

Assessing marine phytoplankton eco-evolutionary
dynamics and physiological responses to
environmental change

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Summary

Anthropogenic emissions of carbon dioxide (CO₂) resulting from fossil fuel burning and changes in land use are affecting our marine environment. Due to the dissolution of CO₂ in the oceans and its associated chemical reaction with water, hydrogen ions are produced that decrease the pH in the water, a process called ocean acidification (OA). Another indirect effect of increased CO₂ concentrations in the atmosphere is global warming, owing to the greenhouse effect. Ocean warming, in turn, will affect ocean mixing patterns and enhance stratification. As a consequence, primary producers will increasingly face nutrient limitation, with far-reaching effects for marine food-webs.

Phytoplankton are a highly important group of microbes in marine and freshwater ecosystems, responsible for about 50% of primary production and hence photosynthetic oxygen production on our planet. Additionally, they play a crucial role in the cycling of nutrients (nitrate, phosphate, silicate (diatoms only)) in the ocean (biogeochemical cycles). They are unicellular, dividing up to a couple of times a day and can reach huge population sizes. Aside from their overwhelming ecological and biogeochemical importance they also lend themselves to short- and long-term experimentation to study how changes in our climate and the environment may affect planktonic ecosystems in the future (Reusch and Boyd, 2013). In my thesis I focused on representatives of the group of coccolithophores, *Gephyrocapsa. oceanica* and *Emiliana. huxleyi* as well as one species of the group of diatoms, *Chaetoceros. affinis*.

In order to understand how climate change will affect biological communities we need to understand all levels of biological responses. Community change as a response to environmental drivers are composed of three components: the physiological responses within an organism, described by its phenotypic plasticity or reaction norm and the ecological and evolutionary responses which are associated with changes on the species and genotype level, respectively. Moreover, there may be eco-evolutionary coupling, thus either ecological interactions such as competition that modify evolutionary responses to physico-chemical changes, or evolutionary change that feeds back to change ecological interactions.

Here I study for the first time over the long-term (up to 220 generations) how among two competing phytoplankton species the different response types play out, and whether or not coupling of ecological and evolutionary processes can be found. Additionally I inves-

tigated the short-term inter- and intraspecific responses of three phytoplankton species to increased CO₂ and what role competitive interactions play on the short-term in a two-species 'community'.

In the first short term study I found that CO₂ responses varied between and within species and highlighted that it is important to investigate intraspecific responses to environmental stressors because this variability includes the potential of adaptation of a species. The second short term study where the eco-evolutionary changes in a competing phytoplankton two species community were assessed using a Lotka-Volterra model, showed how inter- and intraspecific competitive parameters were important to drive changes on the species and genotype level. On the long-term I found that in the competing phytoplankton system of *E. huxleyi* and *C. affinis* the competitive ability and the ability to cope with high CO₂ of the coccolithophore increased within 200 generations and as such the species interactions changed over time. This ecological change was associated with strong genotypic selection which was likely due to experimental conditions. Therefore, I could show that eco-evolutionary coupling in a two-species phytoplankton system is possible. I also found that the entire thermal reaction norm of *E. huxleyi* changed upon adaptation to a stressful high temperature level in combination with increased CO₂.

I have shown that to address long-term changes on the species and genotype level separately and simultaneously and thus investigate potential eco-evolutionary coupling in laboratory experiments is indeed possible. However, the 'community' I investigated is completely underestimating the complexity of natural systems. Thus, one of the big questions that remain, is how to bridge the gap between findings from simple laboratory experiments to findings in natural communities (e.g. mesocosm or field studies).

Deutsche Zusammenfassung

Anthropogener Ausstoss von Kohlendioxid (CO₂), als Folge der Verbrennung fossiler Brennstoffe und Änderungen von Landnutzung, beeinflusst unsere marine Umwelt. Durch die Auflösung von CO₂ in den Ozeanen und die damit einhergehende chemische Reaktion mit Wasser, werden Wasserstoff Ionen produziert, die den pH-Wert im Wasser senken, dieser Vorgang nennt sich Ozeanversauerung. Eine indirekte Auswirkung von erhöhten CO₂ Konzentrationen in der Atmosphäre, die durch den Gewächshauseffekt hervorgerufen wird, ist die globale Erwärmung. Die daraus resultierende Ozeanerwärmung beeinflusst die Ozeanvermischung und kann somit zu erweiterter Stratifizierung führen. Als Konsequenz werden Primärproduzenten immer mehr mit Nährstofflimitation zu kämpfen haben, welche weitreichende Auswirkungen auf das marine Nahrungsnetz mit sich ziehen können.

Phytoplankton sind eine wichtige Gruppe von Mikroorganismen im marinen und Süßwasser Ökosystem, verantwortlich für ca. 50% der gesamten Primärproduktion und der damit verbundenen Sauerstoff Produktion unseres Planeten. Zusätzlich spielen sie eine wichtige Rolle bei der Nährstoffzirkulation (Nitrat, Phosphat, Silikat (nur Diatomeen) im Ozean (biogeochemische Zyklen). Phytoplankter sind meist einzellig, können sich bis zu mehreren Malen am Tag teilen und erreichen sehr hohe Populationsgrößen. Neben ihrer überwältigenden ökologischen und biogeochemischen Bedeutung, eignen sie sich für Kurz- und Langzeit Experimente, um herauszufinden, wie Veränderungen unseres Klimas und der Umwelt das pelagische Ökosystem beeinflussen werden. In meiner Thesis konzentrierte ich mich sowohl auf Vertreter der Gruppe der Coccolithophoren, *Gephyrocapsa oceanica* und *Emiliana huxleyi*, als auch eine Art der Gruppe der Diatomeen, *Chaetoceros affinis*.

Um zu verstehen, wie sich Klimawandel auf biologische Gemeinschaften auswirkt, müssen wir sowohl ökologische als auch evolutionäre Prozesse betrachten. Gemeinschaftsveränderungen als Antwort auf umweltbedingte Einflussfaktoren beinhalten drei Komponenten: die physiologische Antwort innerhalb eines Organisms, die durch phänotypische Plastizität oder Reaktionsnormen beschrieben wird und ökologische und evolutive Antworten, die mit Veränderungen auf der Spezies und Genotypen Ebene assoziiert sind. Zusätzlich kann es öko-evolutionäre Kopplung geben. In anderen Worten handelt es sich hierbei entweder um ökologische Interaktionen (z.B. Konkurrenz), die evolutionäre Pro-

zesse modifizieren oder evolutionäre Veränderungen, die auf ökologische Interaktionen rückwirken.

In dieser Studie habe ich erstmals über eine längere Zeit (ca. 220 Generationen) untersucht, wie sich innerhalb einer konkurrenzierenden Phytoplankton Gemeinschaft die unterschiedlichen biologischen Antworten zeigen und ob eine Kopplung von ökologischen und evolutiven Prozessen gefunden werden kann. Zusätzlich habe ich mir die Kurz- und Langzeit inter- und intraspezifischen Antworten von drei Phytoplankton Arten auf erhöhte CO₂ Konzentrationen angeschaut und welche Rolle Konkurrenz-Interaktionen spielen.

Im ersten Kurzzeit-Experiment habe ich gezeigt, dass sich die physiologischen Antworten innerhalb und zwischen den untersuchten Arten unterscheiden und habe festgestellt, dass es wichtig ist diese zu untersuchen, da die dadurch vorhandene Variabilität wichtig für das Potential der Anpassung einer Art ist. Das zweite Kurzzeit-Experiment zeigte, dass kompetitive Interaktionen zwischen zwei Arten und innerhalb einer Art wichtig sind für die Veränderungen der Spezies- und Genotypen Zusammensetzung. Dies habe ich mit einem Lotka-Volterra Modellierungsansatz herausgefunden. In dem Langzeit-Experiment einer Phytoplankton Gemeinschaft von *E. huxleyi* und *C. affinis*, habe ich gezeigt, dass die Konkurrenzfähigkeit und die Fähigkeit mit erhöhtem CO₂ umzugehen in *E. huxleyi* sich über die Zeit von 220 Generationen erhöhen. Somit haben sich die Interaktionen der beiden Arten auch verändert. Diese ökologischen Veränderungen waren mit einer starken Genotypen-Selektion assoziiert, die wahrscheinlich von den experimentellen Bedingungen getrieben wurde. Somit konnte ich in dieser Studie zeigen, dass öko-evolutionäre Kopplung in einem zwei-Arten Phytoplankton System möglich ist. Ich habe auch herausgefunden, dass sich die gesamte Temperatur Reaktionsnorm von *E. huxleyi* verändern kann, getrieben durch die Langzeitanpassung an eine stressige Umgebung von erhöhter Temperatur in Kombination mit erhöhtem CO₂.

Zusammenfassend konnte ich Lang- und Kurzzeitveränderungen auf der Spezies und Genotypen Ebene einzeln und simultan untersuchen und somit zeigen, dass potentiell öko-evolutionäre Kopplung in Laborexperimenten möglich ist. Allerdings unterschätzt die "Gemeinschaft", die ich untersucht habe, die Komplexität natürlicher Systeme bei weitem. Deswegen bleibt eine der grossen Fragen offen, wie die Lücke zwischen den Forschungsergebnissen von Laborexperimenten oder natürlichen System überbrückt werden kann.

Introduction

Climate change

Anthropogenic emissions of carbon dioxide (CO₂) from burning fossil fuels and changes in land use are affecting our climate and environment (Caldeira and Wickett, 2003; Doney et al., 2009). Under the worst case scenario, the IPCC report of 2014 (IPCC Fifth Assessment Report - Synthesis Report, 2014) predicts an increase of CO₂ up to 1100μatm until the end of this century (Figure 1 left IPCC CO₂). As a direct consequence, CO₂ from the atmosphere will continuously dissolve into the ocean because of a concentration disequilibrium between the air and water (Caldeira and Wickett, 2003). CO₂ reacts in the water to form carbonic acid, which then further dissociates into bicarbonate and carbonate ions:



By increasing the supply of dissolved CO₂ on the left-hand side of the equation, the reaction equilibrium is pushed to the right hand side, with the end result of more hydrogen ions being produced that decrease the pH in the water. This process is called ocean acidification (OA) (Caldeira and Wickett, 2003), the "other CO₂ problem". All carbonate species are a carbon source for primary producers and could potentially affect competitive dynamics (Connell et al., 2013). Overall, the oceans are a huge sink for CO₂ because dissolved CO₂ is transported away from the surface to deeper waters by different processes. Ocean circulation transports dissolved CO₂ into deeper waters mainly in polar regions; this is called the physical carbon pump. The uptake of CO₂ by primary producers and the associated transport into the deep ocean through food web interactions and sinking of organic matter represent the biological carbon pump (Raven and Falkowski, 1999). Both processes favor a net transport of CO₂ into the ocean.

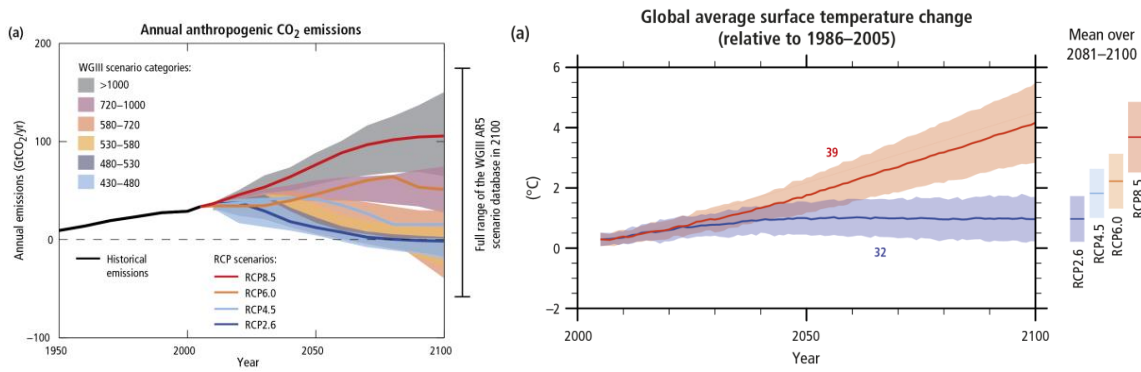


Figure 1. Predicted increase of atmospheric CO₂ concentrations within the next 100 years depending on different emission scenarios (left panel), and associated predictions of warming of sea surface temperatures (right panel) (Data source: (IPCC Fifth Assessment Report - Synthesis Report, 2014)).

Another indirect problem of increased CO₂ concentrations in the atmosphere is the associated increase in temperature owing to the greenhouse effect, commonly called global warming. Due to net energy transport into the ocean, heat from the warmed up atmosphere will be transported into the ocean and thus increase ocean temperatures (Levitus et al., 2001). The IPCC report of 2014 predicts that on average oceans could warm up to 3°C within the next hundred years (IPCC Fifth Assessment Report - Synthesis Report, 2014) (Figure 1 right IPCC SST). Rising temperatures in surface waters result in a more stable stratification of water layers and less mixing of the surface with mid-layer nutrient rich waters (Behrenfeld et al., 2006). Consequently, regions, (e.g. tropical ocean gyres) are prone to become nutrient depleted which likely plays an important role for primary producers that rely on the availability of nutrients to grow (Boyd et al., 2013; Moore et al., 2013).

Phytoplankton

The focus of my thesis is the response of phytoplankton to the already ongoing changes in ocean environment, in particular warming and ocean acidification. Phytoplankton are a highly important group of organisms in our waters. These primary producers are at the base of pelagic food webs and responsible for about 50% of primary production and photosynthetic oxygen production on our planet (Field et al., 1998) (see also biological carbon pump). Additionally, they play a crucial role in the cycling of nutrients (nitrate, phosphate, silicate (diatoms only)) in the ocean (biogeochemical cycles) (Falkowski et al., 2004, 2008). They are unicellular organisms dividing up to a couple of times a day and can reach huge population sizes (Lynch et al., 1991). Aside from their overwhelming eco-

logical and biogeochemical importance they also lend themselves to short- and long-term experimentation to study how changes in our climate and the environment may affect planktonic ecosystems in the future. In addition to a large number of short-term studies, various long term evolutionary studies have been successfully performed with phytoplankton and yielded novel insights on the potential of adaptation to abiotic drivers (Collins et al., 2014; Lohbeck et al., 2012; Schlüter et al., 2014). However, some molecular characteristics of phytoplankton are still unclear or may present difficulties in evolution experiments. Some phytoplankton have rather large diploid genomes including genomic elements of their organelles that had been acquired through endosymbiosis (reviewed in Reusch and Boyd 2013). Additionally, Reusch and Boyd (2013) point out that

Emilinia huxleyi:

One species I focused on in my thesis is the coccolithophore *Emiliana huxleyi*. *E. huxleyi* has become a much-used model species to investigate effects of environmental change life cycle studies or even cellular processes (Paasche et al. 2001) and it has become a popular microorganism to investigate the adaptive potential of environmental change as well (Lohbeck et al. 2012, Schlüter et al. 2014). *E. huxleyi* thrives naturally in many environments and is hence naturally adapted to very different conditions. In my studies I have used relatively fresh isolates (year of isolation 2012 and 2015) of *E. huxleyi* from the pelagic region off the coast of Gran Canary (Gran Canary, 27°59'N 15°22'W). *E. huxleyi* has been characterized with relatively high growth rates (Tyrrell and Merico, 2004) but depending on the environment (e.g. high silicate availability) it still grows slower than other phytoplankton species (e.g. diatoms). In my thesis I used *E. huxleyi* as a model organism to look at short term responses to increased CO₂ (Chapter I) and competition (Chapter II). Additionally, I investigated long term change in the physiological temperature response in response to increased CO₂ (Chapter III) as well as the long-term response to increased CO₂ and competition (Chapter IV).

Gephyrocapsa oceanica

Gephyrocapsa oceanica is also part of the family of the coccolithophores but slightly larger than *E. huxleyi*. Similar to *E. huxleyi* it has also been used in various studies about the effects of climate change and physiological studies (Jin et al. 2013, Meyer and Riebesell 2015). *G. oceanica* is a globally occurring phytoplankton species and the isolates I used came from the pelagic region off the Coast of Gran Canary (Gran Canary, 27°59'N 15°22'W) as well. In my thesis I used *G. oceanica* to investigate its short-term response to increased CO₂ (Chapter I) and compared this response to the closely related coccolithophore *E. huxleyi* and a more distantly related diatom *C. affinis*.

Chaetoceros affinis:

One more species that I focused on in this thesis is *Chaetoceros affinis*. It belongs to the group of diatoms. There have been various studies done on *C. affinis* concerning cellular responses but so far not much is known about its response to ocean acidification or capability of adaptation to increased CO₂. In my thesis I used *C. affinis* to investigate the short-term response to increased CO₂ (Chapter I) and competition (Chapter II) as well as the long-term response and potential to increased CO₂ and competition (Chapter IV).

sexual reproduction is poorly understood and has only very rarely been observed in laboratory conditions. Therefore, when performing (long term) evolution experiments with phytoplankton recombination during sexual reproduction may add another level of complexity that is however currently largely ignored.

In my thesis I have worked with phytoplankton species from two different groups, two coccolithophore species and one diatom. Coccolithophores produce calcite scales, the coccoliths, and the associated calcification has been shown to be negatively impacted by the decrease of pH in the ocean (Riebesell et al., 2000). In contrast, diatoms produce silicate shells around their cells with the consequence that silicate is a prerequisite for them to thrive (Egge and Aksnes; Ragueneau et al., 2000). Silicate rich environments can appear when there are upwelling events that transport nutrient and silicate rich waters up to the surface waters, normally during the mixing events before blooms (Abrantes et al., 2016).

Phytoplankton responses to environmental change

A central concept of this thesis was the integration of the different levels of biological responses to environmental change including physiological, ecological and evolutionary responses. This includes the investigation of species and genotype responses on the short as well as on the long term. As was described above, phytoplankton lend themselves as a useful and important ecological group to investigate different levels of biological responses.

Phenotypic plasticity

The expression of different phenotypes of the same genotype in different environments is called phenotypic plasticity. It is best described by the shape of the reaction norm of a trait at different environments. No visible change in a focal trait despite a change in the environment (horizontal reaction norm) is defined as phenotypic buffering (Pigliucci, Massimo Müller, 2010). This does not preclude changes in other traits or on the molecular level. How phenotypic plasticity interacts with evolutionary adaptation is contentious (Lande, 2009); it is discussed to be both a non-mutual alternative to evolutionary adaptation and a strong driver for adaptation. In the plasticity-first scenario, a population/species survives environmental change due to pronounced plasticity until genetic mutations may occur and potentially fix the previously plastic trait such that the fitness under the new conditions increases (Levis and Pfennig, 2016). When the genotypic composition of a

species is unknown it is still possible to measure reaction norms to environmental change on the species level. However, these responses can differ to the variation in reaction norms on the genotype level (Des Roches et al., 2018) and affect the predictions that can be made. What is often overlooked is that it is the plastic response of species (and genotypes) in the sense of a reaction norm that will change in the process of adaptation (Pigliucci, 2005). In other words, if there is evolutionary change, it is defined as a change in the reaction of the control lines, under the novel conditions, compared to the evolved lines, which can be best depicted by a two-point reaction norm.

Ecological winners and losers

Species differ in their traits such as (cell) size, (nutrient) resource requirements and growth (Litchman et al., 2007) and are likely to respond differently to environmental change such as ocean acidification (Connell et al., 2013; Doney et al., 2009) and ocean warming (Doney, 2006). Increased CO₂ mostly negatively affects calcifying organisms which include coccolithophores (Meyer and Riebesell, 2015) but can also be used as a carbon source by most other phytoplankton species including diatoms, because the pressure of DIC limitation is alleviated (Sabia et al., 2018). The effects of increasing temperatures have also been shown to be species specific (Huertas et al., 2011) and will likely influence the geographical distribution of phytoplankton in the future (Thomas et al., 2012).

Experimental findings on single species would predict that under future environmental conditions communities will change. Species compositional change (or ecological sorting) has already been investigated in response to several environmental factors and found varying short term responses: Interestingly, in a Baltic autumn bloom experiment for example it was shown that increased CO₂ and temperature did not affect the community composition very much and small changes were only found in the rare taxa (Sommer et al., 2015). In contrast, in an Atlantic spring bloom experiment strong changes in community composition could be found: precisely, increased CO₂ concentrations favored diatom species while the combination of increased temperature and CO₂ favored coccolithophore species (Feng et al., 2009). These studies investigated short term responses, however it is unclear how community composition would change if species would adapt.

Adaptation to environmental change

In addition to species sorting, community change can happen by selection on the genotype level (aka evolutionary sorting) provided that multiple genotypes are present in the population. Examples from phytoplankton where selection on the genotype level have been shown and monitored are rare (e.g. Lohbeck 2012). Nevertheless, it is important to include standing genetic variation in an experiment because such variation in natural systems provides a target to selection. Under new environmental conditions the genetic composition of a species should change in favor of the genotypes whose fitness is highest in the respective conditions. Despite the limited investigation of genotype sorting as an evolutionary response there have already been various studies on the potential of adaptation to environmental change in different phytoplankton species. Collins et al (2014) have nicely summarized various studies of experimental evolution in phytoplankton to ocean acidification that include experimental durations of below 50 up to 1000 generations but since then more evolutionary adaptation studies on phytoplankton have been done (Schlüter et al., 2014) that include up to 2200 generations. Concerning diatoms there are several studies that have investigated the potential of adaptation to increased CO₂ and found that there are for example changes growth rates, photosynthesis and respiration among the responses (Crawford et al., 2011; Li et al., 2016; Tatters et al., 2013). As a calcifying organism *E. huxleyi* was one of two coccolithophore species investigated and responded with an increase in growth rates when exposed to high CO₂ concentrations (Lohbeck et al., 2012). In contrast there are not as many experimental evolution studies on ocean warming; nevertheless, it has been shown in *E. huxleyi* that it can adapt to warming and the combined effects of warming and increased CO₂ (Schlüter et al., 2014).

The different biological responses described above vary conceptually: while physiological responses manifest themselves within an organism in response to different environments, ecological and evolutionary responses to different environments are based on physiological responses. Precisely, under new environmental conditions the genetic composition of a species and species composition of a community should change in favor of the genotypes and species whose fitness is highest in the respective conditions which depends on their respective physiological reaction norms. The theoretical integration of biological responses to environmental change may give an idea on how communities will change in the future and to what extent ecological and evolutionary as well as physiological processes play a role. One such theoretical integration approach has for example been

described by Collins and Gardner (2009) as the “price equation” approach where the different contributions of physiological, evolutionary and ecological change are calculated when considering whole community change. However, investigating and as such integrating all of the biological responses in experiments simultaneously is challenging and has only begun to be the focus of recent studies. In my thesis the simultaneous investigation of ecological and evolutionary processes was one important goal.

