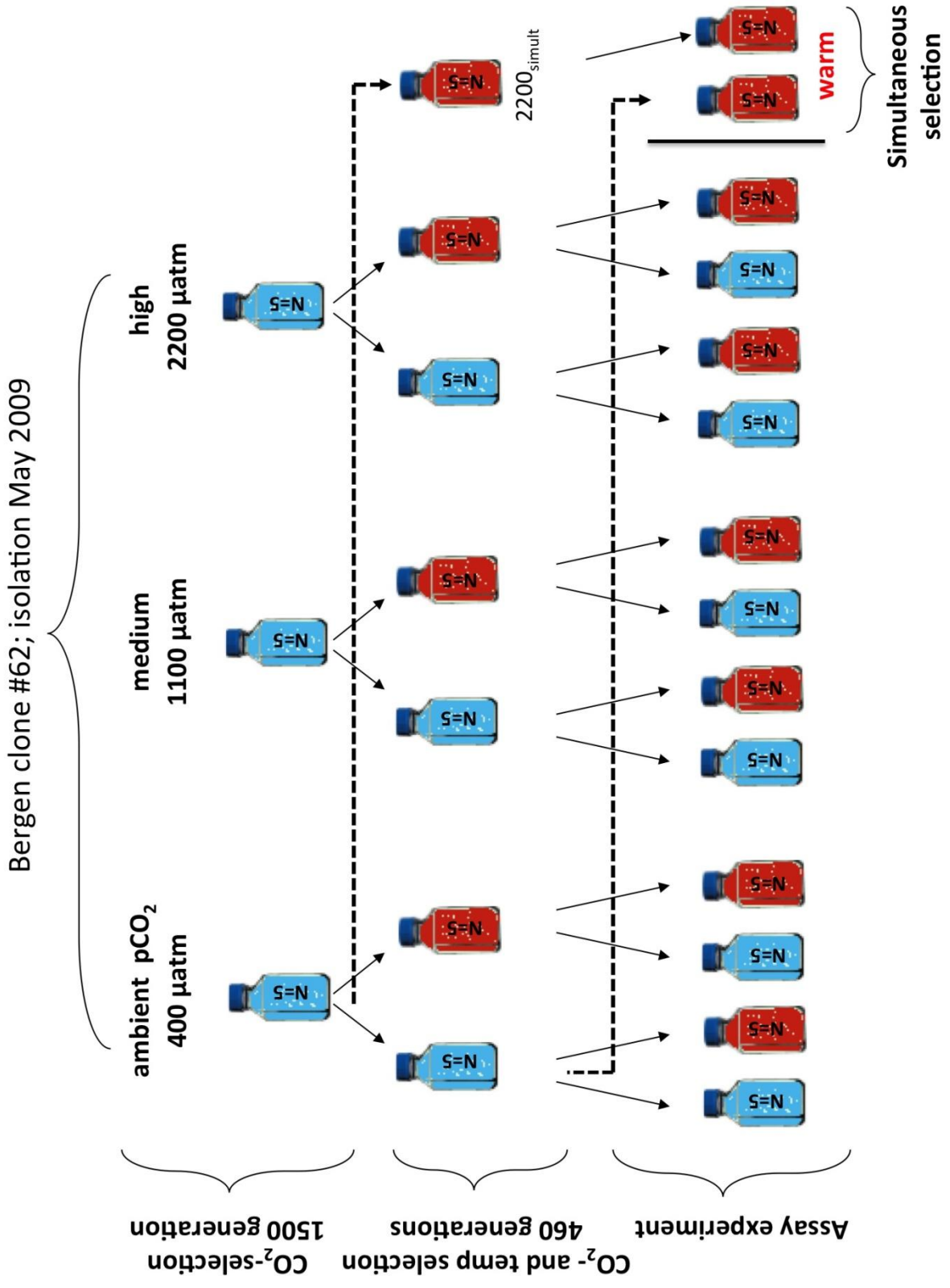