Eco-evolutionary coupling

Investigating the short and long-term responses of species and genotypes of species simultaneously includes potential changes on the ecological and evolutionary level. In recent years it has been recognized that ecological and evolutionary processes can happen on similar time scales and that they likely interact with each other (Pelletier et al., 2009). Either ecological interactions such as competition or predation can modify evolutionary responses or evolutionary change can feed back to change ecological interactions. Such eco-evolutionary coupling has recently been demonstrated and investigated experimentally (Becks et al., 2010) as well as described mathematically (Yoshida et al., 2003) in a rotifer-algae systems as well as in host-parasite systems (Frickel et al., 2016). For example, in the rotifer-algae system the algae population changed between a single cell population and clump forming population depending on the rotifer abundance (eco→evo). This evolutionary change in the algae from single cell to clump formation in turn fed back on the rotifer abundance and as such the species interactions between rotifers and algae (evo→eco). It is however unclear, how environmental change may affect eco-evolutionary coupling and how this plays out in phytoplankton communities.

In my thesis I wanted to address all of the introduced levels of community change first on their own and then in combination, as well as on the short and long term. From this follow four objectives that I addressed throughout my thesis. My first objective was to investigate the short-term response and phenotypic plasticity of three different phytoplankton species to increased CO₂. Following, with the second objective I wanted to characterize the thermal reaction norm of *E. huxleyi* in response to 2000-generations of adaptation to a high, stressful temperature, in combination three levels of CO₂ concentrations. Next, I moved to the community level in the simplest imaginable "community" with two species. To investigate species compositional changes, the requirement is the coexistence of a

minimum of two species which was possible for *E. huxleyi* and *C. affinis* as they display fundamentally different nutrient requirements and different nutrient uptake related strategies. Here, increased CO₂ was used as an environmental driver to see in the third objective how the species and genotype composition simultaneously changed in a short-term experiment. The short-term changes were assessed by applying a Lotka-Volterra modeling approach to assess inter- and intraspecific interactions. In a similar long-term study I addressed the fourth objective of how the potential of adaption to increased CO₂ as well as the presence of another species differed and whether with this approach I could find eco-evolutionary coupling in a phytoplankton 'community'.

Thesis outline

This thesis contains four chapters, each of which is organized in manuscript form and contains the sections abstract, introduction, material and methods, results and discussion. Here I briefly introduce the research questions of the single chapters and explain the reasons for the respective approaches.

Chapter I:

In the first chapter I investigated the inter- and intraspecific plasticity to CO₂ of three major phytoplankton species: *Chaetoceros. affinis*, *Emiliana. huxleyi* and *Gephyrocapsa. oceanica*. I studied how varying the response to CO₂ between closely and more distantly related species is and how large this variation within each species is.

Chapter II:

In the second chapter I applied a Lotka-Volterra modeling approach including measured parameters of growth and carrying capacities as well as estimated parameters of competition to qualitatively assess inter- and intraspecific competition in a two-species phytoplankton community in response to CO₂. In the first Chapter it was shown that the two species *C. affinis* and *E. huxleyi* were different in their response to CO₂ but in the second chapter I wanted to investigate how these differences play out as simultaneous species and genotype sorting over ca 40 generations.

Chapter III:

In the third chapter I assessed whether and how the entire thermal reaction norm of *E. huxleyi* had changed over time due to the adaptation to a high and stressful temperature, in combination with two levels of increased CO₂ and a control. It had been previously shown that *E. huxleyi* was able to adapt to increased CO₂ and temperature, but it is unknown how this adaptation affects the entire reaction norm at multiple temperatures that were not the selection conditions.

Chapter IV

In the last chapter I investigated the adaptive potential of *C. affinis* and *E. huxleyi* to increased CO₂ and to the presence of the respective other species, and to both selection regimes in all combinations. I also tested whether there is eco-evolutionary coupling in both directions, thus if the ecological environment (i.e. presence of the other species) affected

the evolutionary response and if evolutionary change could in turn change species interactions. Thus far long-term evolution studies had been mostly done on single species or even just single strains of species. Here I wanted to use a novel approach to include genotypic variation and two phytoplankton species in a long-term experimental evolution study.

Chapter I

Inter- and intra-specific phenotypic plasticity of three phytoplankton species in response to ocean acidification

Inter- and intra-specific phenotypic plasticity of three phytoplankton species in response to ocean acidification

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Abstract

Phenotypic plasticity describes the phenotypic adjustment of the same genotype to different environmental conditions and is best described by a reaction norm. We focus on the effect of ocean acidification (OA) on inter – and intraspecific reaction norms of three globally important phytoplankton species (*Emiliana huxleyi*, *Gephyrocapsa oceanica*, *Chaetoceros affinis*). Despite significant differences in growth rates between the species, they all showed a high potential for phenotypic buffering (similar growth rates between ambient and high CO₂ condition). Only three coccolithophore genotypes showed a reduced growth in high CO₂. Diverging responses to high CO₂ of single coccolithophore genotypes compared to the respective mean species responses, however, raise the question if an extrapolation to the population level is possible from single genotype experiments. We therefore compared the mean response of all tested genotypes to a total species response comprising the same genotypes, which was not significantly different in the coccolithophores. Assessing species reaction norm to different environmental conditions on short time scale in a genotype-mix could thus reduce sampling effort while increasing predictive power.

Key words: *E.huxleyi*, *G.oceanica*, *C.affinis*, OA, plasticity, reaction norm.

Introduction

The expression of different phenotypes of a genotype in different environments is called phenotypic plasticity. It is described by the shape of the reaction norm of a trait value at different environments. No visible change in a focal trait despite a change in the environment (horizontal reaction norm) is defined as phenotypic buffering [1]. This does not preclude changes in other traits or on the molecular level. How phenotypic plasticity interacts with evolutionary adaptation is contentious [2]; it is discussed to be both a non-mutual alternative to evolutionary adaptation and a strong driver for adaptation. In the plasticity-first scenario, a population/species survives environmental change due to pronounced plasticity until genetic mutations may occur and potentially fix the previously plastic trait such that the fitness under the new conditions increases [3]. Provided that there is standing genetic/genotypic variation, mean population fitness can also increase at the level of populations, as a result from alteration of gene/genotypic frequencies over time caused by selection.

One prominent environmental change is ocean acidification (OA) [4], describing changes in the carbonate system due to anthropogenic CO₂ dissolving in the ocean, which potentially affects organisms, species and communities [5]. In marine phytoplankton different effect sizes and signs in response to OA (i.e. varying reaction norms) have been observed between and within different taxa [6]. A reason for within species differences can be adaptation to different geographical regions [7]. Little is known, however, about inter- and intraspecific variation in reaction norms of populations and communities originating from one geographical region. Additionally, Valladares et al. (2014) summarize, that current mathematical models predicting alterations in communities due to climate change lack data on intraspecific genetic and phenotypic variation. Largely diverging responses to OA of different *E. huxleyi* genotypes among studies [9], raise the question if responses derived from one or a few genotypes can be directly extrapolated to the population and community level.

We compare (i), the intra- and interspecific reaction norms of three phytoplankton species in response to two different CO₂ conditions and (ii) the total multi-genotype species response to the mean intraspecific CO₂-response of the respective species. The species used include two common bloom forming coccolithophores, *Emiliana huxleyi* and *Gephyrocapsa oceanica*, and a diatom, *Chaetoceros affinis*, originating from one region. We

expect that (i) the coccolithophores show a zero to negative reaction norm as a result of OA [10] compared to a positive slope for the diatom as a result of profitable DIC use [11]. Furthermore (ii) genotypes of a species should differ in their growth response and (iii) the total species reaction norm is unequal to that of single genotypes but similar to the calculated mean reaction norm of all genotypes together.

Methods

From *C. affinis*, *E. huxleyi* and *G. oceanica* nine different genotypes each and one mix of all genotypes with equal initial abundances (supplementary, S1) were immediately exposed to ambient and high CO₂ concentration in order to obtain a two-point reaction norm within the acclimation phase. All cultures used were field isolates (2014- 2015) originating from one geographical region (Gran Canary, 27°59'N 15°22'W). This design allowed us to compare the within and among species plasticity of one community and the effect of intraspecific interaction on the short term CO₂-response by contrasting the multi-genotype total species (mixculture) to the mean intraspecific plastic (monoculture) response. All treatment combinations were three-fold replicated resulting in 180 experimental units (0.5 L polycarbonate bottle). Due to space limitation each species was tested separately (June to July 2016; Figure S1).

The ambient and high CO₂-treatment was manipulated by aerating the artificial-seawater (35 salinity; after [12]) for 24h with CO₂-enriched air (400 and 1250ppm, respectively) prior to the experiment. The dissolved inorganic carbon [13] were 2164.68±27.76 and 2307.94±51.59 μmol*kg⁻¹ with a total alkalinity (following [14]) of 2442.04±20.72 and 2456.30±20.63 μmol*kg⁻¹ for ambient and high CO₂, respectively. Nutrients were added to the final concentrations of 19.98±0.39 μmol*L⁻¹ nitrate, 1.01±0.07 μmol*L⁻¹ phosphate and 4.40±0.24 μmol*L⁻¹ silicate for coccolithophores and 34.16±0.30 silicate for the diatoms. The excess of silicate added to media used for diatoms ensured that all species were limited by nitrate in the experiment, a prerequisite to compare results among species. Vitamin and trace metals were added in f/8 concentration [15]. The prepared medium was sterile filtrated (0.2 μm) into the experimental units. Each experimental unit was inoculated with an initial total biovolume of 8280 μm³*ml⁻¹ of exponentially growing cells balancing the substantial differences in cell size of the species used.

The experiment was carried out under constant rotation (0.75 min⁻¹) at 20 °C and a 17:7 day:night cycle reaching a maximum light intensity of 350 μmol*m⁻²*s⁻¹ after 3h dusk

and dawn. The development of each culture was followed by daily cell counts for the coccolithophores (Z2TM COULTER COUNTER®) and fluorescence measurements for the diatom (10AU FIELD AND LABORATORY FLOUREMETER by TURNER DESIGNS). The total sampling volume was below 10%. Cultures were terminated at the third day in stationary phase (experimental duration: 9-16 d).

For statistical analysis the software R was used (R core team 2016). Growth rates were determined for each replicate by fitting an exponential growth model inbuilt in the package “growthrates” [16]. The overall effect of CO₂, species and genotype on growth rate was tested using a nested ANOVA (growthrate~ CO₂*Species*(Genotype/Species)). Subsequent analysis of intraspecific plasticity and the effect of genotype was tested by separate ANOVAs for each species (growthrate~CO₂*Genotype) and genotype (growthrate~CO₂) and visualized as the difference in growth rates between ambient and high CO₂ [17]. The difference between mean interspecific plastic effects and the multi-clonal total species response was tested for each species separately (growthrate~Mono/Mix-culture). Parametric assumptions were explored graphically.

Results

The growth rates (μ) between the species were significantly different ($F_{(2,132)}= 355.586$, $p<0.001$) with *E. huxleyi* and *G. oceanica* showing a 44 % and 28 % lower μ than *C. affinis* (figure 1). Across all species μ was generally lower in high CO₂ and significantly depended on genotype ($F_{(1,132)}= 8.433$, $p=0.004$; $F_{(24,132)}= 6.161$, $p<0.001$; respectively). Analysis on the species level revealed that only the μ of *G. oceanica* was significantly lower in high CO₂ ($F_{(1,56)}= 20.659$, $p<0.001$). Further, the magnitude of the difference in μ between the CO₂-treatments was not uniform among all tested genotypes within each species (figure 2.). While the mean difference of *C. affinis* genotypes ranged from 0.109 to -0.273 with a substantial standard error, those of *E. huxleyi* had a narrow range from 0.029 to -0.097, with one genotype (C30) showing a significant lower μ under high than ambient CO₂ ($F_{(1,4)}= 48.64$, $p=0.002$). The general negative mean difference in *G. oceanica* genotypes ranged from -0.17 to -0.21. Two genotypes (GC59, GC58) were significantly negatively affected by CO₂ ($F_{(1,4)}= 10.7$, $p=0.031$; $F_{(1,4)}= 42.12$, $p=0.003$) which drove the overall significant negative effect of CO₂ on the μ of *G. oceanica*. Finally, the difference in μ between mono and mixcultures was significantly different only in *C. affinis* ($F_{(1,22)}= 8.405$, $p=0.008$) with a higher μ in the mix than in the monocultures.

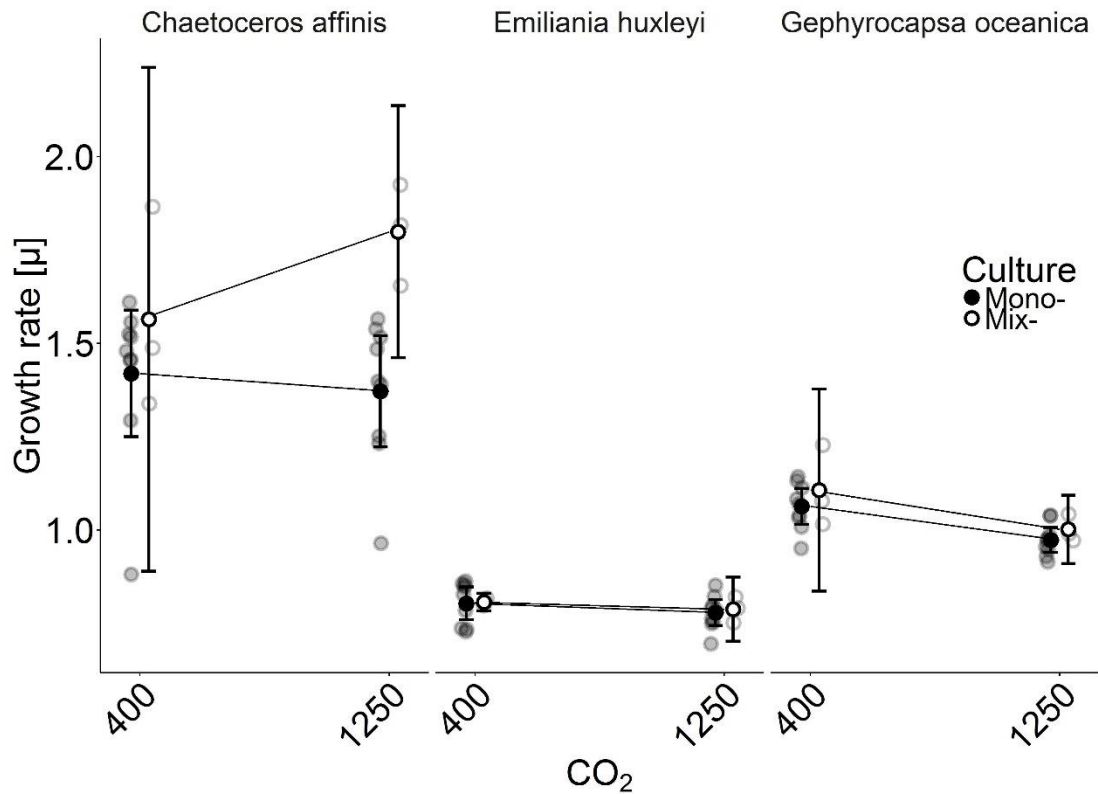


Figure 1: Two-point reaction norm of growth rates in ambient and high CO₂ across mean of each genotype grown in mono-culture (closed circle, N=9 (9 genotypes)) and a mix-culture of all genotypes (open circle, N=3 (3 replicates)) for each species. Mean and 95% CI.

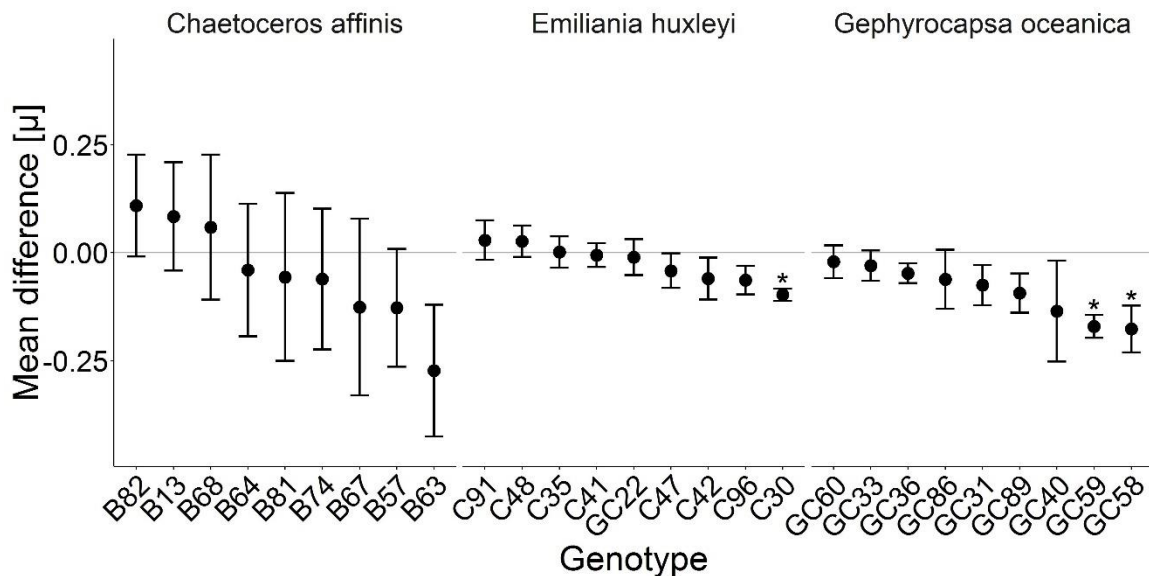


Figure 2: Mean difference and its standard error of growth rates (μ) between high and ambient CO₂ of each genotype and species. Grey line indicates no difference in growth between CO₂-treatments and * highlights genotypes where growth rate was significantly affected by CO₂.

Discussion

We assessed the variation of phytoplankton acclimation reaction norms in two potential "loser" and one potential "winner" species under ocean acidification. Interestingly, all three species mostly buffered the effect of CO₂ and thus, showed a mean reaction norm slope similar or close to zero. However, within species, the response range varied. *C. affinis* showed the largest range in growth rates among genotypes tested. *E. huxleyi* and *G. oceanica* are ecologically more alike compared to *C. affinis* which could explain a more similar negative response among them. Due to the extensive literature [18] showing negative effects of OA on coccolithophores we expected to see more genotypes showing a significant negative effect in growth under high CO₂, but note that most of those measurements were taken after acclimation, while our study was designed to address exactly the acclimation phase. Nevertheless in line with literature we found that *G. oceanica* was most and significantly negatively affected by high CO₂ [10]. Overall the weak effect of CO₂ could partly be due to the high variability among replicates masking a potential difference in growth rate between the two treatments. Additionally the experimental level of CO₂ in this study may be within the natural range (daily fluctuations, upwelling) species experience and can be phenotypically buffered [19].

The effect of CO₂ on single genotypes differed compared to the mean species response. *E. huxleyi* for example showed no overall effect of CO₂ on growth even though one genotype grew significantly slower under high CO₂. We observed the opposite in *G. oceanica* with an overall negative effect of high CO₂ on growth rate even though seven out of nine genotypes showed no difference. Our findings highlight the importance of testing many genotypes compared to the use of single genotypes, as has been done in most studies so far [18], to avoid over- or underestimation of a species reaction norm to climate change.

The question remains how to minimize the sampling effort needed to study reaction norms of a representative set of genotypes of a species. We here show that the reaction norm of a culture containing the full set of genotypes compared to the mean of all genotypes cultured singly was similar in both coccolithophores but not in the diatom. The significant effect of culture condition on the slope in *C. affinis* could be driven by the high variability within the three replicates in the mixcultures. Nevertheless, our results suggest that the use of a mixculture of genotypes is sufficient to assess a species reaction norm on short time scales. This largely suggests that the total species reaction norm obtained from

the mixcultures reflects the mean species plasticity if, as assumed here, genotype loss due to sorting is likely negligible (supplementary S2).

Our experiments highlight the importance of investigating species reaction norms rather than reaction norms of single genotypes to better predict reactions to short-term environmental change. We suggest that analyzing a mix of genotypes is potentially an achievable and feasible way to identify more realistic species reaction norms. Nevertheless, future studies should assess final genotype sorting to fully understand the species reaction norms.

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Additional information for publication I

Ethics

This research did not make use of organisms covered by the German Animal Welfare Act (TierSchG).

Data accessibility

PANGEA <https://doi.pangaea.de/10.1594/PANGAEA.865212> [20]

Authors' contributions

Study designed by GH, LL, TR, BM. Lab work carried out by GH, LL, JR, DO; data analysis by GH, LL. All authors drafted or revised the manuscript, gave final approval and agree to be held accountable for the content.

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Supplementary Material Chapter I:*S1: Genotype isolation and genotyping*

All genotypes used were isolated from the same geographical region in the North Atlantic (south-east off Gran Canary, Spain, 27°59'N 15°22'W) between spring 2014- spring 2015. All genotypes of one species, with one exception, were isolated at once from a 2L seawater sample. More specifically, the *C. affinis* genotypes were isolated in December 2014, while *G. oceanica* was isolated in February 2015 and *E. huxleyi* were collected and isolated in February 2014 (except for GC22 which was isolated in February 2015). All genotypes used in this experiment will be deposited in the Roscoff Culture Collection (<http://roscoff-culture-collection.org/>; Table S1)

All coccolithophore strains (i.e. *E. huxleyi* and *G. oceanica*) were genotyped using microsatellite (msat) analysis to assure that each culture used is a different strain (genotype). For *C. affinis* the msats are still under development. We nevertheless assume that all isolated single strains belong to a different genotype. Firstly, isolation of genotypes of all species followed the same protocol. Secondly, literature has shown that isolation of the same genotype from an amount of water, like we sampled in this study, is unlikely [1,2]. For microsatellite analysis DNA was extracted by adding 10 µL of TE-buffer to resuspend pellet of coccolithophores. Samples were then sonicated for 3 minutes at 100% and then incubated for 1h at 56°C. Microsatellite amplification was done in the following reaction mix: 2.5µL multiplex mastermix (Qiagen), 0.25 µL 5pM forward and reverse primer (primers EHMS15b and P02E10b for *E. huxleyi* [3], primers GE06 and GE07 (Table S2.) for *G. oceanica*), 1 µL Q solution (Qiagen), 0.5 µL H₂O and 0.5 µL of DNA template. The PCR reaction run for msat amplification was set up as follows: an initial phase of 15min at 95°C, 30 cycles of 30sec at 94°C, 90sec at 57°C and 1min at 72°C and final step of 30 min at 60°C. 1 µL PCR products was then added to a mix of ROX and Hidi (Qiagen) of 0.25 µL and 8.75 µL respectively and incubated for 3min at 94°C to denature double stranded products. The sequencer 3130xl Genetic Analyzer (Applied Biosciences) was then used to analyse microsatellite composition of each sample and data was analysed using GeneMarker software.

Table S1: All genotypes used are listed in the column “manuscript” under the name used in here and will be deposited in the Roscoff Culture Collection (RCC) under the name shown in the “RCC” column.

| <i>C. affinis</i> | | <i>E. huxleyi</i> | | <i>G. oceanica</i> | |
|-------------------|--------------|-------------------|-----------------|--------------------|---------------|
| Manuscript | RCC | Manuscript | RCC | Manuscript | RCC |
| B13 | EHGLL13 B | C48 | EHGKLC48_2 0 | GC31 | EHGLLGC3 1 |
| B75 | EHGLL57 B | C30 | EHGKLC30_2 0 | GC33 | EHGLLGC3 3 |
| B63 | EHGLL63 B | C35 | EHGKLC35_2 0 | GC36 | EHGLLGC3 6 |
| B64 | EHGLL64 B | C91 | EHGKLC91_2 0 | GC40 | EHGLLGC4 0 |
| B67 | EHGLL67 B | C96 | EHGKLC96_2 0 | GC58 | EHGLLGC5 8 |
| B68 | EHGLL68 B | C47 | EHGKLC47_2 0 | GC59 | EHGLLGC5 9 |
| B74 | EHGLL74 B | C41 | EHGKLC41_2 0 | GC60 | EHGLLGC6 0 |
| B81 | EHGLL81 B | C42 | EHGKLC42_2 0 | GC86 | EHGLLGC8 6 |
| B82 | EHGLL82 B | GC22 | EHGLLGC22 | GC89 | EHGLLGC8 9 |

Table S2: Primers used for *G. oceanica* msat analysis (strains N=9). Transcriptome as basis stems from Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP) [4] and is called Gephyrocapsa-oceanica-RCC1303

| Primer name | Sequence | Product length (from test) | Msat repeat unit | No. Alleles |
|---------------|-----------------------|----------------------------|----------------------|-------------|
| GE06Fm | GTAATTGTCGTACGCCCG | 162 | (CT) _{17.5} | 8 |
| GE06Rm | CCAGGAA-TAGACTTAGGCCG | | | |
| GE07Fm | TGTCTCAGAGTCTCGCGG | 189 | (TC) ₁₉ | 8 |
| GE07Rm | GAGTTTGTGGCTGTCCTTTC | | | |

S2: Relative contribution of genotypes in mixcultures

The mixcultures consisted of all genotypes of one species that initially contributed the same number of cells/mL. The relative contribution of each genotype was therefore 11 % at the start of the experiment (Figure S1). To avoid differences in initial concentrations of the respective genotypes’, the mixcultures were inoculated from a stock mixculture prepared just before the start of the experiment. As in the monoculture each experimental unit was inoculated with an initial total biovolume of 8280 $\mu\text{m}_3 \cdot \text{ml}^{-1}$ cells, resulting in

an initial concentration of 20 cells*ml⁻¹, 180 cells*ml⁻¹ and 24 cells*ml⁻¹ for *C. affinis*, *E. huxleyi* and *G. oceanica*, respectively.

Change in the relative contribution of genotypes in the mixcultures was not tested over the course of the experiment. While we assumed that the relative genotypic contribution changes over time [5], we, however, did not expect an actual loss of genotypes in this experiment with only one batch cycle. Personal experience with the same *E. huxleyi* genotypes showed that a significant change in relative abundance of the genotypes occurs after an experimental duration of four batch cycles while only one genotype was actually lost after this time. This suggests that genotypic exclusion in *E. huxleyi* takes longer than the experimental duration of this study. It also concurs with Lohbeck et al. (2012) demonstrating that after 160 days (corresponding to ~ 30 batch cycles) only half of the initially present six *E. huxleyi* genotypes were lost [6]. The maintenance of genotypes in comparatively short-termed experiments can, however, also depend on the number of initially present genotypes. Sjöqvist and Kremp (2016) showed that all genotypes in *Skeletonema marinoi* mixcultures remained present if the cultures started with lower genotypic richness (i.e. 5 genotypes). In contrast higher initial genotypic richness (i.e. 20 genotypes) enhanced exclusion [7]. The use of nine genotypes in this study corresponds to a mid-range genotype richness compared to Sjöqvist and Kremp's study, and thus supports that genotype exclusion is expected to be low or almost negligible in our short term experiment.

However, in future studies the negligible sorting and loss of genotypes over a short period of time (as argued above) should be assessed by analysis of the final genotype contribution after the experiment. This way the species reaction norm can be fully understood.

3 Species with each 9 genotypes (date of experiment 2016)

C. Affinis (11th-22nd July)



E. Huxleyi (9th-21st June)

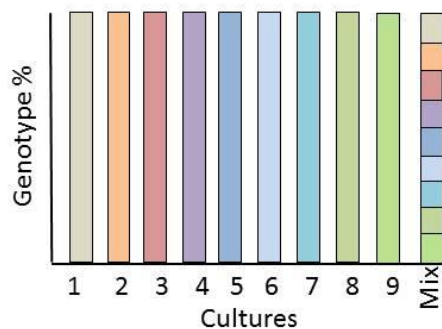


G. Oceanica (27th June- 12th July)



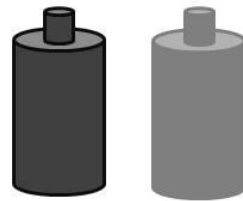
Each genotype grown in a monoculture and in a mix under ambient and high CO₂

Relative contribution of the genotypes in the mono- and mix-cultures of each species:



All cultures are exposed to a CO₂ treatment:

ambient (400ppm) high (1250ppm)



3X Replicated

Culture volume: 660ml

Medium: N-limited

Rotation : 0.75 min⁻¹

Temperature: 20 °C

Light intensity:
350 μmol*m⁻²*s⁻¹

Daily sampling and experimental length

The development of each culture was followed:

- fluorescence measurements -diatom
- cell counts - coccolithophores

Not more than 10% of the volume sampled through the experiment.

Termination of cultures on 3rd day in stationary phase (9-16 days experimental duration).

Figure S1: Overview of the experimental set up. Species and genotypes at the top, followed by general information of the cultivation as well as the CO₂ treatments and genotype composition in Mono- and Mixcultures. In the initial phase of the mixculture each of the nine genotypes contributes equally and is present with 11%. At the bottom are information about daily sampling as well as experimental length.

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Chapter II

Competition drives eco-evolutionary sorting among a two-species marine phytoplankton community

Competition drives eco-evolutionary sorting among a two-species marine phytoplankton community

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Abstract

Eco-evolutionary responses comprise species compositional change (an ecological process) and changes in genotypic composition within species (an evolutionary process). We studied eco-evolutionary marine phytoplankton responses to increasing dissolved CO₂ concentrations ("ocean acidification") by establishing an experimental system with co-existing members of two major phytoplankton groups, the coccolithophore *Emiliana huxleyi* (focal species) and the diatom *Chaetoceros affinis*, each composed of nine genotypes. We predicted a shift of dominances among species, making the diatom competitively superior because it is fertilized by CO₂ while the calcifying coccolithophore will be negatively impacted. Similarly, we hypothesized that genotypes of *E. huxleyi* show frequency increases as a result of selection to the same principles, i.e. gaining opportunity against conspecifics and being more tolerant to enhanced CO₂. In order to investigate the effect of competitive interactions in different CO₂ treatments, we compared our findings to simulations of a Lotka-Volterra growth model. In line with our expectations, we found *C. affinis* rapidly dominated the community and that there was a difference between the relative contributions in ambient and high CO₂. In contrast, genotype sorting in *E. huxleyi* did not differ between ambient and high CO₂ concentrations. Nevertheless, we found rapid genotypic selection causing the same three genotypes of *E. huxleyi* to remain in the population across replicates. Measured differences among genotypes in growth rate and carrying capacities were insufficient to adequately model time courses. Instead, after model parameter adjustment to simulate experimental data, we demonstrated that an inclusion of estimated competitive parameters was key to explain species and genotype sorting. Competitive processes were less strong in high CO₂ concentrations, which may help to compensate for potentially negative effects of enhanced CO₂ in marine phytoplankton communities.

Keywords: Eco-evolutionary sorting; CO₂; ocean acidification; Lotka-Volterra; competition; phytoplankton; *C. affinis*; *E. huxleyi*

Introduction

Community responses to a changing environment play out on two fundamental levels, either via species compositional change, an ecological process, or genotype selection, an evolutionary process. Both processes occur on similar timescales and mutually influence each other (Barraclough, 2015; Fussmann et al., 2007; Van Doorslaer et al., 2010). The interdependence of ecological and evolutionary processes has mainly been described in predator-prey or host-parasite systems (Ellner and Becks, 2010; Koch et al., 2014) and only recently attention shifted to systems where primary producers, such as phytoplankton, competitively interact (Litchman et al., 2012). Phytoplankton are major primary producers that constitute the basis of most pelagic food webs and play an important role in the global biogeochemical cycles of nitrogen, phosphate and silicate (diatoms only) (Falkowski et al., 2004, 2008). Due to their ecological importance and relatively short generation times, phytoplankton represent an ideal study system to understand eco-evolutionary dynamics.

Phytoplankton species as well as single genotypes of species differ in many traits, for example in (cell) size, (nutrient) resource requirements and uptake strategies and growth rates (Dutkiewicz et al., 2015; Fu et al., 2007; Hattich et al., 2017) and hence, react differently to environmental change. Diatoms, as most phytoplankton species (e.g. chlorophytes and cyanobacteria) are little affected by elevated CO₂ (Li et al., 2016; Schaum et al., 2012). Often, they even profit from elevated CO₂ due to enhanced photosynthesis and growth since dissolved inorganic carbon (DIC) limitation is alleviated. Calcifying species, such as coccolithophores, have been shown to respond with reduced growth and calcification rates (Riebesell et al., 2000). Findings on single species and genotypes are a first order indication how communities potentially change under future environmental conditions. However, such an approach largely excludes potential effects of inter- and intraspecific competition, which likely influences how environmental factors are affecting community structures.

Most natural phytoplankton populations are composed of many different interacting species and genotypes within species (Krueger-Hadfield et al., 2014; Rengefors et al., 2017). Hence, realistic phytoplankton experiments need to include diversity on the species and genotype level where selection on the species level is likely to play out first followed by prompt selection on the genotype level (Hairston et al., 2005). Diversity among and with-

in species requires coexistence and can be explained by resource competition theory. It posits that coexistence is only possible when different resource niches, dependent on nutrient uptake strategies and requirements, are occupied at least along one particular axis (Chesson, 2000; Sommer, 1984a; Tilman, 1977). Any environmental change moves available ecological niches, which implies that competitive dynamics change (Irwin et al., 2015; Litchman et al., 2012) with the prediction that species, which cope better with environmental change, should become competitively superior. Additionally, standing genetic variation allows intraspecific competition to play out as selection given that genotypic specific traits confer different fitness (Kremp et al., 2012).

In this study, we investigated the response of a phytoplankton community to increased seawater CO₂ allowing for intra- and interspecies competition and selection. We established a two-species community with the coccolithophore *Emiliana huxleyi*, and the diatom *Chaetoceros affinis*, representing two important phytoplankton groups. Each species consisted of nine genotypes. We exposed this community to ambient and increased CO₂ (predicted end of the century concentrations, (IPCC Fifth Assessment Report - Synthesis Report, 2014)). The two study-species were selected because they display fundamentally different nutrient requirements and different nutrient uptake related strategies, which enabled their coexistence in semi-continuous batch cycles – a prerequisite for the investigation of species compositional change. Diatoms (i.e. *C. affinis*) have high nutrient uptake rates and are consequently characterized by high maximum growth rates (Litchman et al., 2007). At the same time, diatoms have high half saturation constants for nutrient uptake (Litchman et al., 2007). As such they represent the ‘velocity-adapted’ species (Sommer, 1984b) and thrive in nutrient replete and fluctuating conditions, being able to rapidly monopolize nutrients. Coccolithophores (i.e. *E. huxleyi*) in contrast, have lower maximum nutrient uptake rates. Hence, after a nutrient pulse they initially lose out against diatoms. Their lower half-saturation constants, however, makes them the better competitors under nutrient poor conditions occurring at later successional stages. To further understand observed changes in the community, we applied a Lotka-Volterra model with growth rate and carrying capacities from our two species (Hattich et al., 2017), as well as estimated parameters of competitive strengths.

Our objectives were to understand (1) how a two-species community of marine phytoplankton responds on the species and genotype level to increased seawater CO₂ and (2) qualitatively assess the major interactive processes underlying the observed eco-

evolutionary dynamics. We predict that *C. affinis* will dominate the community with an even higher contribution under high CO₂. To examine the dynamics of genotype sorting as a function of CO₂ concentrations, we focused on changes in *E. huxleyi* genotype composition. Given that differences among clones in their susceptibility to CO₂ have been described (Hattich et al., 2017; Lohbeck et al., 2012), we predict different selection dynamics, visible as divergent genotype sorting, among both CO₂ treatments. In order to assess the contribution of competition to the observed dynamics (second objective), we applied a simple Lotka-Volterra model to describe the temporal dynamics. It was parametrized based on experimental data and estimations. By including or excluding the interspecific and intraspecific competition terms, we compared the ability of the model to simulate species and genotype sorting in ambient and high CO₂ conditions.

Material and Methods

Experimental set-up and culturing conditions

The experiment was conducted with a simplified community comprising of two species, the diatom *C. affinis* and the coccolithophore *E. huxleyi*. Each species was present with nine different genotypes. All genotypes used were isolates originating from one geographical region (Gran Canary, 27°59'N 15°22'W, isolated 2012 and 2015) and are deposited in the culture collection in Roscoff (see Hattich et al 2017). Three replicate cultures were each exposed to ambient and high CO₂ conditions (400 and 1200ppm, respectively; 1200 ppm represents predicted end of the century conditions) for 4.3 months (16 batch cycles each lasting 8 days, minimum of 70 generations). We followed species sorting over time by light microscopy as well as genotype sorting in *E. huxleyi* via reisolation at three time points during the experiment.

The ambient and high CO₂ treatment was manipulated by aerating the artificial-seawater (35PSU salinity; after (Kester et al., 1967)) for 24h with CO₂-enriched air (400 and 1200ppm, respectively) prior to each batch cycle. The dissolved inorganic carbon (Hansen et al., 2013) varied between 2196.37±58.05 and 2331.50±50.26 μmol*kg⁻¹ with a total alkalinity (following (Matthiessen et al., 2012)) of 2448.61±36.03 and 2435.85±16.31 μmol*kg⁻¹ for ambient and high CO₂, respectively. Nutrients were added to the final concentrations of 19.94±0.47 μmol*L⁻¹ nitrate, 1.06±0.19 μmol*L⁻¹ phosphate and 4.40±0.38 μmol*L⁻¹ silicate. Vitamin and trace metals were added in f/8 concentration (Guillard, 1975). The prepared medium was sterile filtrated (0.2 μm) into the exper-

imental units (0.5L polycarbonate bottle). At the start of the experiment each experimental unit was inoculated with an initial total biovolume of $8280 \mu\text{m}^3 \cdot \text{mL}^{-1}$. The different species were added at a ratio of 1:1 of biovolume with all genotypes contributing equally. The experiment was carried out under constant rotation (0.75 min^{-1}) at 20°C and a 17:7 day:night cycle reaching a maximum light intensity of $350 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ 3h after dusk and dawn.

At the end of each batch cycle cell number and cell biovolume were determined using microscopy (Zeiss Axiovert Observer) to calculate the contribution of each species to the culture and the amount needed to transfer to the next batch cycle; again $8280 \mu\text{m}^3 \cdot \text{mL}^{-1}$. A sample of each experimental unit was photographed five times and biovolume of both species determined after Hillebrand et al. (1999). *E. huxleyi*'s biovolume was calculated as $\frac{1}{6} \times \pi \times d^3$ (d=diameter) and *C. affinis*' biovolume was calculated as $\pi \times \left(\frac{b}{2}\right)^2 \times a$ (a=length, b=width). Biovolume/mL was then calculated by multiplying cells/mL with the mean biovolume of the five measurements.

Reisolation of E. huxleyi genotypes and microsatellite analysis

E. huxleyi genotypes could be unambiguously identified via microsatellite genotyping (Hattich et al. 2017, supplementary), allowing us to monitor genotype shifts in the different treatments. For the quantification of *E. huxleyi* genotypes a maximum of 20 cells per culture were re-isolated by dilution in 48 well plates. This provides a detection limit of 5% difference between the contribution of the genotypes to the *E. huxleyi* population. When left unmoved in culture plates, a single *E. huxleyi* cell forms a bacterial-like "benthic" colony, which can be easily detected after ten days. Fresh medium (500 μL) was added to each well with growing colonies and cells were grown for another ten days. Cells were then centrifuged and the pellets of *E. huxleyi* were used for DNA extraction and further microsatellite analysis. *E. huxleyi* DNA was extracted by adding 15 μL of TE-buffer to re-suspend the pellet. Samples were then sonicated for three minutes at 100% and then incubated for 1h at 56°C . Microsatellite amplification was done following the protocol described in (Hattich et al., 2017, supplementary material) with the following primers: Primer S15 (Access. No. AJ487032 (F) AJ487305 (R)) and Primer E10 (Access. No. AJ487314 (F) AJ487315 (R)). The relative contribution of specific genotypes to the total population of *E. huxleyi* was calculated as (no. of certain genotype/no. of genotypes analyzed per culture).

Statistics

For both the analysis of the ecological sorting and the evolutionary sorting relative data (i.e. relative contribution of species' to total biovolume and relative *E. huxleyi* genotype abundance to *E. huxleyi* biovolume, respectively) was used. For the analysis of the relative biovolume we used a repeated measures analysis of variance (anova). Before starting the analysis, we tested for the homogeneity of variance using a Fligner Killeen Test (Fligner and Killeen, 1976) and for the normality of the residuals using a Shapiro-Wilk Normality Test (Shapiro and Wilk, 1965). Another prerequisite for a repeated measures anova is sphericity, which was tested using a Mauchly's Test for Sphericity (Mauchly, 1940). All assumptions were met so that we could continue with the analysis without data transformation. We started with the overall analysis of the effects of batch cycle (i.e. time) and CO₂ treatment on the relative biovolume. Since we only had two species in the community, this test was only necessary for one species because the relative biovolumes depended on each other. we focused on three time points during the experiment (batch cycle (BC)4 “short-“, BC8 “mid-“ and BC16 “longterm”, respectively) and started with a complete data set analysis (aov((rel_BioV)~(Time*CO₂)+Error(Replicate/(Time))). We then continued analyzing the time points separately by subsetting the dataset into BC4, BC8 and BC16 (aov(rel_BioV)~CO₂)). A permutational multivariate analysis of variance (permanova, with 999 permutations) was used to test for the differences in genotype compositional change in *E. huxleyi* between the treatments. The assumption of equal multivariate spread between the treatments was tested using an anova comparing the beta diversities of the treatment groups. All modelling and statistical analysis was done using the software R (R Coreteam 2016). The following packages were used for the analysis, plotting and modelling: ggplot2, deSolve, vegan, ez.

Model simulation

In order to differentiate between inter- and intraspecific competition driving the temporal change under ambient and high CO₂ conditions, we applied a simple Lotka-Volterra model and compared the outcome of the modeling data with experimental data. Lotka Volterra introduced already in 1928 (Volterra, 1928) mathematical solutions to one-to multispecies growth models, where they compete for resources or feed on each other.

R core software (R core team 2016) was used to write a competitive Lotka-Volterra-model:

$$\frac{dx_i}{dt} = r_i x_i \left(1 - \frac{(\sum_{i=1}^N \alpha x_i) + (\sum_{j=1}^N \beta x_j)}{K_i} \right)$$

$$\frac{dx_j}{dt} = r_j x_j \left(1 - \frac{(\sum_{i=1}^N \gamma x_i) + (\sum_{j=1}^N \delta x_j)}{K_j} \right)$$

consisting of the following components:

$$x_i = \text{species } i, \text{ genotype } 1 - 9$$

$$x_j = \text{species } j, \text{ genotype } 1 - 9$$

$$r_i = \text{growth rates of species } i, \text{ genotype } 1 - 9$$

$$r_j = \text{growth rates of species } j, \text{ genotype } 1 - 9$$

$$K_i = K \text{ for species } i, \text{ genotype } 1 - 9$$

$$K_j = K \text{ for species } j, \text{ genotype } 1 - 9$$

$$\alpha = \text{intraspecific competitive effect of species } i$$

$$\beta = \text{interspecific competitive effect of species } j \text{ on } i$$

$$\gamma = \text{intraspecific competitive effect of species } j$$

$$\delta = \text{interspecific competitive effect of species } i \text{ on } j$$

The model included two species ($i=Emiliana\ huxleyi$, $j=Chaetoceros\ affinis$) consisting of nine genotypes each, in essence we then had a model of 18 individuals that affected each other differently (see inter- and intraspecific competitive effect). For each genotype per species, a single function was formulated including specific growth rates (r), carrying capacity (K) and estimations for the inter- and intraspecific competitive parameters. Growth rates and carrying capacities stem from measured data in ambient and high CO_2 from a previous experiment (Hattich et al 2017). The inter- and intraspecific competitive parameters reflect in a phenomenological sense an interactive depression among competitors, which are not the performance of K and r . We estimated and adjusted these parameters such that the model output would reflect the experimental data. The functions were solved numerically for 16 iterations (batch cycles) using time steps of one and the length of eight for one batch cycle using the package 'deSolve' (Soetaert et al., 2010). To compare the numerical output of the model with the experimental data we calculated the 95%

confidence interval (95% CI) of the experimental data and checked, if the model output fell within the limits of the 95% CI. For each of the three time points, the treatments were assessed separately. This was done for the relative contributions of the species to the community and the relative contributions of *E. huxleyi* genotypes to the *E. huxleyi* population. By assessing the congruence of the model data, first without and then with an explicit competition term, with the experimental data, we intended to draw qualitative inferences of underlying processes in our experimental system. Here we focused on the effects of intra- and interspecific competition on differential species and genotype sorting in both CO₂ treatments.