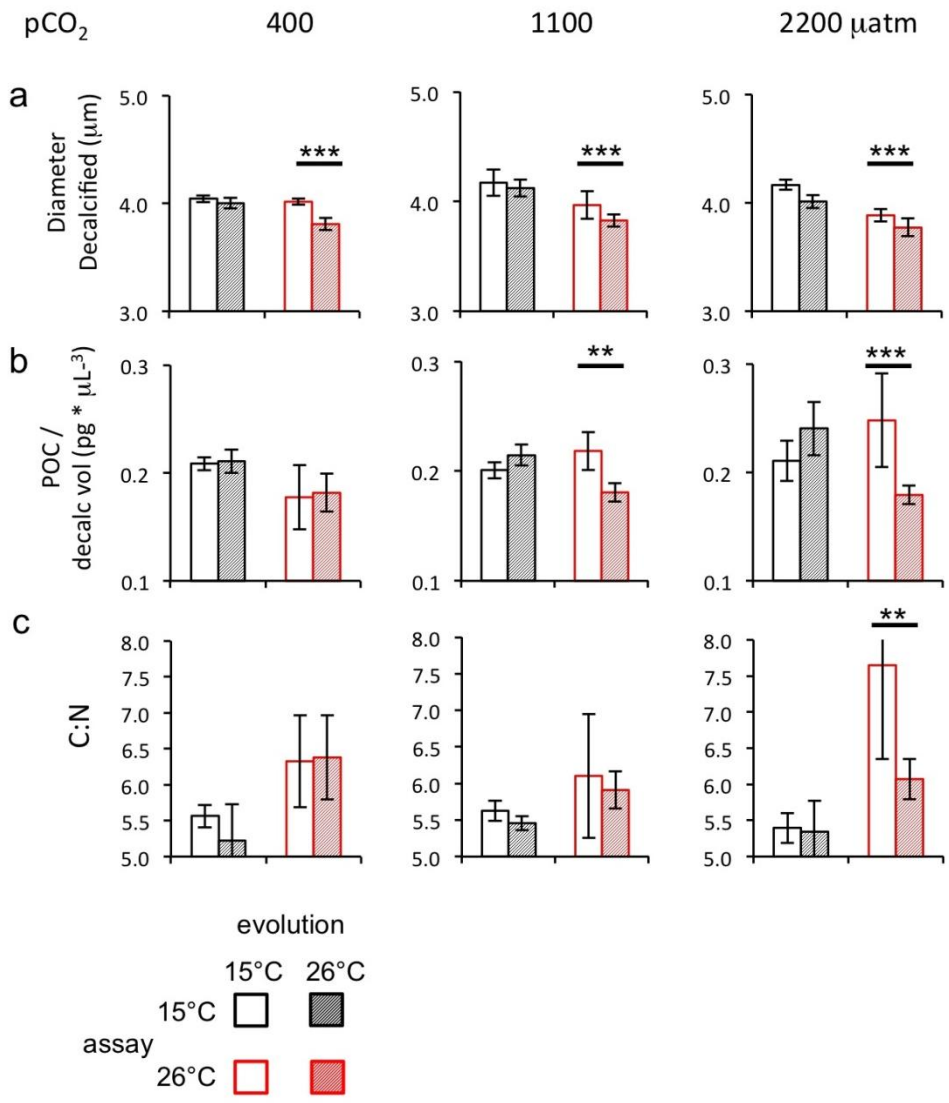