Results

Ecological (species) sorting

The diatom *C. affinis* dominated throughout the 16 batch cycles and contributed considerably more biovolume (as proxy for biomass) than the coccolithophore *E. huxleyi*, sometimes up to nine-fold (in high CO₂) (Figure 1a)). In the high CO₂ treatment, *C. affinis* was as expected more dominant than in the ambient CO₂ treatment, whereas the opposite naturally was true for *E. huxleyi* (Table 1 relative biovolume “CO₂”, $F_{1,4}=12.28$, $p=0.023$). This pattern did not change between the three time points analyzed (Table 1 relative biovolume no effect of “Time”). After initial marked divergence of biovolumes during the first batch cycles, *E. huxleyi* biovolumes continually increased to be at 30% and 14% (ambient and high CO₂, respectively) of the total biovolume at batch cycle 16. After four batch cycles the species sorting had reached a first separation between ambient and high CO₂ (Table 1 relative biovolume BC4: “CO₂” $F_{1,4}=8.947$, $p=0.040$), which is reduced towards batch cycle 8 and then increased again towards batch cycle 16 (Table 1 relative biovolume BC16: “CO₂” $F_{1,4}=8.862$, $p=0.041$).

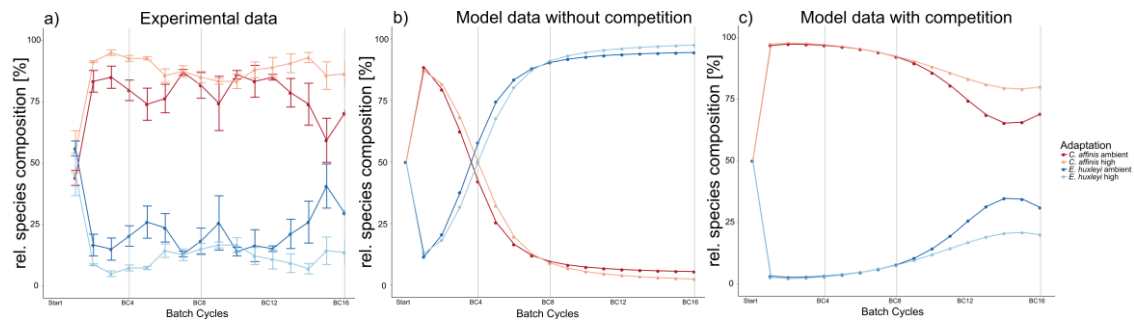


Figure 1: Relative species contribution to community over 16 batch cycles (BC). Dark and light line colors of the relative biovolume of *C. affinis* (red) and *E. huxleyi* (blue) refer to species sorting in ambient and high CO₂ concentrations, respectively. **a)** shows experimental data whereas **b)** and **c)** show modeling data without and with competition, respectively.

Evolutionary (genotype) sorting

For the genotypes of *E. huxleyi* competitive exclusion was swift during the course of the experiment. We hypothesized that the genotypes of *E. huxleyi* show varying responses to altered CO₂, resulting in differential genotype sorting but we did not find any differences in the sorting between ambient and high CO₂ treatment. We could only detect significant effect of time (Table 1 Genotype sorting: “Time” $F_{3,16}=14.3203$, $p=0.001$): after four batch cycles four and three of nine genotypes (ambient and high CO₂, respectively) were still detectable with mostly similar contributions (Figure 2a). The strong contribution of the three genotypes C42, C41 and GC22 remained until batch cycle 16 indicating strong genotype sorting at the beginning of the experiment that eventually resulted in competitive exclusion of six genotypes.

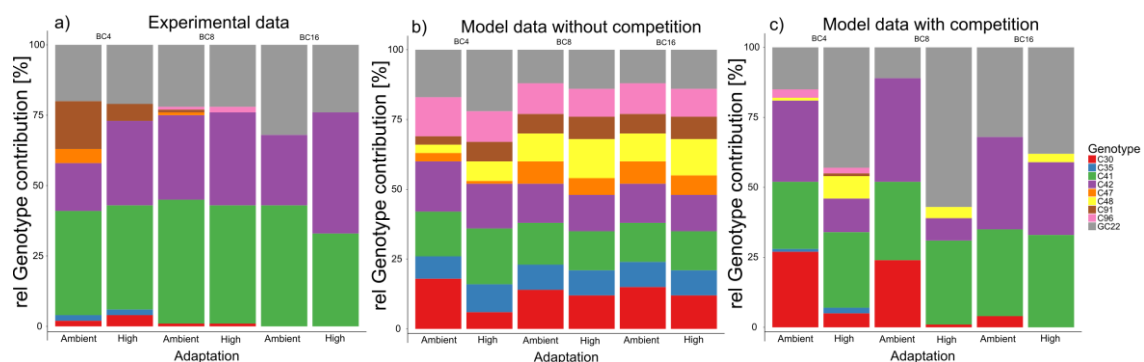


Figure 2: Relative genotype contribution to community. Bar charts show *E. huxleyi* relative genotype contributions to community at three time points over the course of the experiment in ambient and high CO₂ concentration. **a)** shows experimental data whereas **b)** and **c)** show modeling data without and with competition, respectively.

Table 1: statistical analysis results

| <i>Relative Biovolume</i> | | | | | <i>Genotype sorting</i> | | | | | |
|-------------------------------------|----|--------|---------|---------|----------------------------------|----|---------|----------|----------------|---------|
| <i>Error: Replicate</i> | df | MS | F-value | P-Value | | df | MS | F Mod-el | R ² | P-Value |
| <i>CO₂</i> | 1 | 522.7 | 12.28 | 0.023 | <i>Time</i> | 3 | 0.36015 | 14.3202 | 0.67204 | 0.001 |
| <i>Residuals</i> | 4 | 42.6 | | | <i>CO₂</i> | 1 | 0.05350 | 2.1272 | 0.03328 | 0.124 |
| <i>Error: Replicate: × Time</i> | | | | | <i>Time × CO₂</i> | 3 | 0.02379 | 0.9459 | 0.04439 | 0.473 |
| <i>Time</i> | 2 | 94.39 | 2.412 | 0.151 | <i>Residuals</i> | 16 | 0.02515 | | 0.25029 | |
| <i>Time × CO₂</i> | 2 | 65.72 | 1.679 | 0.246 | | | | | | |
| <i>Residuals</i> | 8 | 39.14 | | | | | | | | |
| BC4 | | | | | | | | | | |
| <i>CO₂</i> | 1 | 253.50 | 8.947 | 0.040 | | | | | | |
| <i>Residuals</i> | 4 | 28.33 | | | | | | | | |
| BC8 | | | | | | | | | | |
| <i>CO₂</i> | 1 | 16.67 | 0.339 | 0.592 | | | | | | |
| <i>Residuals</i> | 4 | 49.17 | | | | | | | | |
| BC16 | | | | | | | | | | |
| <i>CO₂</i> | 1 | 384.0 | 8.862 | 0.041 | | | | | | |
| <i>Residuals</i> | 4 | 43.3 | | | | | | | | |

Inferences from Lotka-Volterra simulations

Only when we included growth rate (r) and carrying capacity (K) derived from a previous experiment (Hattich et al., (2017) (Table 2), the model runs revealed that *E. huxleyi* dominated the community without any difference between ambient and high CO₂ (Figure 1b). After 16 batch cycles the genotype composition of *E. huxleyi* was different. (Figure 2b). The remaining genotypes were C30, C41, and C42 in ambient CO₂ and C41 and GC22 in high CO₂ conditions. These genotypes have the highest growth rates and carrying capacities (supplementary Figure 1 and 2).

A critical improvement of model runs was observed only when we included a dedicated interspecific competition term (Table 2) into our model. We were able to simulate the dominance of *C. affinis* as in the experimental data throughout all 16 batch cycles. As in the experimental data, differences between the CO₂ treatments emerged after eight batch cycles (Figure 2c) (model data in 95% CI of experimental data in BC 9-16). The estimated interspecific competition of *C. affinis* on *E. huxleyi* was higher than interspecific competition of *E. huxleyi* on *C. affinis* in both CO₂ treatments. These findings resemble differences in attainable biovolume of *C. affinis* and *E. huxleyi* growing with and without the respective other species. The congruence of model and experimental data of the relative contributions of the *E. huxleyi* genotypes between the CO₂ treatments (Figure 2.c) when intraspecific competition (Table 1) was included only appeared at the last point of comparison (BC 16): we could simulate the dominance of genotypes C41, C42 and GC22 in

ambient and high CO₂. Intraspecific parameters were different in the CO₂ treatments and also between the dominating genotypes.

Table 2: Parameters used for the Lotka-Volterra model and their data source. In the first model runs only growth rate and carrying capacity were included. In the subsequent model runs all competition parameters were included

| CO ₂ Treatment | Growth rate r measured in Hattich et al. 2017 | | Carrying capacity K measured in same experiment as Hattich et al. 2017 | Growth rate r measured in Hattich et al. 2017 | | Carrying capacity K measured in same experiment as Hattich et al. 2017 | |
|---------------------------|---|-----------------------|---|--|-----------------------|---|-----|
| ambient | C30 | 0.847 | 4.807*10 ⁷ | B13 | 0.881 | 0.343*10 ⁷ | |
| | C35 | 0.785 | 3.267*10 ⁷ | B57 | 1.517 | 0.500*10 ⁷ | |
| | C41 | 0.828 | 4.801*10 ⁷ | B63 | 1.525 | 0.942*10 ⁷ | |
| | C42 | 0.857 | 4.446*10 ⁷ | B64 | 1.557 | 0.528*10 ⁷ | |
| | C47 | 0.737 | 3.119*10 ⁷ | B67 | 1.611 | 1.309*10 ⁷ | |
| | C48 | 0.729 | 4.018*10 ⁷ | B68 | 1.480 | 0.737*10 ⁷ | |
| | C91 | 0.735 | 2.842*10 ⁷ | B74 | 1.294 | 0.453*10 ⁷ | |
| | C96 | 0.853 | 3.431*10 ⁷ | B81 | 1.455 | 1.273*10 ⁷ | |
| GC22 | 0.863 | 3.940*10 ⁷ | B82 | 1.456 | 1.273*10 ⁷ | | |
| high | C30 | 0.750 | 4.170*10 ⁷ | B13 | 0.965 | 0.383*10 ⁷ | |
| | C35 | 0.786 | 2.800*10 ⁷ | B57 | 1.390 | 0.500*10 ⁷ | |
| | C41 | 0.823 | 4.094*10 ⁷ | B63 | 1.252 | 1.141*10 ⁷ | |
| | C42 | 0.797 | 3.895*10 ⁷ | B64 | 1.517 | 0.738*10 ⁷ | |
| | C47 | 0.695 | 3.122*10 ⁷ | B67 | 1.485 | 1.182*10 ⁷ | |
| | C48 | 0.755 | 4.526*10 ⁷ | B68 | 1.538 | 0.886*10 ⁷ | |
| | C91 | 0.764 | 2.708*10 ⁷ | B74 | 1.233 | 0.614*10 ⁷ | |
| | C96 | 0.790 | 3.084*10 ⁷ | B81 | 1.399 | 0.613*10 ⁷ | |
| GC22 | 0.852 | 3.940*10 ⁷ | B82 | 1.565 | 0.613*10 ⁷ | | |
| CO ₂ Treatment | α (intraspecific competition <i>E. huxleyi</i>) adjusted to reproduce experimental genotype sorting | | β (interspecific competition <i>C. affinis</i> on <i>E. huxleyi</i>) adjusted following Carrara et al 2015 and to reproduce experimental species sorting | γ (interspecific competition <i>E. huxleyi</i> on <i>C. affinis</i>) adjusted following Carrara et al 2015 and to reproduce experimental species sorting | | δ (intraspecific competition <i>C. affinis</i>) estimated; mean value because not focal species | |
| ambient | C30 | -8 | 0.54 | 0.3 | | B13 | 0.1 |
| | C35 | 0.1 | | | | B57 | 0.1 |
| | C41 | 0.7 | | | | B63 | 0.1 |
| | C42 | 0.2 | | | | B64 | 0.1 |
| | C47 | 0.1 | | | | B67 | 0.1 |
| | C48 | 0.1 | | | | B68 | 0.1 |
| | C91 | 0.1 | | | | B74 | 0.1 |
| | C96 | 0.1 | | | | B81 | 0.1 |
| GC22 | 1 | B82 | 0.1 | | | | |
| high | C30 | 0.2 | 0.27 | 0.1 | | B13 | 0.1 |
| | C35 | 0.2 | | | | B57 | 0.1 |
| | C41 | -0.3 | | | | B63 | 0.1 |
| | C42 | 3 | | | | B64 | 0.1 |
| | C47 | 0.2 | | | | B67 | 0.1 |
| | C48 | 0.2 | | | | B68 | 0.1 |
| | C91 | 0.2 | | | | B74 | 0.1 |
| | C96 | 0.2 | | | | B81 | 0.1 |
| GC22 | -0.4 | B82 | 0.1 | | | | |

Discussion

In this study, we investigated how elevated CO₂ concentrations modulated the composition of a two-species community on the species and genotype level and what possible interactive mechanisms affected this sorting. The community consisted of two members of important phytoplankton groups, one diatom (*Chaetoceros affinis*) and one coccolithophore (*Emiliania huxleyi*). As expected, we found that despite marked fluctuations the

dominance of *C. affinis* was favored by high CO₂. In contrast, genotype sorting was similar in both CO₂ environments. Furthermore, we have successfully applied a Lotka-Volterra model to understand what modulates species and genotype sorting and thus competitive dynamics in the experiment. Species and genotype sorting in the model only matched the experimental data when competition parameters were included.

On the interspecific level, we have shown that the environmental driver CO₂ can influence community compositional change. When we excluded competitive parameters the model results were only driven by data derived from monoculture experiments, but this was not enough to explain the experimental sorting. Here we showed that species interactions are essential to predict changes in community structure under future environmental conditions. The differences between the competitive parameters were similar to the difference in attainable biovolume of the species when growing with and without the respective other species. The concept of differences in attainable biovolume relating to competition (Carrara et al., 2015) was here transferred from terrestrial plants to a marine phytoplankton community and worked very well indicating a fundamental concept of resource division among community players. With the diatom as a competitor *E. huxleyi* attains lower carrying capacity than without (supplementary Figure S1). In contrast, for *C. affinis* the carrying capacity is the same with and without *E. huxleyi* as a competitor. In order to ensure coexistence of the two species we limited the silicate concentrations to ca 5µM in our study. It is possible that the low silicate concentrations constrained the growth of *C. affinis* and as such was unaffected by the coccolithophore. From this followed that interspecific competition of *C. affinis* on *E. huxleyi* (β) should be higher than the interspecific competition of *E. huxleyi* on *C. affinis* (γ) which was comparable to the estimated interspecific competitive parameters that we included in the model. CO₂ can be used as a carbon resource and thus alleviate DIC limitation on *C. affinis* (Sabia et al., 2018) but due to calcification processes it has a slightly negative effect on *E. huxleyi* (Riebesell et al., 2000). These opposing effects on the phytoplankton species may have enhanced the divergence in the community in high CO₂ concentrations.

Contrary to our expectations, genotype sorting in the experiment was similar in both CO₂ environments. The investigation of the intraspecific competition from the simulated model data was then used to understand this similarity. From previous data, we have seen that the growth rates and carrying capacities vary between genotypes and CO₂ treatments leading to different reaction norms for CO₂ treatments (Hattich et al., 2017) (Figure S3).

The genotypes with the highest growth rates and carrying capacities in the respective CO₂ concentrations were the ones that the simulations predicted when we only included these measurements. Nevertheless, to find congruence between model and experimental data, we needed to include competitive parameters that reflect direct negative effects of genotypes onto one another. One process determining the competitive term could be the usage of nitrate, which always became limiting at the end of the experimental batch cycle. To substantiate this, we compared data for nitrate affinities (a) in ambient and high CO₂ (supplementary figure S4) obtained in earlier pilot experiments (supplementary material S1) with the actual rank order of competitiveness of *E. huxleyi* genotypes. We focused on the genotypes that the model predicted to remain until batch cycle 16 in ambient and high CO₂ (C30, C41, C42 and GC22): in ambient CO₂ genotype C30 displayed the lowest affinity for nitrate while GC22 one of the highest, indicating weak and strong competitive abilities, respectively. In high CO₂, the competitiveness was low for genotype GC22 and C41 but one of the highest for genotype C42. These orders can be found in the nitrate affinities as well. So even though we did see different reaction norms for CO₂ regarding carrying capacities, growth rates and nutrient affinities we argue that these effects could have been too small to play out against other regulating factors such as allelopathy (Legrand et al., 2003) that lead to similar genotype sorting in the experiment. Seeing the parallels between nitrate affinity and competitive ranking in the model indicates that further understanding of nutrient uptake related strategies and mechanisms should be focused on in future studies. These may play a very important role in eco-evolutionary dynamics and how communities react to environmental change.

A limitation of our study is the time scale of less than 100 generations and that we did not find the expected effect of CO₂ on the genotype level. It is unclear how a community will change when evolution, owing to novel mutations, is allowed under 100+ generations but also how another environmental driver would affect eco-evolutionary dynamics. Evolutionary adaptation in single strains of phytoplankton have already been shown in different studies including thermal adaptation (Listmann et al., 2016) and adaptation to CO₂ (Lohbeck et al., 2012) in coccolithophores and effects of elevated temperature and nutrients in other phytoplankton species (Thomas et al., 2017). These experimental evolution studies investigated adaptation over time scales of more than one year, corresponding to 400 asexual generations or more. Interestingly, a three months study in large outdoor mesocosms (Scheinin et al., 2015) demonstrated an evolutionary response of a diatom

species to acidification on a much faster scale: when re-isolating diatom genotypes and measuring their reaction to CO₂ strong fitness increases were seen. The authors explain this with fast genotype sorting for the fittest genotypes. Therefore, it is important to expand experimental evolution in the laboratory to simulate a more natural setting (i.e. two or more interacting species and standing genetic variation) to assess how species and genotypes can adapt under realistic conditions.

We have here shown a first approximation of how a two species/several genotypes-community is affected by the environmental driver CO₂ and how useful the combination of experimental and model data is to understand underlying eco-evolutionary processes. Only when we combined model simulations with experimental data to understand competitive dynamics, we were able to explain why expectations from monoclonal experiments were not seen in the experimental community.

Overview supplementary material

Supplementary material S1: K_s-value experiment

Supplementary material S2: supplementary figures

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Additional information Chapter II

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Supplementary Material Chapter II:

Supplementary figures to Chapter II:

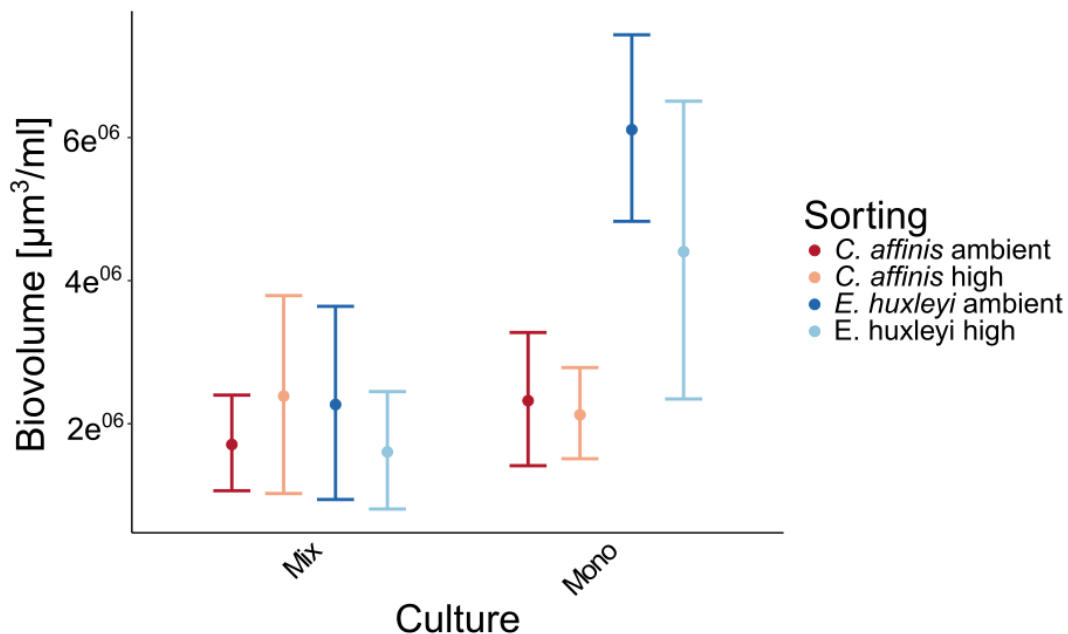


Figure S1: Biovolume in mono and mix cultures after batch cycle 1: Dark and light colors of the biovolume of *C. affinis* (red) and *E. huxleyi* (blue) refer to attainable biovolume in ambient and high CO₂ concentrations, respectively. On the left the biovolume in a mix culture is shown whereas on the right the biovolume in a mono culture is shown.

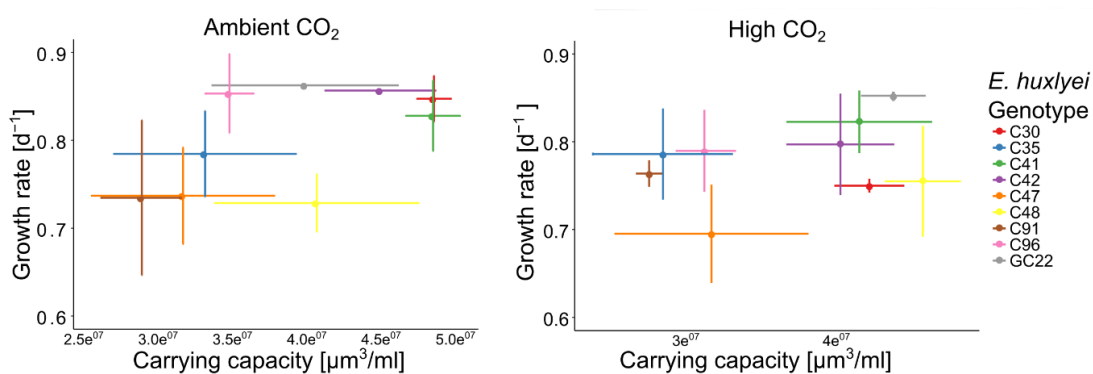


Figure S2: Growth rate and carrying capacities for each *E. huxleyi* genotype in ambient (left) and high (right) CO₂

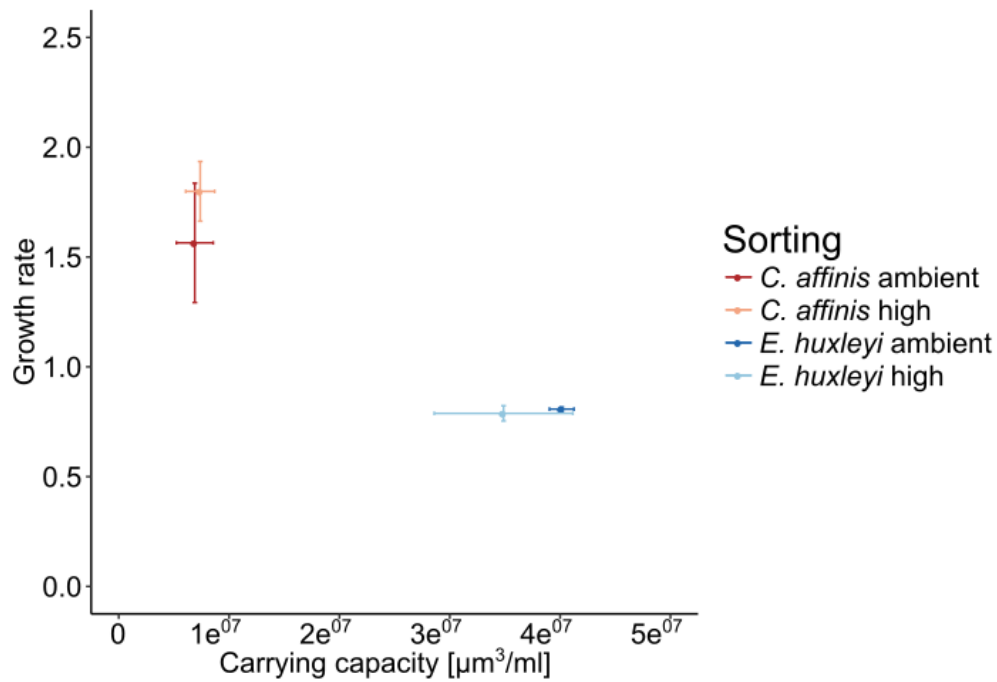


Figure S3: Growth rate and carrying capacities for *E. huxleyi* and *C. affinis* in mono culture.

Dark and light colors refer to the *C. affinis* (red) and *E. huxleyi* (blue) attainable biovolume and growth rates when they are growing without a competitor.

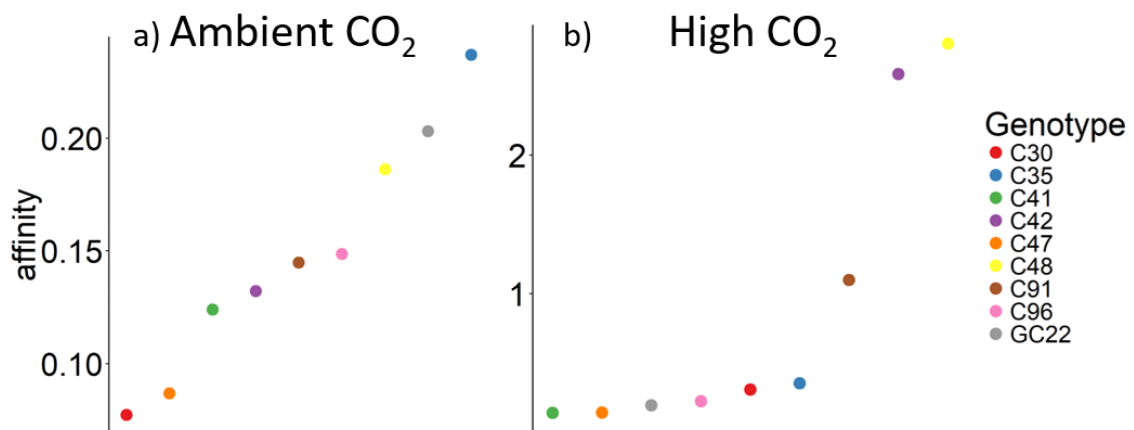


Figure S4: Affinity for nitrate for each *E. huxleyi* genotype in ambient (left) and high (right) CO₂:

Affinities were calculated from monod curve fitting (see supplementary material affinity for nitrate). Note that the axes are different indicating higher overall affinities to nitrate when genotypes are grown in high CO₂. Higher affinities indicate stronger competitive abilities.

Supplementary material S1 – nutrient affinity experiment

For each genotype of *Emiliana huxleyi* (*E. huxleyi*) we calculated a K_S value for nitrogen (N). We measured the growth rates for each genotype at eight different nitrogen concentrations (0; 2.09; 4.55; 9.81; 14.83; 20.07; 30.79; 41.23 $\mu\text{mol/l}$). Each culture was inoculated with a biovolume of 8280 $\mu\text{m}_3 \cdot \text{ml}^{-1}$ resulting in 1900 cells/ml in ambient CO_2 and 2300 cells/ml in high CO_2 . The experiment was carried out under constant rotation (0.75 min^{-1}) at 20 °C and a 17:7 day:night cycle reaching a maximum light intensity of 350 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ after 3h dusk and dawn. The development of each culture was followed throughout the experiment by daily cell counts for the coccolithophores (Z2TM COULTER COUNTER®).

Growth rates were determined by fitting an exponential growth model inbuilt in the R package “growthrates” (Maintainer & Petzoldt 2016) ($N \geq 9.81$) and manual calculation of growth rates by fitting a regression line through \ln plotted cell numbers ($N \leq 4.55$ in ambient and $N >$ in high CO_2). For very low nutrient concentrations growth was difficult to detect. Thus to avoid under- or overestimation we decided to only consider cultures where the cell number had doubled over the experimental duration.

K_S values were calculated by fitting a monod model inbuilt in the R package “FME” (Soetaert & Petzoldt 2010) to the measured nutrient concentrations and calculated growth rates for each genotype separately. The K_S value was then calculated with the following formula:

$$K_S = \frac{\left(\frac{y_{max}}{2} * p_2\right)}{\left(p_1 - \frac{y_{max}}{2}\right)}$$

using the parameters p_1 and p_2 and $\frac{y_{max}}{2}$ from the model fit. Figure S1 shows the model fits to each genotype in R in ambient and high CO_2 . The affinity was then calculated as $a = \frac{y_{max}}{K_S}$ (Figure S2).

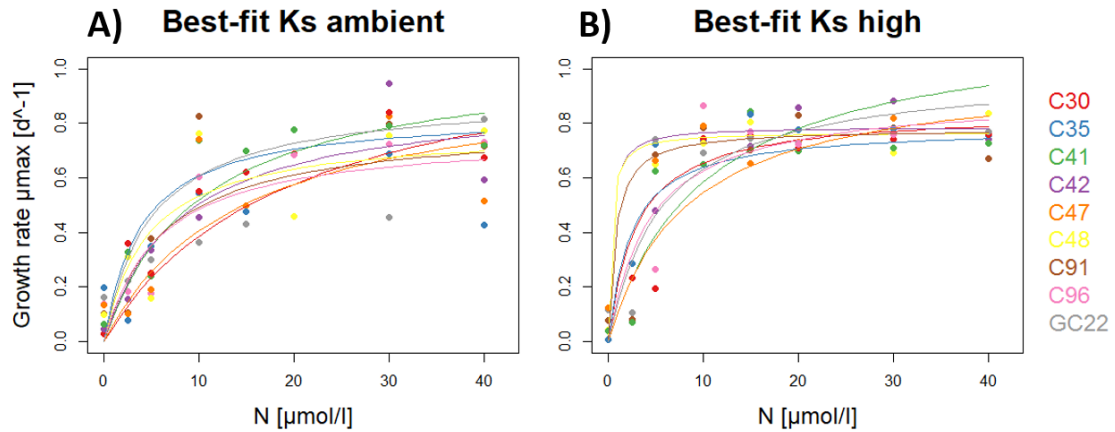


Figure S1: Model fit of all genotypes to calculate K_s values in ambient (A) and high (B) CO_2 conditions.

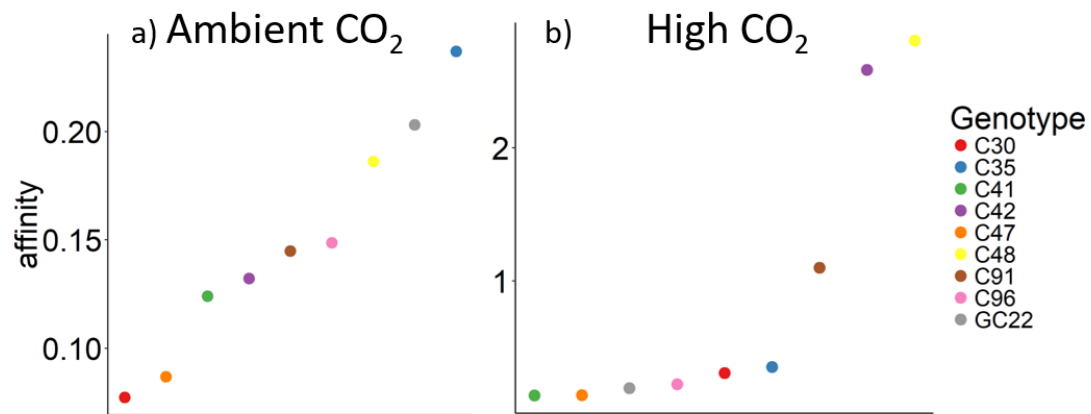


Figure S2: Affinity for nitrate for each *E. huxleyi* genotype in ambient (left) and high (right) CO_2 :

Affinities were calculated from monod curve fitting (see supplementary material affinity for nitrate). Note that the axes are different indicating higher overall affinities to nitrate when genotypes are grown in high CO_2 . Higher affinities indicate stronger competitive abilities.

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Chapter III

Swift thermal reaction norm evolution in a key marine phytoplankton species

Swift thermal reaction norm evolution in a key marine phytoplankton species

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Abstract

Temperature has a profound effect on the species composition and physiology of marine phytoplankton, a polyphyletic group of microbes responsible for half of global primary production. Here we ask whether and how thermal reaction norms in a key calcifying species, the coccolithophore *Emiliana huxleyi*, change as a result of 2.5 years of experimental evolution to a temperature $\approx 2^\circ\text{C}$ below its upper thermal limit. Replicate experimental populations derived from a single genotype isolated from Norwegian coastal waters were grown at two temperatures for 2.5 yrs before assessing thermal responses at 6 temperatures from 15-26°C, with pCO₂ (400/1100/2200 μatm) as a fully factorial additional factor. The two selection temperatures (15°/26.3°C) led to a marked divergence of thermal reaction norms. Optimal growth temperatures were 0.7°C higher in experimental populations selected at 26.3°C than those selected at 15.0°C. An additional negative effect of high pCO₂ on maximal growth rate (8% decrease relative to lowest level) was observed. Finally, the maximum persistence temperature (T_{max}) differed by 1-3°C between experimental treatments, as a result of an interaction between pCO₂ and the temperature selection. Taken together, we demonstrate that several attributes of thermal reaction norms in phytoplankton may change faster than the predicted progression of ocean warming.

Key words: adaptation, coccolithophore, experimental evolution, global warming, phytoplankton, reaction norm, temperature

Introduction

Temperature has an overriding effect on species composition, photosynthetic performance and growth rates of marine phytoplankton (Eppley 1972; Raven and Geider 1988; Thomas et al. 2012; Boyd et al. 2013). The thermal physiology of phytoplankton species broadly corresponds to mean temperature values within their climate zone. For example, in tropical species, optimal temperatures and upper thermal limits are higher compared to temperate or polar species (Thomas et al. 2012; Boyd et al. 2013; Thomas et al. 2016). There is also preliminary evidence that temperature niche width is correlated with the annual temperature variation, with temperate species displaying a wider temperature range than both tropical / subtropical and polar species (Boyd et al. 2013) although this pattern was not observed in a recent literature compilation (Thomas et al. 2016).

At the within-species level, there are also appreciable differences in thermal responses on growth and photosynthesis rates (Brand 1982; Wood and Leatham 1992) that likely have a heritable basis (Zhang et al. 2014). In the light of global change impacting all marine and terrestrial ecosystems today, such within-population diversity may provide essential standing genetic variation for populations to track climate change via genotypic selection and hence adaptive evolution at the population level (Reusch and Boyd 2013; Collins et al. 2014).

Although there have been some recent evolution experiments in the phytoplankton to ocean acidification and warming (e.g. Lohbeck et al. 2012; Schlüter et al. 2014; Hutchins et al. 2015), we currently do not know the time scales over which thermal reaction norms evolve, the response in growth and other traits to a range of environmental temperatures. The question of how rapidly biologically meaningful differences in these reaction norms can arise via adaptive evolution is highly important to understanding future ocean biogeochemical cycles, as the environment for phytoplankton is changing on the time-scale of decades (e.g. Boyd et al. 2010). As a tool relatively new to marine science, long-term experiments can directly address whether and how organismal responses to global change may evolve at the population level (Sunday et al. 2014; Collins et al. 2014). Here, fast-dividing marine microbes (approx. 1 cell division day⁻¹) are prime examples for observing evolution in action over timescales of several months to years (Reusch and Boyd 2013; Collins et al. 2014; Hutchins et al. 2015).

The response of a given phytoplankton species or genotype to temperature is described by a thermal reaction norm, which is typically unimodal and left- (negatively) skewed (Huey and Kingsolver 1989) since above the optimal temperature T_{opt} , growth rates decline more rapidly than below it. The maximum of the curve depicting the optimal growth temperature is correlated with (and generally higher than) the mean environmental temperature at the locale from which a population or genotype has been isolated for laboratory cultivation, reflecting adaptation to local temperature conditions (Thomas et al. 2012; Thomas et al. 2016). As in other ectothermic species (Huey and Kingsolver 1989), phytoplankton thermal reaction norms are evolutionarily constrained by thermal trade-offs that prevent any one species from dominating across all temperatures found in the world's oceans (Boyd et al. 2013). For example, species can be categorized as having a narrow or a wide thermal niche (specialist vs. generalist), with maximal growth rates that are traded off against generalist growth performance and vice versa (Angilletta Jr. et al. 2003; Izem and Kingsolver 2005). Other conceivable trade-offs could be envisaged between maximal persistence temperature, thus stress tolerance, and maximal growth rates, but experimental data from phytoplankton demonstrating such trade-offs are lacking.

Our model species is the world's most abundant calcifying microalgae, the coccolithophore *Emiliania huxleyi* (Paasche 2002), that is one of the most intensely studied eukaryotic phytoplankton species with a near-worldwide distribution. Recently, this species has also become a model for combining experimental evolution and phytoplankton ecology (Reusch and Boyd 2013). Selection experiments subjecting this important phytoplankton species have shown that rapid adaptation to ocean acidification is possible within the time frame of one year either through genotypic sorting or via the occurrence of novel mutations within asexually dividing replicate populations (Lohbeck et al. 2012). In terms of temperature adaptation, previous experiments have shown that this species can adapt to a temperature only 1-2°C below the maximal growth temperature by rapid adaptive evolution within a timeframe of one year (corresponding to ≈ 500 asexual generations). Interestingly, increases in fitness relative to control populations were amplified by simultaneous exposure to ocean acidification levels of 1100 and 2200 μatm (Schlüter et al. 2014). The work by Schlüter et al. (2014) only tested two temperatures in the assay experiment, a control temperature (15.0°C) and one a few degrees below the lethal threshold (26.3°C). It is thus currently unknown how the entire thermal reaction norm may have changed upon thermal adaptation in this species, as well as in any other phytoplankton species.

Here we address whether or not selection for a single temperature close to the upper thermal limit (26.3°C) resulted in a reconfiguration of the entire reaction norm shape relative to populations evolving at 15°C, the approximate isolation temperature of the coccolithophore ecotypes at Bergen, Norway (Lohbeck et al. 2012). Note that all genotypes / replicate populations had been grown previously for 4 yrs at 15°C such that the warm temperature is a novel environment, while we do not deny that there is also some long-term adaptation to 15°C still ongoing during the experimental phase of the present study. This was studied in full factorial combination with two levels of ocean acidification (1100 and 2200 $\mu\text{atm pCO}_2$) along with ambient controls (400 $\mu\text{atm pCO}_2$). We were particularly interested if adaptation to high temperature also changed the optimal growth temperature T_{opt} , maximum persistence temperature T_{max} (i.e. the temperature above which growth rate becomes negative), and maximal growth rates μ_{max} (Boyd et al. 2013; Thomas et al. 2016). Moreover, we studied possible trade-offs, for example with respect to T_{max} , T_{opt} and μ_{max} . Evolution experiments are particularly suited to address trade-offs because trait correlations, among the above three attributes of thermal reaction norms, have to evolve within an identical genetic background (Fry 2003).

Material and Methods

Study species, culturing and experimental design

The coccolithophore *Emiliana huxleyi* is the most abundant calcifying organisms in the world oceans, distributed from subpolar to subtropical waters (Paasche 2002). When forming blooms, their areal extent can be seen from outer space owing to the calcite platelets that reflect a proportion of the incoming solar radiation. Previous studies in *Emiliana huxleyi* have demonstrated swift evolutionary adaptation to ocean acidification and warming in asexual populations within the time frame of one year (approx. 500 asexual divisions) (Schlüter et al. 2014). Here we built upon a previous CO_2 (Lohbeck et al. 2012) and temperature selection experiment (Schlüter et al. 2014) and ask whether and how the entire thermal reaction norm differs in two sets of asexual experimental populations that evolved for 2.5 years under a control and one high temperature close to the upper thermal limit. Note that maximal water temperatures off Bergen, Norway, are at most 19°C (see August maxima at <http://www.seatemperature.org/europe/norway/>), thus while the control temperature is within the conditions encountered by the culture genotypes, this was not the case for the high temperature.

The temperature evolution experiment started in February 2013 when *Emiliana huxleyi* semi-continual batch cultures at three CO₂-levels ($N=5$) were subdivided into a "cold" (15.0°C) and a "warm" (26.3°C) treatment, resulting in six fully factorial "temperature by CO₂ treatment" combinations (Fig. 1). Phenotypic changes after one year (approx. 500 asexual generations) of temperature selection, tested at only two assay conditions, i.e. the two selection regimes, have already been published elsewhere (Schlüter et al. 2014). The exact level of the "high" temperature was determined in pilot experiments because initially, daily specific growth rates were approximately similar at both temperatures, thus the elapsed number of generations would also be similar across any occurring evolutionary adaptation (Schlüter et al. 2014). The temperature treatment was run for 1200 asexual generations or 2.5 years, thus ≈ 700 generations longer than the results reported in Schlüter et al. (2014). The original selection lines were founded in 2009 from a single cell isolated from a natural phytoplankton assemblage in the coastal waters off Bergen (Lohbeck et al. 2012). *Emiliana huxleyi* cultures were uni-algal but not axenic as checked by monthly light microscopy and flow cytometry.

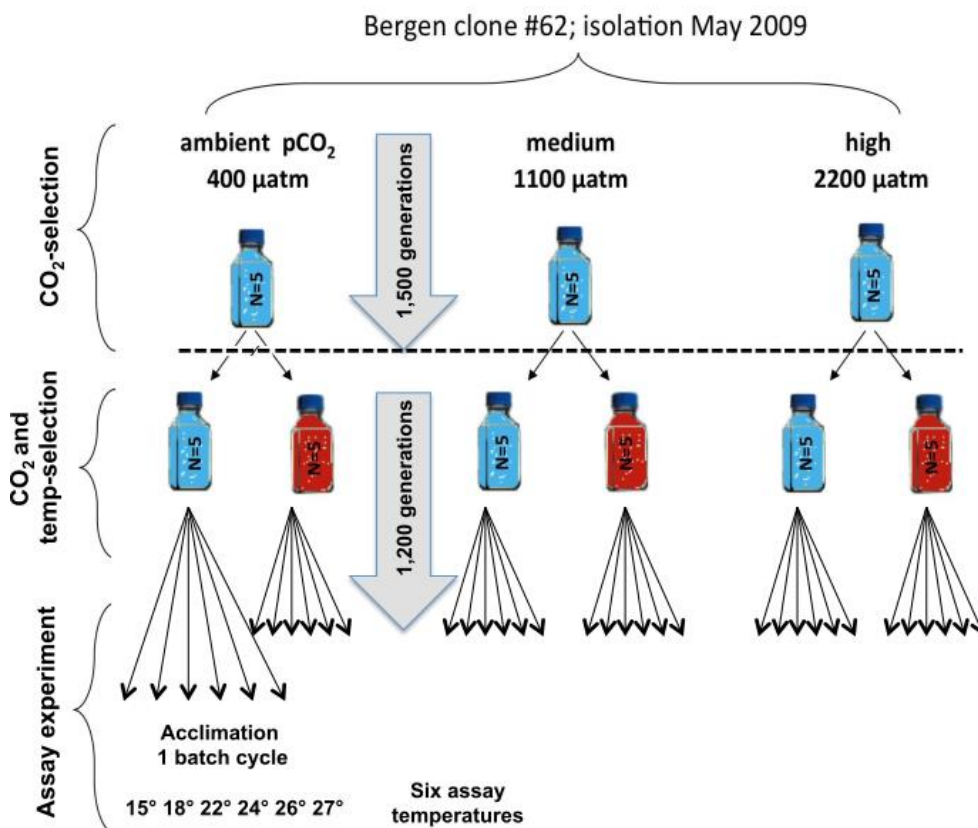


Figure 1. Five years of experimental evolution in *Emiliana huxleyi* in semi-continuous batch cultures. Schematic representation of the experimental design and the selection history.