Fig S2 (overleaf). Long-term responses of *Emiliania huxleyi* batch cultures to temperature selection (2 levels, 15.0°C and 26.3°C) under three levels of ocean acidification. Temperature adapted (hatched bars) vs. non-adapted (open bars) populations of *E. huxleyi* are compared within three different CO_2 environments, when exposed to cold and warm temperatures (black and red bars, respectively). (a) decalcified cell diameter (b) particulate organic carbon (POC) standardized to cell volume (excluding the coccosphere) (c) carbon to nitrogen (C:N) ratio. If significant, statistical results of ANOVA contrasts are indicated by bars and asterisks (*0.05≥*P*>0.01, **0.01≥*P*>0.001, ***P<0.001) only for the temperature adaptation response, i.e. for treatments adapted to high temperature vs. those selected at 15.0°C, both assayed at 26.3°C. Error bars are ±1SD. Full ANOVA results are given in Supplementary Table S5.



Supplementary Table S1. Results of a pilot experiment on growth rates of *Emiliania huxleyi* at the upper thermal limit. The high pCO_2 corresponds to 2200 µatm. SD = standard deviation (N=5).

CO_2-environment	temperature (°C)	Mean growth rate	SD
ambient	26.3	1.106	0.105
ambient	26.5	1.130	0.065
ambient	27.0	0.654	0.047
high	26.3	0.986	0.061
high	26.5	0.861	0.116
high	27.0	0.276	0.061

Supplementary Table S2. Autoregressive time series analysis of exponential growth rates in *Emiliania huxleyi* over one year as a function of temperature and CO_2 selection. SE = standard error of the mean (N=5).

selection treatment

pCO_2 (µatm)	Temp (°C)	Growth rate increase (µ batch cycle^{-1})	SE	*t*	*P*
400	15.0	0.000099	0.000102	0.97	0.337
1100	15.0	0.00031	0.000163	1.9	0.061
2200	15.0	0.00013	0.000078	1.66	0.101
400	26.3	0.002491	0.000372	6.69	<0.0001
1100	26.3	0.00252	0.000212	11.87	<0.0001
2200	26.3	0.003002	0.000273	11	<0.0001
2200_{simult}	26.3	0.003826	0.000358	10.7	<0.0001

Supplementary Table S3. Pairwise quasi *F*-tests comparing slopes of autoregressive time series models among the 4 treatments selected at high temperature (26°C) under different CO_2 selection/assay conditions (in µatm). Above diagonal depict *P*-values, below diagonal give $F_{1,143}$-values.

	400	1100	2200	2200_{simult}
400	x	>0.9	0.377	0.0134*
1100	<0.01	x	0.338	0.0193*
2200	0.79	0.93	x	0.285
2200_{simult}	6.27	5.6	1.15	x

Supplementary Table S4. Comparison of different selection regimes to temperature and ocean acidification in *Emiliania huxleyi*. Populations were either adapted to high CO_2–concentration (= ocean acidification) and high temperature selection sequentially, or simultaneously (2200_{simult}).

	high pCO_2 /warming sequential		high pCO_2 /warming simultaneous		
response	mean	SD	mean	SD	t-test *P**
growth rate	1.2020	0.0315	1.2372	0.0386	0.155
diameter total (μm)	4.4053	0.1303	4.4551	0.0514	0.46
diameter (decalc μm)	3.7738	0.0815	3.8732	0.0439	0.052
PIC $cell^{-1}$ (pg)	4.2125	0.2452	3.6757	0.2245	0.007
POC $cell^{-1}$ (pg)	5.0552	0.4879	5.4670	0.1284	0.133
PIC:POC ratio	0.8373	0.0657	0.6723	0.0365	0.0024
C:N ratio	6.0730	0.2832	5.9974	0.2659	0.67

* *t*-test assumes unequal variances

Supplementary Table S5: Statistical analyses of the selection response of *Emiliania huxleyi* to temperature selection in factorial combination with CO_2 condition and assay temperature condition using ANOVA and non-parametric tests. Data transformations are given if applicable. In case of heterogeneous variances despite transformation (growth rate), an additional Welch ANOVA was performed, followed by Kruskal-Wallis comparisons of treatment means among adapted and non-temperature-adapted populations under high temperature. *P*-values >0.1 are not given. The corresponding treatment means are presented in Figures 2, 3 and S2. One replicate of the POC/PON filters was lost, hence the denominator of *F* for all elemental composition variables had df = 47.

Response/Trait	Evolution temperature	Assay temperature	CO_2-condition	Evol_temp* assay_temp	Evol_temp*CO_2-condition	Assay_temp* CO_2-condition	Evol_temp* assay_temp* CO_2
Growth rate (log-transformed)	$F_{1,48}$=6.43 P=0.015	$F_{1,48}$=56.99 P<0.0001	$F_{2,48}$=27.24 P<0.0001	$F_{1,48}$=56.46 P<0.0001	$F_{2,48}$=1.92 ns	$F_{2,48}$=1.41 ns	$F_{2,48}$=1.917 ns
	Welch ANOVA: P<0.0001; Wilcoxon rank comparison of adapted /non-adapted treatments under high temperature: all P=0.0154.						
Cell diameter	$F_{1,48}$=27.52 P<0.0001	$F_{1,48}$=305.9 P<0.0001	$F_{2,48}$=4.96 P=0.011	$F_{1,48}$=0.980 P=0.327	$F_{2,48}$=1.5 ns	$F_{2,48}$=6.489 P=0.0032	$F_{2,48}$=5.09 P=0.0099
Particulate organic carbon (POC*cell^{-1}, log-transformed)	$F_{1,47}$=23.85 P<0.0001	$F_{1,47}$=88.65 P<0.0001	$F_{2,47}$=5.90 P<0.0052	$F_{1,47}$=28.11 P<0.0001	$F_{2,47}$=1.44 ns	$F_{2,47}$=0.197 ns	$F_{2,47}$=2.717 P=0.07
Particulate inorganic carbon (PIC*cell^{-1}, log-transformed)	$F_{1,47}$=2.04 ns	$F_{1,47}$=48.59 P<0.0001	$F_{2,47}$=0.52 ns	$F_{1,47}$=3.51 P=0.067	$F_{2,47}$=2.43 P=0.099	$F_{2,47}$=0.335 ns	$F_{2,47}$=0.854 ns