Three CO₂ levels were combined with the two temperatures in a full factorial way: along with a control treatment at 400 µatm we subjected *E. huxleyi* to 1100 µatm as simulation of an end-of-the-century levels and 2200 µatm as the highest possible future level of ocean acidification in the year 2300 (Caldeira and Wickett 2003). CO₂ levels were reached by bubbling the medium with CO₂-enriched air before inoculating the experimental populations at the respective temperature treatments for 24h, see (Schlüter et al. 2014) for details on the seawater chemistry and CO₂ /carbonate measurements.

For long-term experiments and temperature assays we used artificial seawater ASW according to (Kester et al. 1967) with the following nutrient additions: 64 µmol kg⁻¹ of nitrate, 6 µmol kg⁻¹ phosphate, trace metals and vitamins according to f/8 composition, 10 nmol kg⁻¹ of selenium and 2 ml kg⁻¹ filtered North Sea water to avoid limitation by micronutrients.

Cultures were kept under continuous rotation (0.5 min⁻¹ in two Sanyo MLR-351 light cabinets) at 150 µmol m⁻² s⁻¹ photon flux density under a 16:8 light:dark conditions during each 5 day cycle. To start the next batch cycle, and renew the medium, every five days 10⁵ cells were transferred from cultures into fresh medium. The cell abundance and diameter were measured in triplicate before each transfer, using a Beckman Coulter Z2 Particle and Size Analyzer. Daily specific growth rates (μ) were calculated from cell abundance as $\mu = (\ln N_d - \ln N_0)/d$. All culture work, including the ASW preparation, was done under sterile conditions (laminar flow) (Schlüter et al., 2014).

Assay experiment to determine the thermal reaction norm

The reaction norm of all 30 replicates was determined at six assay temperatures after one full cultivation cycle of transfer of 100 000 cells to a new culture flask for acclimation (5 days =5-8 cell divisions), namely at 15°C, 18°C, 22°C, 24°C, 26°C and 27°C. While the two lower temperature values are attained at Bergen (Norway) coastal waters, the isolation location of the tested *E. huxleyi* genotype, we were particularly interested in the possible changes of upper range of temperature tolerance. This also implies that our design inappropriate to address correlated responses at low temperatures (i.e. <15°C). Batch cycles to assess growth rates differed in length because we needed to maintain approximately similar maximal cell numbers in the assays. In order to accommodate for the different growth rates batch cycles lasted 6 days in the 15°C treatment, 5 days in the 18°C treatment, 4 days in the 22°C, 5 days for 24°C, 7 days (15°C) and 5 days (26.3°C) for 26°C

and 10 days (26.3°C) in the 27°C treatment. Preparation of ASW was similar to the long-term culturing phase, except that during CO₂ manipulation via were bubbling the medium, the seawater was kept at the planned assay temperature (15°C, 18°C, 22°C, 24°C, 26°C, and 27°C (±3°C)) for 24h, and not at the respective temperature selection (15.0 and 26.3°C). Nutrients were never limiting given the cell abundance achieved at the end of the batch cycles. At the end of each growth cycle the cell densities and cell diameter were determined triplicate by a Z2 Coulter Particle Count and Size Analyzer (Beckman®), specific daily growth rates (μ) were calculated from cell densities as above.

Statistical analyses

Where strains exhibited no growth at either 26 or 27°C, we removed the 27°C measurement from our calculations. The absence of growth can indicate either zero or negative growth rate, but we were unable to measure negative growth rates. Therefore, zero is almost certainly an overestimate at 27°C in these cases.

Thermal reaction norm parameter estimation

Thermal reaction norms are typically left-skewed, and have been described using a variety of functions. We used the equation and parameter and bootstrap-based uncertainty estimation procedures described in Thomas et al. (2012) and Boyd et al. (2013).

$$f(T) = ae^{bT} \left[1 - \left(\frac{T-z}{w/2} \right)^2 \right] \quad (1)$$

where specific growth rate f depends on temperature, T , as well as parameters z , w , a and b . w is the temperature niche width (the range of temperatures over which growth rate is positive), while the other three (z, a, b) possess no explicit biological meaning but interact to influence the rate of increase in growth rate with temperature, the maximum growth rate and the optimum temperature for growth. We fit (1) to the growth data for each strain using maximum likelihood to obtain estimates for parameters z , w , a and b . In addition, we estimated the optimum temperature for growth (T_{opt}) and maximum persistence temperature (T_{max}) through numerical estimation. For maximum growth rate μ_{max} , we instead used the highest growth rate measured in our growth assays, as we are less confident in these estimates from our fitted reaction norms.

Estimating the influence of temperature selection and pCO₂ levels on traits

We tested whether the three thermal traits changed as a result of selection at different temperatures and pCO₂ levels while accounting for uncertainty in our estimates of these traits using a parametric bootstrapping approach. For each replicate, we fitted the thermal reaction norm function to the growth rate measurements and extracted the residuals from this fit. We then performed 1000 residual bootstraps, a procedure in which the residuals are randomly ‘reassigned’ to the predicted values (each of which corresponds to a growth rate measurement) and added to them, thereby generating a slightly different thermal reaction norm. For each iteration, we refitted the function and estimated the parameters (w , a , b) and also two of the derived traits, T_{opt} and T_{max} .

Examining the distribution of the traits (T_{opt} and T_{max}) over the 1000 bootstraps allowed us to quantify the uncertainty in our trait estimates, which we then incorporated in models seeking to explain how selection temperature and pCO₂ influenced them. For each set of bootstraps of all 30 experimental units, we fitted a linear model explaining each trait as a function of selection temperature (coded as a categorical variable), pCO₂ level and their interaction. pCO₂ level was standardized by subtracting the mean and dividing by two standard deviations to improve fitting procedures and generate readily comparable model parameter values (Gelman 2008; Gelman and Su 2015). We then examined the distribution of linear model parameter estimates over the 1000 bootstraps to determine whether temperature selection, pCO₂ or their interaction significantly influenced the traits. If the 95% confidence interval of a linear model parameter did not overlap zero, we concluded that the model parameter had a significant influence on the trait. If the interaction between selection temperature and pCO₂ was an important predictor, we did not draw conclusions about significance of the main effects.

In the case of μ_{max} , we fitted the linear model only the highest measured growth rate of each strain (no bootstraps), using selection temperature (coded as a categorical variable), pCO₂ level and their interaction as explanatory variables. As with the other two traits, we standardized pCO₂ level by subtracting the mean and dividing by two standard deviations. After this, we estimated the 95% confidence intervals on the fitted model parameters.

Parameter estimation and bootstrapping was performed in the R Statistical Environment 3.2.2. (R Core Team 2015).

Results

Fitted reaction norms of all 5 replicates within the two selection treatments are depicted in Figure 2, grouped by selection treatment and CO₂ condition. 2.5 years of selection at 15°C and 26.3°C and three CO₂ levels resulted in marked differences in reaction norm shape, which we capture in important temperature traits (Fig. 3). The effect sizes of the selection conditions (i.e. the regression coefficients of the parameters) on T_{opt} , T_{max} and μ_{max} are presented in Fig. 4. The variance explained (adjusted R^2) by the models for these three traits was 0.37, 0.74, and 0.65, respectively. Temperature had a strong effect on T_{opt} and T_{max} , while CO₂ was important for determining the maximal growth rate G_{max} and for T_{max} . Both μ_{max} and T_{max} were influenced by interactions between CO₂ and selection temperature. We discuss the changes in the three traits below. We do not interpret our estimates of thermal niche width as we lack critical values at the lower temperature threshold to characterize them accurately.

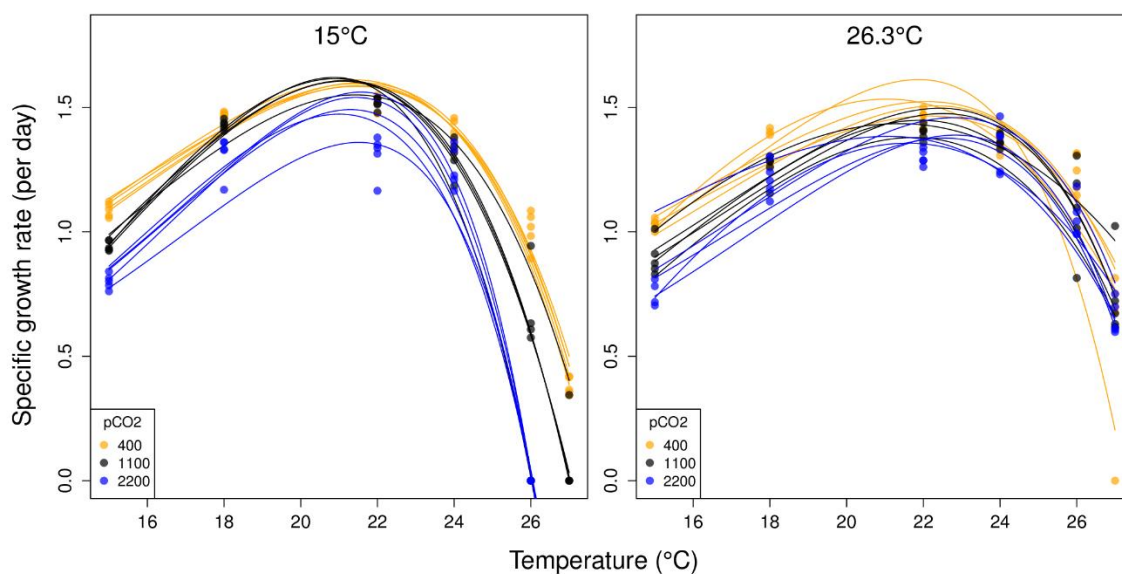


Figure 2. Fitted temperature reaction norms for individual replicate asexual populations according to equation (1) in *Emiliana huxleyi*. Curves are grouped according to two different selection temperatures, 15.0°C (left) and 26.3°C (right panel) fully crossed with three CO₂ environments, depicted by colour (orange = 400 μ atm, black = 1100 μ atm, blue = 2200 μ atm). Note that the CO₂ environment was similar during selection and temperature assay.

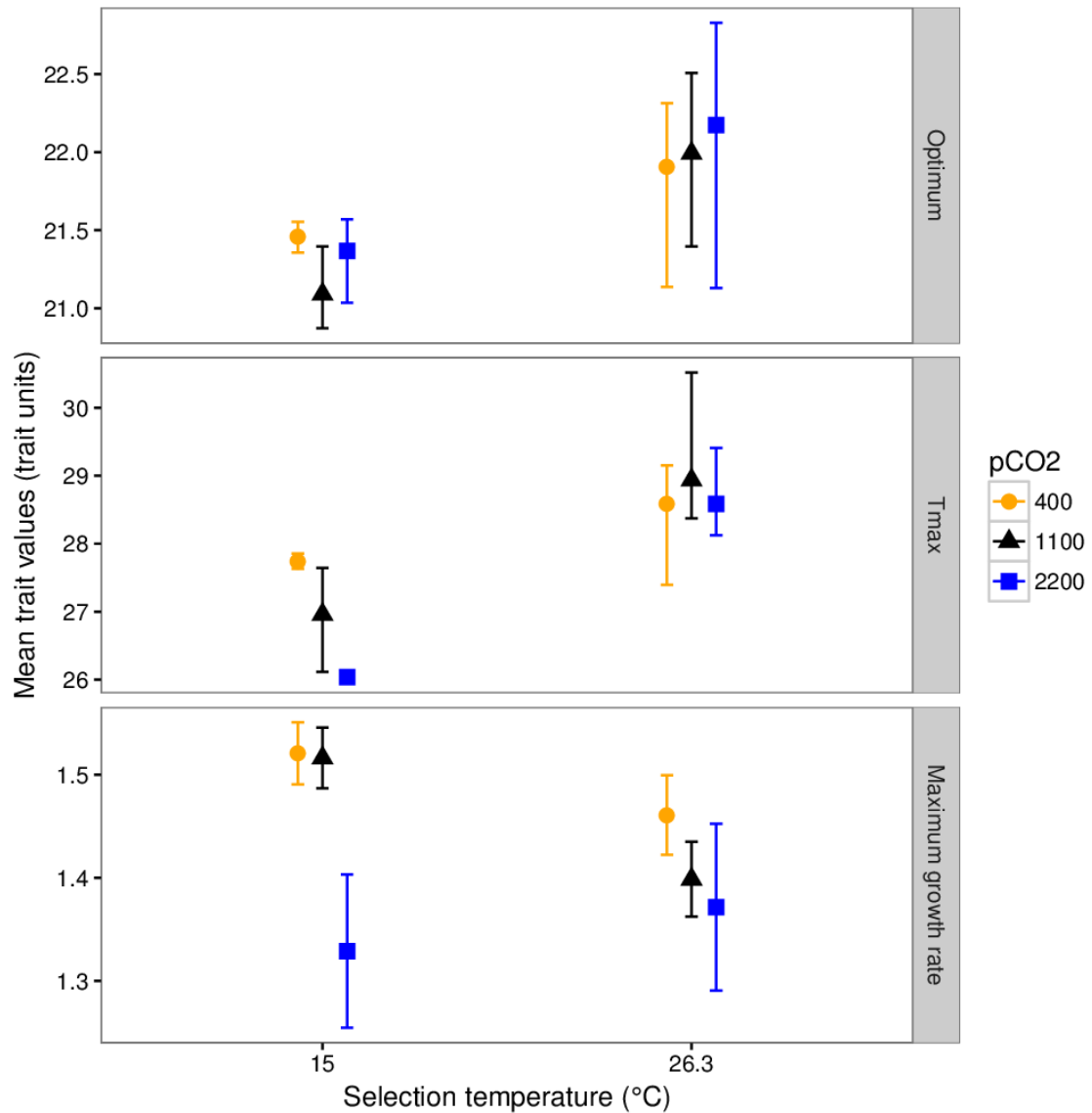


Figure 3. Mean temperature traits (T_{opt} , T_{max} and μ_{max}) in the different treatments. T_{opt} and T_{max} were estimated from reaction norm fits depicted in Fig. 2, while μ_{max} was calculated from the measured growth rates. Confidence intervals (+/-95%) are based on residual bootstraps of the fitted reaction norms for the first two traits; for μ_{max} , calculations were based on the empirically measured growth rates and assumed normality of the data.

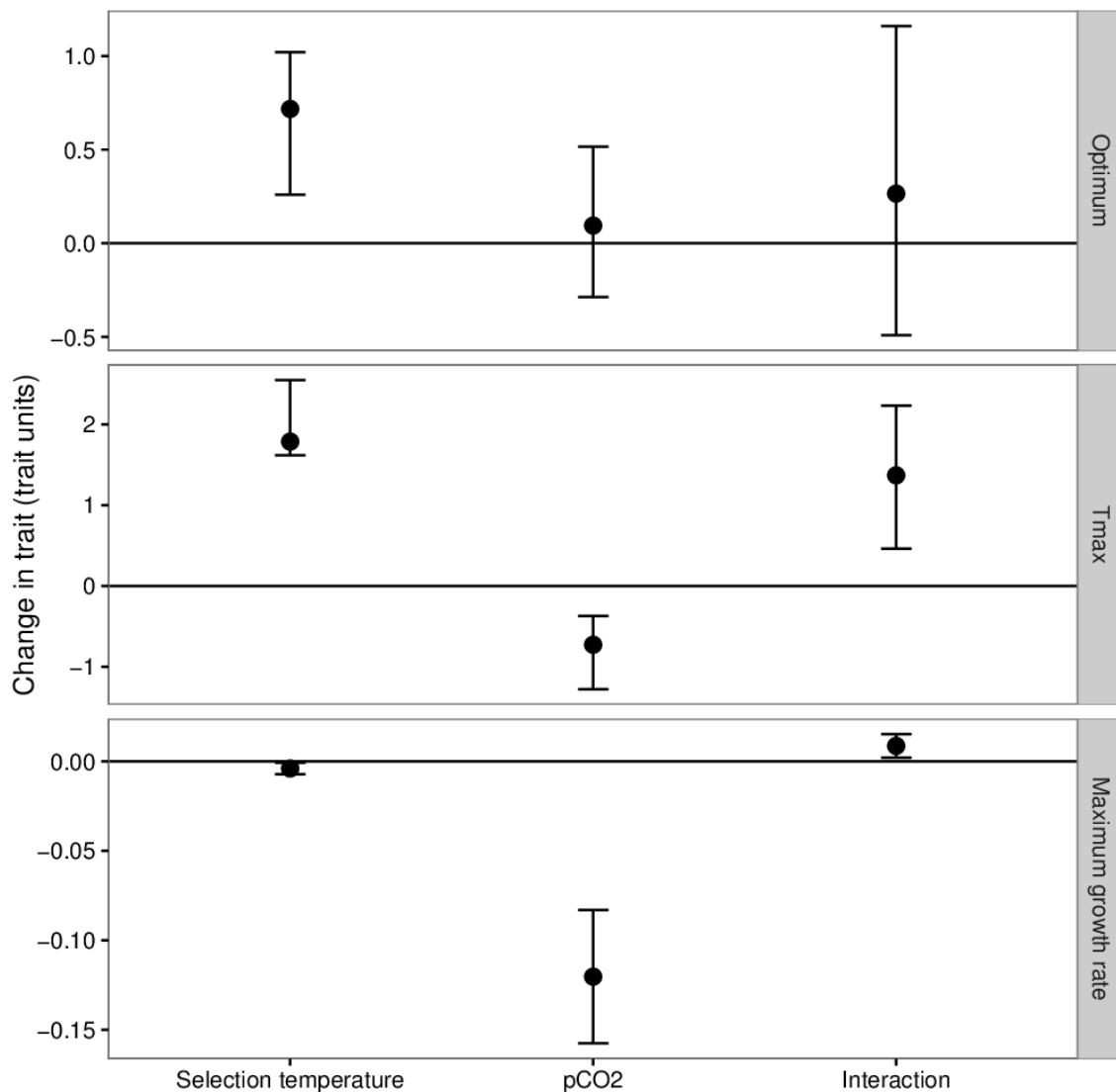


Figure. 4. Changes in *Emiliana huxleyi* temperature traits T_{opt} , T_{max} (based on the non-linear curve fit according to equation (1)) and μ_{max} (based on measured growth rates) as a function of selection temperature, pCO_2 and their interaction. Standardized trait coefficients and their interaction are depicted; if confidence intervals do not overlap with zero, effects are statistically significant.

Optimum temperature for growth (T_{opt})

T_{opt} differed by $0.7^\circ C$ on average between the low and high-temperature selection treatments (Fig. 3), being $21-21.5^\circ C$ in the former and $22^\circ C$ in the latter (Fig. 4). pCO_2 did not have a detectable influence on T_{opt} either directly or as part of an interaction with temperature selection (Fig. 3, 4).

Maximum persistence temperature (T_{max})

T_{max} varied substantially between treatments and was influenced by an interaction between temperature selection and pCO_2 level (Fig. 4). In the high-temperature selection

treatments, T_{\max} reached 28.5 - 29°C regardless of pCO₂ level (Fig. 3). However, in the low-temperature selection treatments, T_{\max} varied by 2°C depending on pCO₂ level (Fig. 4). At 400 μatm (control), T_{\max} was nearly 28°C, but this decreased by 1°C at 1100 μatm and 2°C at 2200 μatm (Fig. 3).

Maximum growth rate (μ_{\max})

μ_{\max} was reduced strongly at higher pCO₂ levels, but the extent of the reduction was influenced by an interaction with temperature (Figs. 3 and 4). μ_{\max} decreased more strongly with increasing pCO₂ at 15°C (by nearly 0.2 per day at 2200 μatm relative to 400 μatm) than at 26.3°C (by approximately 0.09 per day).

Discussion

As a response to climate forcing via emission of greenhouse gasses, in particular CO₂, atmospheric warming has already produced pronounced ocean warming during the past few decades in the world's oceans, and even down to depths of 2000m (Roemmich et al. 2015). As a result, among diverse plankton species, observed range shifts have been attributed to mean ocean warming (or increased variability) and concomitant pole-ward shifts of distributional ranges (Poloczanska et al. 2013). Adaptive evolution is a non-mutually exclusive process to range shifts that allows populations to "stay" within their geographical range and has recently come into the research focus (Hoffmann and Sgro 2011).

Here, we have addressed how the rate at which adaptive evolution will produce different thermal reaction norms in a genetically near-uniform base population. We started our experimental evolution with a single isolate from Bergen, Norway. Thus, due to the lack of standing genetic variation, evolutionary adaptation most likely occurred via novel mutations. Mutant genotypes with under positive selection displayed higher Darwinian fitness and rose to (near) fixation in the asexually dividing population. Our scenario thus simulates that sexual reproduction and recombination is rare or absent (Reusch and Boyd 2013), which may actually be the case in some *E. huxleyi* populations (Von Dassow et al. 2015). For our isolates, population-level data indicate that they are composed of many genotypes (unpublished data), which mirror results from the English channel that even in bloom situations genotypes are in Hardy-Weinberg equilibrium (Krueger-Hadfield et al. 2014) suggesting frequent sexual reproduction.

Although we started with one single genotype, we nevertheless found marked divergence among the two temperature selection treatments in several reaction norm parameters such as T_{opt} , T_{max} and μ_{max} . These effects of temperature selection interacted with the CO_2 environment. Specifically, effects on T_{max} , in particular, were markedly negatively affected by medium and high ocean acidification levels but only in high temperature selection treatments. This highlights how important interactions of major projected ocean perturbations are not only to understand future physiology, but also the evolutionary trajectories in plankton populations (Collins 2011; Schlüter et al. 2014). Note that our design cannot strictly test for temporal changes in reaction norms, since we cannot retest our initial base population or genotype as is possible in model systems such as *E.coli* or yeast with the help of cryopreservation (Elena and Lenski 2003). The salient experimental test is therefore the synchronic test of different evolution treatments at the same time, which also provides the necessary control for laboratory adaptation.

We cannot exclude that the genotype #62, originally isolated in 2009 off Bergen, Norwegian coastal waters, is special in terms of adaptation rates or magnitudes towards ocean warming and acidification. In other words, it is highly desirable to compare different genetic backgrounds with respect to their rates and magnitudes of adaptation, including associated phenotypic traits.