PIC:POC ratio (cubic-root transformed)	$F_{1,47}$=2.74 ns	$F_{1,47}$=0.73 ns	$F_{2,47}$=5.47 0.007	$F_{1,47}$=2.121 ns	$F_{2,47}$=5.74 P=0.006	$F_{2,47}$=0.26 ns	$F_{2,47}$=3.11 P=0.054
POC biomass production*cell^{-1} *5d^{-1}	$F_{1,47}$=5.233 P=0.0267	$F_{1,47}$=39.21 P<0.0001	$F_{2,47}$=30.29 P<0.0001	$F_{1,47}$=51.64 P<0.0001	$F_{2,47}$=0.49 ns	$F_{2,47}$=0.307 ns	$F_{2,47}$=0.323 ns
PIC biomass production*cell^{-1} *5d^{-1}	$F_{1,47}$=7.17 P=0.012	$F_{1,47}$=33.14 P<0.0001	$F_{2,47}$=43.58 P<0.0001	$F_{1,47}$=57.14 P<0.0001	$F_{2,47}$=1.31 ns	$F_{2,47}$=0.955 ns	$F_{2,47}$=0.457 ns

Figure S3 in supplementary information

Cell diameter decalcified	$F_{1,48}$=39.76 P<0.0001	$F_{1,48}$=125.6 P<0.0001	$F_{2,48}$=4.58 P=0.0151	$F_{1,48}$=3.857 P=0.055	$F_{2,48}$=0.40 ns	$F_{2,48}$=6.736 P=0.0026	$F_{2,48}$=2.82 P=0.070
POC per cell volume decalcified	$F_{1,47}$=3.51 P=0.067	$F_{1,47}$=11.31 P<0.015	$F_{2,47}$=8.46 P=0.0007	$F_{1,47}$=24.78 P<0.0001	$F_{2,47}$=1.80 ns	$F_{2,47}$=0.189 ns	$F_{2,47}$=8.37 P=0.0008
Carbon to nitrogen (C:N) ratio (log-transformed)	$F_{1,47}$=7.123 P=0.010	$F_{1,47}$=53.16 P<0.0001	$F_{2,47}$=1.55 ns	$F_{1,47}$=0.982 ns	$F_{2,47}$=1.817 ns	$F_{2,47}$=4.449 P=0.017	$F_{2,47}$=3.850 P=0.028

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Als erstes möchte ich mich bei Thorsten Reusch und Ulf Riebesell bedanken. Zum einen, dass sie mir die Möglichkeit dieser Doktorarbeit geboten haben und mich stets unterstützt haben. Viel mehr noch aber für das Vertrauen, dass sie in mich gesetzt haben dieses Projekt umzusetzen.

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Eidesstattliche Erklärung

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

Long-term adaptation of the coccolithophore *Emiliania huxleyi* to ocean acidification and global warming

von mir selbstständig angefertigt wurde. Die Arbeit wurde keiner anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegt. Dies ist mein bisher erstes und einziges Promotionsverfahren.

Ich habe keine als die angegebenen Hilfsmittel und Quellen verwendet und die Arbeit unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft erstellt.

Teile dieser Arbeit wurden als Manuskripte in wissenschaftlichen Fachzeitschriften veröffentlicht:

Publikation II in *Nature Climate Change* mit Kai T. Lohbeck, Magdalena A. Gutowska, Joachim P. Gröger, Ulf Riebesell und Thorsten B. H. Reusch als Koautoren.

Kiel, November 2015 Lothar Schlüter