Our data support results of a shorter (1 yr = 500 generations) evolution experiment reported in Schlüter et al. (2014). He tested thermal adaptation only at the two selection temperatures, 15.0°C and 26.3° over one year and found pronounced adaptation of growth rates to the long-term selection temperature in reciprocal exposure assays. The relative fitness of high temperature adapted replicates compared to low temperature-adapted ones (as quotient of exponential growth rates) was 1.08-1.25, when tested at 26.3°C. The higher the CO_2 level in the experiment of Schlüter et al. (2014), the greater were the fitness gains of adapted populations, relative to the control populations evolving at today's pCO_2 and at a temperature of 15°C. When focusing on the rather similar assay temperature 26°C assessed here, the present data reveal an even stronger thermal adaptation after about 700 additional generations of evolution. Depending on CO_2 , we find a relative fitness of 1.22 and 2.05 in ambient and medium (1100 μatm) pCO_2 . Note that at the highest CO_2 -level (2200 μatm), no growth was observed in the 15°C adapted populations at an assay temperature of 26°C. We also found a pronounced correlated response at 15°C -

low-temperature adapted populations grew faster at 15°C than high-temperature -adapted ones (Fig. 2).

Contrary to Schlüter et al. (2014), at an assay temperature of 26°C, we now find zero growth in some 15°C-adapted replicate populations, which may be due to further adaptive evolution towards low-temperature specialization. However, this may have been influenced by differences in assay methods. In the present study, the initiation of the assay experiment (which started with a one-week acclimation phase in all cases) was abrupt (i.e. within one single day) while the assay temperature was reached at a rate of 1°C per day in the earlier experiment by Schlüter et al. (2014). The concomitant carry-over effects may have negatively affected growth rates in the 2nd assay cycle where the growth rates presented in this paper were measured.

In the ideal case, we would have started the thermal evolution experiment with identical genetic material at the onset. However, replicate lines had already 3 yrs time (about 1500 generations) to accumulate slightly favorable and concomitant hitchhiking mutations (Lang et al. 2013) under long-term CO₂-selection before thermal selection had started. Thus, the starting genetic diversity was likely higher than a pure uni-clonal inoculum derived immediately from a single cell directly before the thermal selection started. This may explain the relatively fast pace and extent of reaction norm evolution compared to previous assessments of adaptation to ocean acidification (Lohbeck et al. 2012). Note, however, that also the CO₂-ambient control lines (at 400 µatm) always evolving under ambient CO₂ levels had also changed their thermal reaction norm relative to the 26.3°C selected replicates, thus previous high-CO₂ selection was apparently not prerequisite for adaptive responses.

In any case, the standing genetic variation in natural phytoplankton populations is much higher: ample standing genetic variation in coccolithophores has often been observed in genetic marker studies (Iglesias-Rodriguez et al. 2006; Krueger-Hadfield et al. 2014) or physiological assessments (Brand 1982; Wood and Leatham 1992; Kremp et al. 2012; Zhang et al. 2014), which provide abundant possibilities for selection to operate.

The major result of evolution at different temperatures was that the optimal growth temperature T_{opt} shifted upwards, which was expected. Since growth rates increased only little during the 5 years of selection at 15°C (Schlüter and Reusch, unpublished data), we attribute most of the divergence in reaction norm shape to changes at high temperature.

This may reflect a trade-off with between low- and high-temperature performance. A second major finding is that T_{\max} shifted upwards very strongly relative to the low-temperature adapted populations, indicating that adaptation to lethal temperatures is possible over monthly-to-yearly time scales even in very small populations (relative to natural populations). While this upward shift was present in all high-temperature selection replicates, $p\text{CO}_2$ had a negative effect on T_{\max} in the low-temperature selection treatments. Currently, we have no explanation for this interaction, but it clearly deserves further testing in this and other phytoplankton species. In contrast to these two traits, μ_{\max} was only weakly affected by temperature selection but strongly decreased with increasing $p\text{CO}_2$, especially at 15°C. Unfortunately, since we could not assess the niche width, our findings cannot be interpreted within a generalist-specialist trade-off scenario (Angilletta Jr. et al. 2003; Izem and Kingsolver 2005). However, it suggests that a general "flattening" and broadening of the reaction norm may have occurred, superimposed onto a right-hand shift of the entire reaction norm curve.

Although our upper thermal selection temperature was very unrealistic with respect to the temperatures *E. huxleyi* may experience throughout the North Atlantic (maximal temperatures at Bergen, the isolation site 19°C in August), our results nevertheless provide a proof-of-principle of swift evolution of reaction norms, and provide first insights into trade-offs of important traits associated with phytoplankton temperature reaction norms. Biogeochemical models of future ocean productivity contain thermal sensitivities of major phytoplankton groups as key parameters (Taucher and Oschlies 2011). Our results show that these thermal traits are parameters that can change by evolution and may to some extent track the expected increases in sea surface temperatures. A big open question is whether in the ocean, thermally sensitive species will be replaced by taxa possessing higher optimal growth temperatures and upper tolerances (i.e. ecological compositional change), or whether *in situ* evolution of thermal reaction norms will occur as a non-exclusive additional response, favouring the persistence of existing species and communities (Collins and Gardner 2009).

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Additonal information for publication III

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Data archiving statement: Primary data are available through the data repository Pangaea (<http://doi.pangaea.de/10.1594/PANGAEA.856736>).

Chapter IV

Rapid experimental selection changes species interactions and the CO₂ response in a two-species phytoplankton community through eco-evolutionary coupling.

Rapid experimental selection changes species interactions and the CO₂ response in a two-species phytoplankton community through eco-evolutionary coupling.

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Abstract

We studied adaptation in two important phytoplankton species, *Emiliana huxleyi* and *Chaetoceros affinis*, in response to long-term exposure to increased CO₂ in combination with the presence of the respective other species. In order to allow for rapid evolutionary adaptation via genotype sorting, populations of both species were experimentally assembled as mixtures of nine genotypes. In adaptation assay experiments we only found adaptation to enhanced CO₂ in *C. affinis* at the end (after 200 generations) of the selection experiment irrespective of the presence of the other species. In *E. huxleyi* no direct adaptation to CO₂ or the other species was detectable. At the same time, we observed rapid and reproducible genotype sorting that left a single winner among the initial nine genotypes of each species. We therefore conclude that the intended experimental conditions of semi-continuous batch cycles with a nutrient limited stationary phase represented a general selection regime overriding the dedicated treatments most of the time. Indirectly, the strong evolutionary shift in genotype composition in both species resulted in an eco-evolutionary coupling, namely in dramatically changed species interactions over time, rendering the once competitively inferior species *E. huxleyi* superior over the diatom and at the same time increasing its ability to cope with high CO₂ concentrations. Thus, we have shown that constrained laboratory "community" experiments can be used to investigate eco-evolutionary coupling. Moreover, the general experimental conditions can impose strong and overriding selection which then indirectly feeds back to species interaction.

Introduction

In order to understand how climate change will affect biological communities we need to understand both ecological and evolutionary processes that include changes on the species and genotype level, respectively (Fussmann et al., 2007; Kremer and Klausmeier, 2013). Ecological and Evolutionary processes can be altered by abiotic environmental factors (e.g. increased CO₂) as well as biotic interactions (e.g. competition, which consequently are likely to play a crucial role in changing communities (Doney et al., 2009; Lawrence et al., 2012). Phytoplankton are important primary producers and owing to their large population sizes contribute to about half of all global primary productivity (Falkowski et al., 2008). Due to their short generation times they are an ideal and important system where responses to environmental change, in terms of ecological and evolutionary shifts, can be understood concurrently.

One prominent environmental change is ocean acidification (OA), describing changes in the seawater carbonate system due to anthropogenic CO₂ dissolving in the ocean which lead to a decrease in ocean pH (Caldeira, 2003). There are plenty of studies suggesting that species and communities will be and are already affected by increased seawater CO₂ concentration (Doney et al., 2009) and consequently that there will be winners and losers (Dutkiewicz et al., 2013). Diatoms, as most phytoplankton species (e.g. chlorophytes and cyanobacteria) (Li et al., 2016; Schaum et al., 2012), are little affected by elevated CO₂. Often, they even profit from elevated CO₂ due to enhanced photosynthesis and growth since dissolved inorganic carbon (DIC) limitation is alleviated. Calcifying species such as coccolithophores have been shown to respond with reduced growth and calcification rates (Riebesell et al., 2000). Nevertheless, due to intraspecific variation genotypes can react differently to environmental change compared to the overall species response (Des Roches et al., 2018). Increased CO₂ has different short-term effects on species and their genotypes (Hattich et al., 2017), thus there seems to be standing genetic variation also reflected in trait diversity that selection can operate with (Lohbeck et al., 2012). Nine different genotypes of the diatom *Chaetoceros affinis* showed varying positive to neutral responses under elevated CO₂ whereas nine different genotypes of the coccolithophore *Emiliania huxleyi* showed neutral to negative responses (Hattich et al., 2017). We therefore expect that these investigated species and genotypes would react to long-term exposure to increased CO₂ by genotypic sorting along their trait-based reaction norms, similar to differential genotype sorting both in *E. huxleyi* (Lohbeck et al., 2012) as well as diatom

species (Scheinin et al., 2015). This differential genotype sorting was associated with adaptation to higher CO₂ by increasing growth rates under high CO₂ compared to ambient conditions.

Investigating single species or genotypes of species is a first approximation to understand how environmental change may affect these. Nevertheless, species do not exist alone and there will always be species interactions as a possible biotic driver of change (de Mazancourt et al., 2008; Lancaster et al., 2017; Lawrence et al., 2012). In phytoplankton the interferences between species are most likely due to resource competition, but it cannot be excluded that other effects such as allelopathy play a role (Birch, 1957; Legrand et al., 2003). We have previously investigated possible species interactions between the diatom *C. affinis* and the coccolithophore *E. huxleyi* in a short-term experiment and found that the interactive effects are important for species compositional change and that they are potentially different in altered CO₂ concentrations (Listmann et al., in prep). Yet, it is unknown how these species interactions between *C. affinis* and *E. huxleyi* change in the long term and how adaptation to increased CO₂ in combination with adaptation to another species mutually influence each other. We posit that over longer term, the change in genotype composition due to the intended selection via another species in combination with CO₂ will ultimately alter the species interaction in a way unforeseeable by experimenting with each of the species in isolation. Having two species in a co-evolving community as well as investigating adaptive processes allows for the observations of potential eco-evolutionary coupling (Post and Palkovacs, 2009). In order to allow for rapid evolutionary adaptation via genotype sorting, populations of both species were experimentally assembled as mixtures of nine genotypes with even frequencies. Reciprocal adaptation assays were designed to test for direct adaptation of the two species to the single and combined effect of enhanced CO₂ and the presence of the respective other species after short and longer selection. Compositional changes in genotype frequencies were followed by re-isolation and microsatellite genotyping over the course of the experiment to monitor the strength of genotypic selection.

Our overall objective was to understand eco-evolutionary coupling in a two-species phytoplankton community consisting of *E. huxleyi* and *C. affinis*. We predict a weak negative effect of increased CO₂ on *E. huxleyi* whereas *C. affinis* should be fertilized by increased CO₂. Based on previous short term experiments we assume that the presence of *C. affinis* should strongly reduce the abundance of *E. huxleyi* and that there is no effect vice versa

(Listmann et al in prep). The combined effect of these treatments depends on the type of interaction of these effects (i.e. synergistic, additive, antagonistic) (Crain et al., 2008). We expect that in *E. huxleyi* the combination of both increased CO₂ and the presence of the diatom species are additive negative stressors leading to increased negative response to CO₂ in the 2-species community. Since the presence of *E. huxleyi* potentially reduces the availability of CO₂ it is possible that there is an antagonistic effect of increased CO₂ leading the disappearance of fertilization of increased CO₂ in *C. affinis* in the 2-species community. Due to adaptation we expect that any negative effects of the treatments should be alleviated over time. We should find differential genotype sorting between the treatments, as we assume a tradeoff between tolerance to increased CO₂ and competitive ability between the different genotypes. Generally, we expect stronger and faster adaptation in *E. huxleyi* because the treatments impose more stressful conditions than for *C. affinis*. Our objectives were thus to understand 1) how the absolute species abundance of *E. huxleyi* and *C. affinis* changes over 200 generations when exposed to increased CO₂, the presence of the respective other species and the combination of both, 2i) how *E. huxleyi* and *C. affinis* genotype composition changes over time in response to the different experimental treatments and 2ii) if the differential genotype sorting is associated with adaptation of *E. huxleyi* and *C. affinis* to increased CO₂, the presence of the respective other species and combination of both and if so; how fast. This study is to our knowledge one of the first on phytoplankton that investigates the long-term response and adaptation to an abiotic driver and biotic species interactions.

Methods

We exposed two important phytoplankton species; the coccolithophore *E. huxleyi* and the diatom *C. affinis* to increased CO₂ and each other over 36 batch cycles, representing approximately 200 asexual generations. We followed absolute species abundance as biovolume at the end of each batch cycle over time to understand how the species change in response to the different treatments. Each species started as a mixture of nine different genotypes to allow for the monitoring of genotype compositional change via re-isolation. All genotypes used were isolates originating from one geographical region (Gran Canary, 27°59'N 15°22'W, isolated 2012 and 2015) and are deposited in the culture collection in Roscoff (see Hattich et al 2017). After ca 50 and 200 generations we conducted reciprocal adaptation experiments that exposed the selection treatments to all possible assay condi-

tions. We were particularly interested in interactions of selection treatment * assay treatment as these would indicate evolutionary adaptation over the course of the experiment.

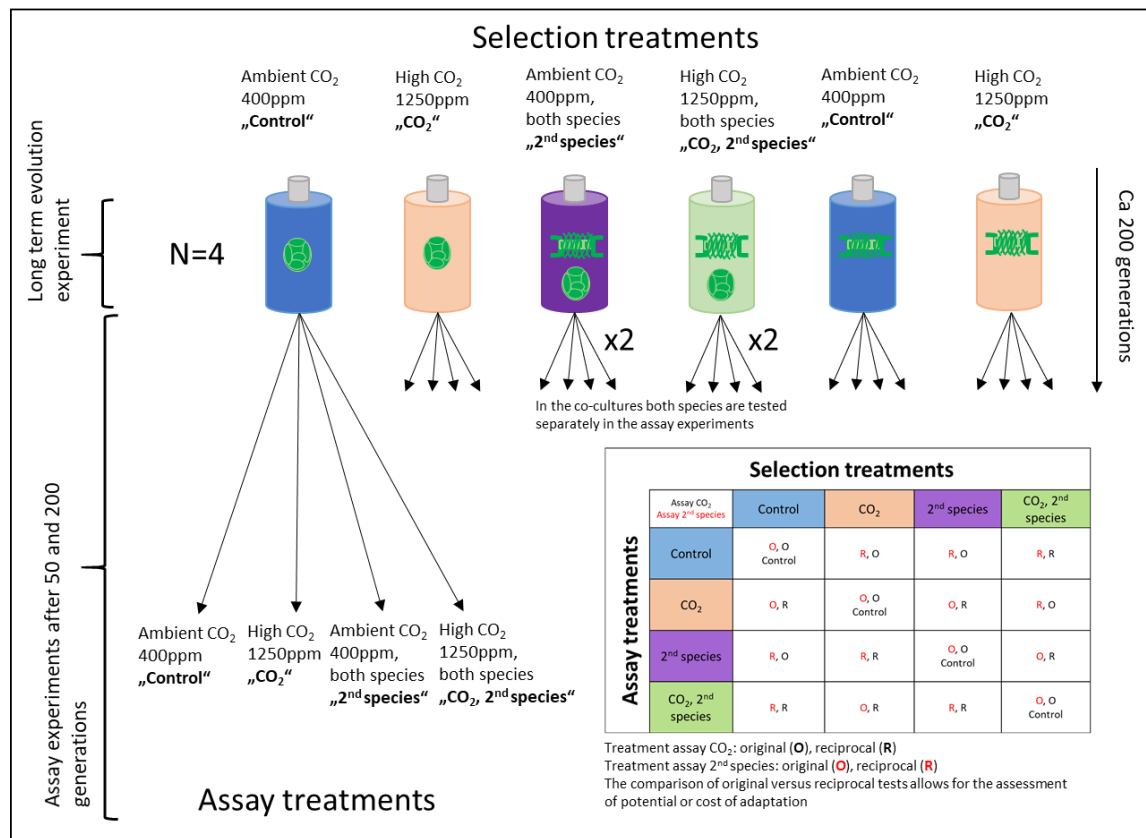


Figure 1: Experimental design of the long-term evolution experiment and its two assay experiments. The phytoplankton species *E. huxleyi* and *C. affinis* and their 9 genotypes each were allowed to adapt to the following selection conditions for 200 generations: “Control”, “CO₂”, “2nd species” and “CO₂, 2nd species”. The cultures of *E. huxleyi* and *C. affinis* where the respective 2nd species was added, were the same since we tested the effect of the two species on each other. The assay experiments were conducted after 50 and 200 generations. Here, every evolved culture was tested in all possible experimental treatments in a full factorial design. The species from the co-evolved communities were tested separately after separating them using a sieve of 20µM.

Experimental set up and culturing conditions

The selection treatments were set-up in a fully orthogonal way with the single factors CO₂ (two levels, control and high) and presence /absence of a 2nd species resulting in 4 treatment combinations. Four replicate cultures of each of the four treatments were kept for 9 months (36 batch cycles, resulting in 210-230 generations in *E. huxleyi* and 195-210 generations in *C. affinis*) in semi-continuous batch cultures. In order to simulate a bloom situation with resource competition under depleted nutrients (Paulton, 1991), we ran the study as semi-continuous batch cultures with both species reaching the stationary phase

(supplementary Figure S2 growth graphs of the different species). This resulted in the almost full depletion of all macronutrients (nitrate, phosphate and silicate).

The ambient and high CO₂-treatment was manipulated by aerating the artificial-seawater (35 salinity; after [12]) for 24h with CO₂-enriched air (400 and 1250ppm, respectively) prior to 2 batch cycles. The dissolved inorganic carbon (Hansen et al., 2013) varied between 2052.39±34.49 and 2202±8.19 μmol*kg⁻¹ with a total alkalinity (following (Matthiessen et al., 2012)) of 2272.56±11.24 and 2273.12±11.43 μmol*kg⁻¹ for ambient and high CO₂, respectively. Nutrients were added to the final concentrations of 19.61±0.09 μmol*L⁻¹ nitrate, 0.99±0.02 μmol*L⁻¹ phosphate and 4.34±0.14 μmol*L⁻¹ silicate. Each experimental unit was inoculated with an initial total biovolume of 8280 μm³*mL⁻¹. When the two species were growing together they were added at a ratio of 1:1 of the biovolume to start the experiment. The experiment was carried out under constant rotation (0.75 min⁻¹) at ~22 °C and a 17:7 day:night cycle reaching a maximum light intensity of 350 μmol*m⁻²*s⁻¹ 3h after dusk and dawn. At the end of each batch cycle the biovolume was determined with microscopy (for details see Listmann et al. (in prep.) for the transfer to the next batch cycle.

Frequency assessments of genotypes

In order to follow genotype frequencies in both species, we re-isolated cells of both species after batch cycles 1, 4, 8, 24 and 36. Genotypes of both species could be unambiguously identified via microsatellite genotyping (see (Hattich et al. 2017, supplementary) for *E. huxleyi* and for *C. affinis* genotype identification see supplementary material S2).

In *C. affinis* we re-isolated a minimum of 20 cells of *C. affinis* by dilution into 48 well plates. When microscopy (magnification 10x) revealed the presence of single cells/chains, these were transferred to new medium to grow for another 8 days. The cells were then centrifuged, and the DNA of the resulting pellet was extracted using the Quiagen® DNeasy Blood & Tissue 96-well plate kit. We found that very small amounts of material were not enough to yield adequate microsatellite analysis results (no usable data). Microsatellite amplification was done following the protocol described in supplementary S2 with the following primers: Primer C.a_LL_8 (Access. No. t.b.a. (F) t.b.a. (R)) and Primer C.a_LL_17 (Access. No. t.b.a. (F) t.b.a. (R)).

Reciprocal adaptation assays

After 8 and 36 batch cycles we tested each species for their evolutionary adaptation to the intended selection regimes, i.e. single and combined effect of CO₂ and the respective other species. Every evolved culture including the co-evolved species were tested in all four experimental treatments in a full factorial way. As a prerequisite to understand the potential of adaptation of both species separately and to make the adequate species combinations in the reciprocal assay experiments, it was necessary to separate the diatom and coccolithophore by a 20µM mesh sieve. This was possible because they differed enough in size: the diameter of *E. huxleyi* was between 2.5 and 4µM whereas the length of *C. affinis* started at ca. 15µM. Additionally, *C. affinis* has long spines that make it bigger than the coccolithophore and kept it from going through the used sieve. We had previously tested the efficiency of the separation and found that after sieving the growth of the “unwanted” species was negligible. In order to test for adaptation and not short-term acclimation we let the cultures acclimatize to the assay conditions for one entire batch cycle prior to measuring adaptation responses. The CO₂ treatment in the assays was manipulated as in the long-term experiment and was therefore a constant manipulated factor in the two assays. As for the selection by the 2nd co-occurring species the abundance of the 2nd species was adjusted in the respective assays to represent the actual presence of the other species at the time point of adaptation testing and this varied among the two assays (Table 2 relative species contributions). Specifically, at the end of batch cycle 8 and 36 the relative contributions of each species to the 2-species-community were determined in both CO₂ treatments. The mean of the relative composition was then used in the assays as the relative species composition in the respective “2nd species”-treatments in both ambient and high CO₂ (Table 2 relative species contributions). The 2nd species we added in the assay treatments came from the same selection treatment as the assay treatment to avoid any confounding responses of the added species. Consequently, the conditions for the treatments “2nd species” and “CO₂, 2nd species” were not equal in strength in both assays.

Table 2. relative species contributions to each co-evolving community at the end of batch cycle 8 and 36.

| Treatment | Species | Batch cycle 8 [% contribution] | Batch cycle 36 [% contribution] |
|---|-------------------|---------------------------------------|--|
| “2 nd species” | <i>E. huxleyi</i> | 21.1 | 72.3 |
| | <i>C. affinis</i> | 78.9 | 27.7 |
| “CO ₂ , 2 nd species” | <i>E. huxleyi</i> | 3.9 | 85.1 |
| | <i>C. affinis</i> | 96.1 | 14.9 |

Depending on the treatment that each adapted species was exposed to in the assays we could test for the potential or the cost of evolutionary adaptation (Collins, 2011). In the statistical analysis this could be identified as significant interactions of the respective selection and assay treatment factors (e.g. selection CO₂ x assay CO₂).

Statistical analyses

For the analysis of the absolute biovolume and the reciprocal assay data we used a repeated measures analysis of variance (rmANOVA). Before starting the analysis we tested for the homogeneity of variance using a Fligner Killeen Test (Fligner and Killeen, 1976) and for the normality of the residuals using a Shapiro-Wilk Normality Test (Shapiro and Wilk, 1965). The assumption of sphericity was not violated because we only had one repeated measure (BC8 and BC36) (Field et al.). All assumptions were met such that we could continue with the analysis without data transformation. The absolute biovolume was first assessed for the overall effects of batch cycle (i.e. Time), “CO₂” and “2nd species”. Precisely, we focused on two time points during the experiment (batch cycle (BC)8 “short-“ and BC36 “longterm”, respectively) and started with a complete data set analysis (aov((BioV)~(Time * CO₂ * 2nd species) + Error(Replicate/(Time))). We then continued analyzing the time points separately by subsetting the dataset into BC8 and BC36 (aov(BioV)~ CO₂ * 2nd species))). The analysis was done separately for each species. A similar approach was used for the analysis of the reciprocal adaptation assay starting with an overall analysis (aov((muExp)~(selection CO₂ * selection 2nd species * Assay CO₂ * Assay 2nd species * Time) + Error(Replicate/Time))) followed by a separate analysis of each assay. A permutational multivariate analysis of variance (permanova, with 999 permutations) was used to test for the differences in genotype compositional change between the treatments. All modelling and statistical analysis was done using the software R (Coreteam, 2016). The following packages were used for the analysis, plotting and modelling: ‘ggplot2’, ‘deSolve’, ‘vegan’, ‘ez’ (Michael A. Lawrence, 2016; Oksanen et al., 2007; Soetaert et al., 2010; Wickham, 2009).

Results

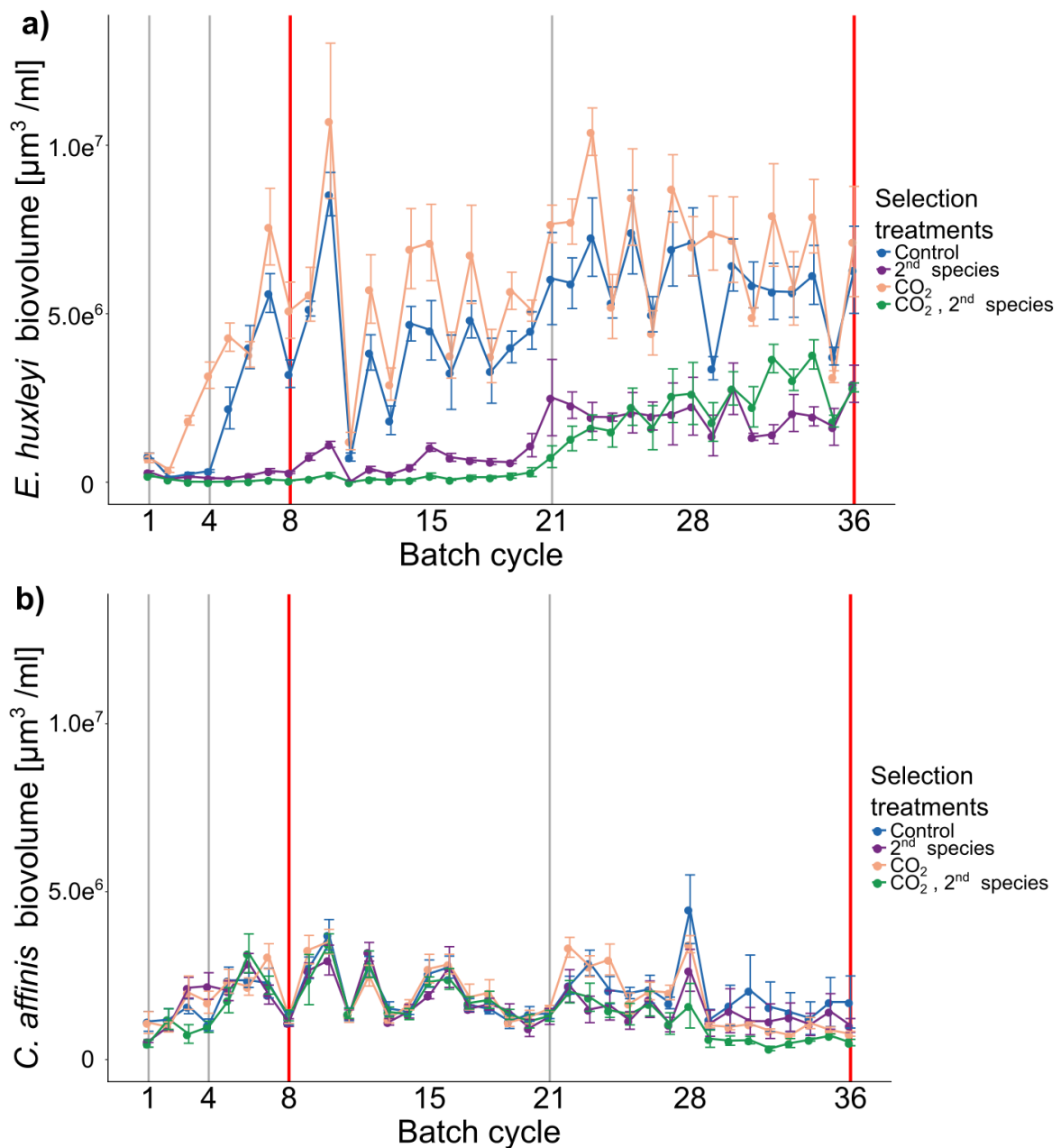


Figure 2. The absolute biovolume of *E. huxleyi* (a)) and *C. affinis* (b)) measured at the end of each batch cycle over 36 batch cycles in the four selection treatments is shown here (mean \pm SE; N=16 per time point). The grey and red lines show the time points where samples were taken for genotype reisolation. The red lines show when the cultures were tested for adaptation in the reciprocal assay test.

Visual assessment shows clear changes in the absolute biovolume of *E. huxleyi* over time in response to all treatments (Figure 2 a), such responses were absent in *C. affinis*, where absolute biovolume was constant over time (Figure 2 b). At the beginning there were large differences between the total biovolume due to the presence of the 2nd species

whereas this difference decreased over time. The absolute biovolume of *E. huxleyi* increased most strongly around BC18-22 about two orders of magnitude in the treatment where the 2nd species was present. When *E. huxleyi* was growing without *C. affinis* the increase in absolute biovolume is seen within the first 10 batch cycles. These temporal changes in *E. huxleyi* were similar between the CO₂ treatments and the treatments where the second species was present. The first reciprocal adaptation assay fell within a phase (BC8) before the most prominent changes, whereas the second assay was conducted at the end of the experiment (BC36). We will use these time points for further elaboration of treatment responses in the experiment. Generally, there was a strong negative effect of the presence of the diatom on *E. huxleyi* which was less strong at BC36 compared to BC8 (Figure 2a) (Table S1. Statistical analysis BC8: “2nd species” $F_{1,12}=355.50$, $p<0.0001$; BC36: “2nd species” $F_{1,12}=12.742$, $p=0.004$). In the first half of the experiment (until ca BC21) the absolute biovolume of *E. huxleyi* was reduced by increased CO₂ when *C. affinis* was present (Figure 2a) (Table S1 Analysis BC8 *E. huxleyi* “CO₂ x 2nd species” $F_{1,12}=34.78$, $p<0.0001$). This effect disappeared at the end of the experiment (Table S1 Analysis BC36 *E. huxleyi*). No effect of *E. huxleyi* on the abundance of *C. affinis* could be seen while the diatom also showed no response to increased CO₂ (Figure 2b) (Table S1 Analysis BC8 and BC36 *C. affinis*).

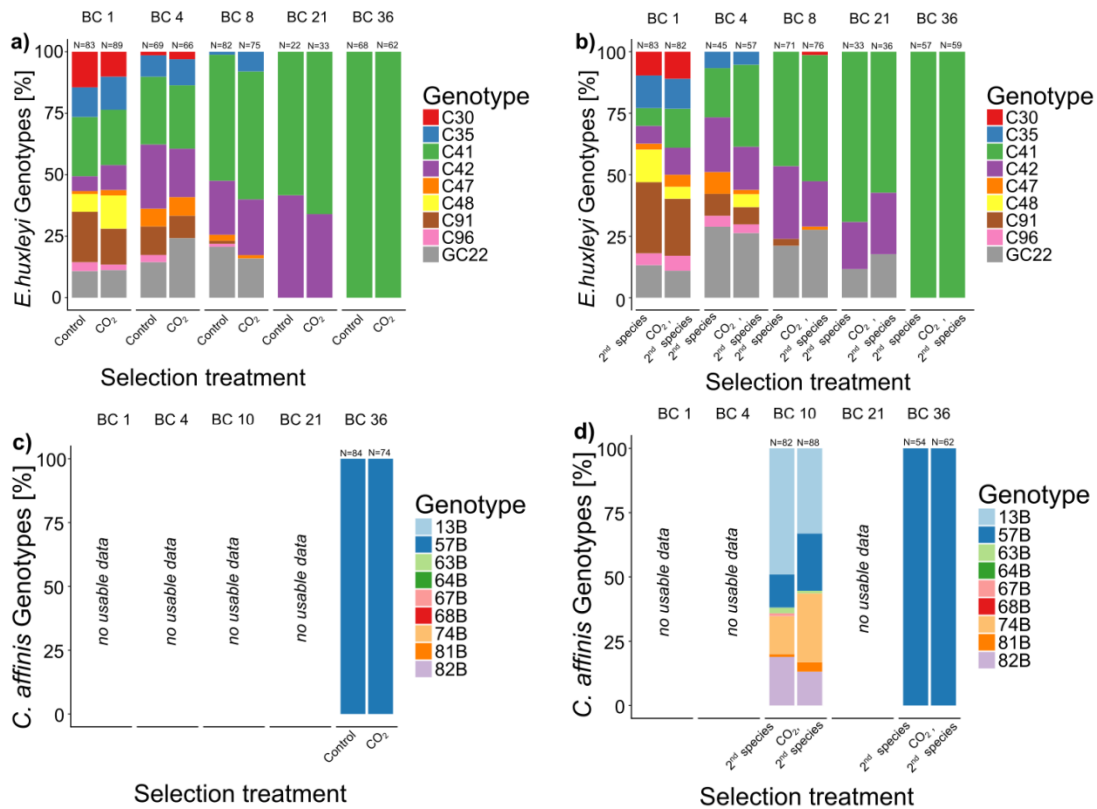


Figure 3. Relative genotype contributions in *E. huxleyi* (a) & b)) and *C. affinis* (c) & d)) experimental populations at 5 and 2 timepoints, respectively, over the course of the experiment in ambient and high CO₂ concentrations. a) and c) show the contribution to the population without the second species whereas b) and d) show the contribution to the population with the respective other species. Re-isolation and DNA extraction of *C. affinis* genotypes proved difficult and we did not obtain usable data. However, the overall picture of strong genotype sorting remained.

We were unable to detect any differential genotype sorting between the treatments for both species using a perMANOVA approach. However, we observed strong overall evolutionary change by marked, non-random sorting of genotypes across treatments which applied to both species, as a result a single genotype of originally nine genotypes remained in *E. huxleyi* and *C. affinis* after 36 batch cycles (Table S2 significant effect of “Time” in both species). There are missing data in Figure 3 c) and d) because the re-isolation of genotypes and subsequent DNA extraction did not yield enough material for microsatellite detection. However, the remaining of only one genotype at the end of the selection experiment in *C. affinis* was in line with the findings in *E. huxleyi*.

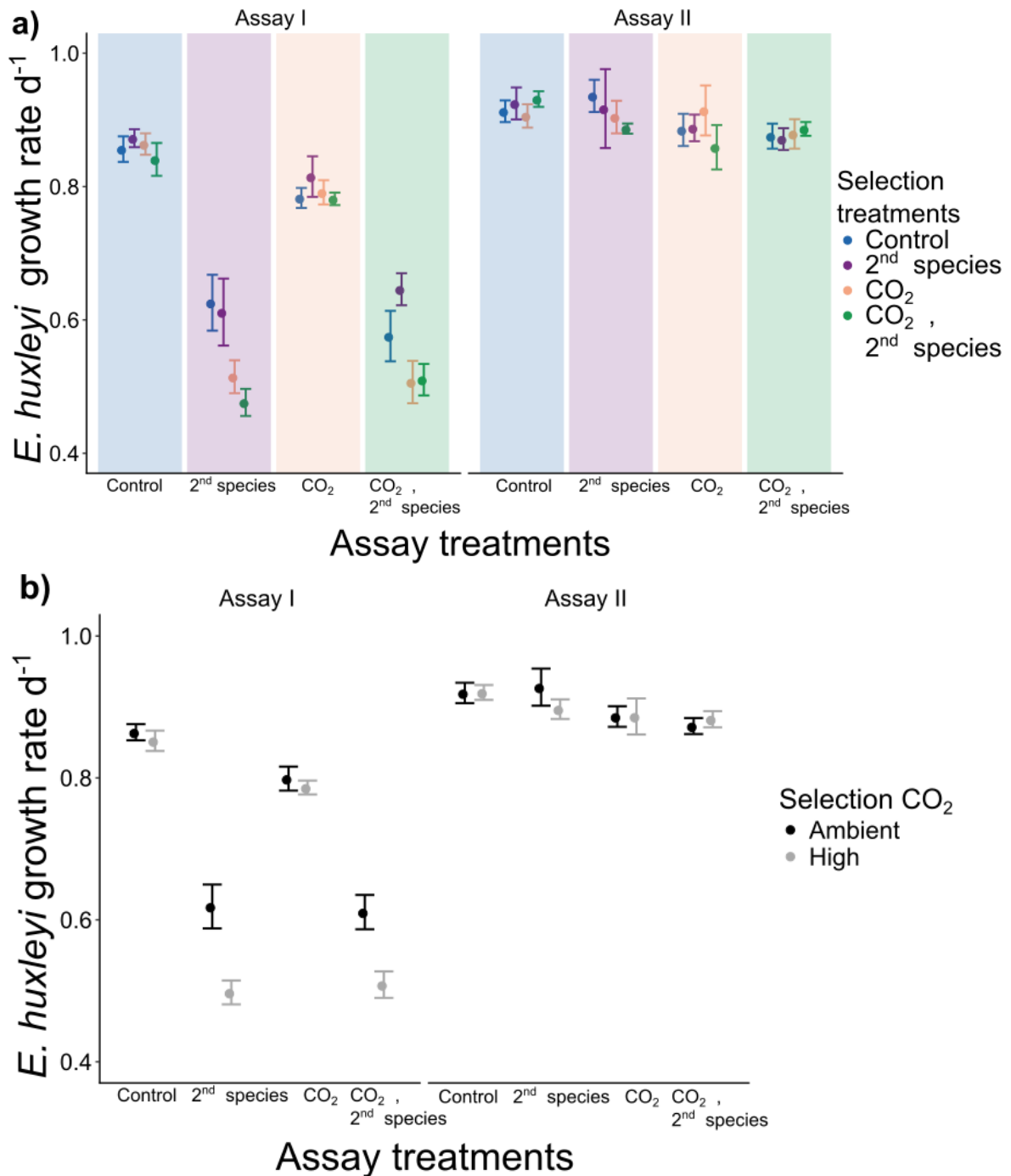


Figure 4. Results of adaptation assay experiments on growth rates of *E. huxleyi* after 8 and 36 batch cycles (assay I and II, respectively). Panel a) shows the growth rates of all cultures from the selection treatments (symbols) in the full factorial way (mean \pm SE, N=4) grouped for the different assay conditions (x-axis). In panel b) we pooled the growth rates of the cultures that had been selected in the 2nd species treatments because they did not have a significant effect (mean \pm SE, N=8) for better understanding of the significant main and interactive effects.

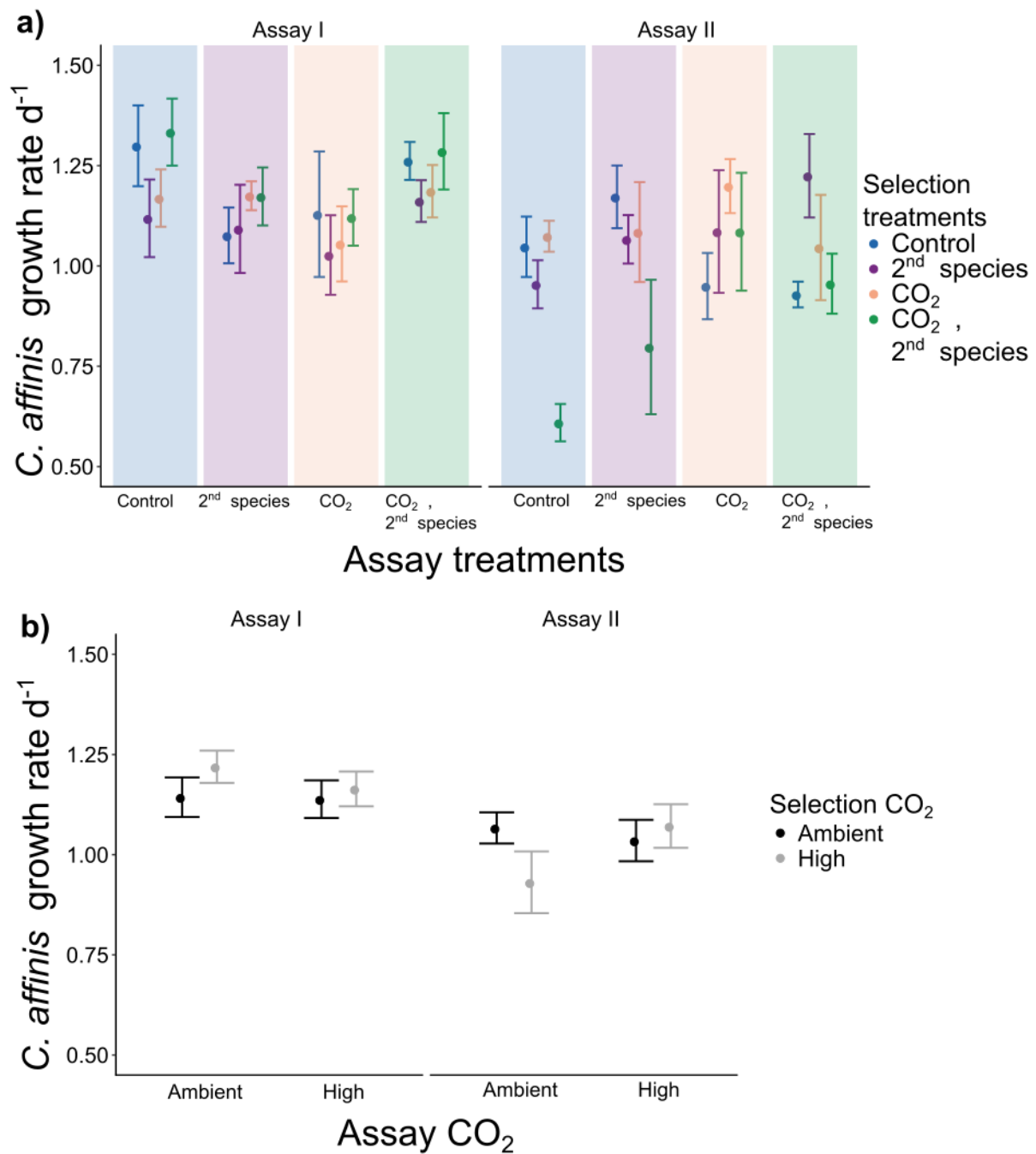


Figure 5. Main results of adaptation assay experiments on growth rates of *C. affinis* after 8 and 36 batch cycles (assay I and II, respectively). Panel a) shows the growth rates of all cultures from the selection treatments (symbols) in the full factorial way (mean \pm SE, N=4) grouped for the different assay conditions (x-axis). In panel b) we pooled the growth rates of the cultures that had been selected in the 2nd species treatments and grew in the 2nd species assay treatments because they did not have a significant effect (mean \pm SE, N=16) for better understanding of the significant interactive effects.

In *E. huxleyi* we found an overall significant effect of CO₂ selection in the first assay which was due to slightly lower growth rates in cultures that had been selected in high CO₂ during the experiment (Table S3 Analysis Assay I *E. huxleyi* “selection CO₂”

$F_{=1,47}=17.807$, $p=0.0001$). This negative effect was not detectable anymore in the second assay (Assay II *E. huxleyi* “selection CO₂” $F_{=1,47}= 0.928$, $p= 0.340$) (Figure 4b). Additionally, in the first assay the growth rates were much lower when the diatom was present (Table S3 Analysis Assay I *E. huxleyi* “Assay 2nd species” $F_{=1,47}=254.708$, $p<0.0001$) whereas in the second assay this decrease in growth rates was not found (Analysis Assay II *E. huxleyi* “Assay 2nd species” $F_{=1,47}= 1.068$, $p=0.307$) (Figure 4b) even though the diatom was still present with a contribution of ca. 15-30% (Table 2). These findings in *E. huxleyi* confirm the weakening of the negative effect of CO₂ and the 2nd species that we have already seen in the absolute biovolume during the experiment.

In *E. huxleyi* the rapid and equal genotype sorting was associated with a no direct adaptation, which would have been seen as the interaction of selection and assay treatments, to increased CO₂ or the presence of the 2nd species after 50 or 200 generations of experimental duration (Figure 4a). However, we did find signs for adaptation to increased CO₂ in *C. affinis* in the second assay (Figure 4 b) (Table S3 Analysis Assay II *C. affinis* “selection CO₂” * “assay CO₂” $F_{=1,47}=17.807$, $p=0.0001$) despite equal genotype sorting and an overall decrease of growth rates over time (Figure 5a) (Table S3 Analysis Assay I and II *C. affinis*: “Time” $F_{=1,46}=14.843$, $p<0.001$). There were other significant higher order interactions in *C. affinis* but following our objectives they do not have a reasonable relevance to be interpreted.

We can use the time effect among the identically executed reciprocal assay experiments to identify general potential adaptation to the laboratory batch cycle environment as a uniform change in both species. Interestingly, in *E. huxleyi* we observed a uniform increase in growth rates over time in all selection treatments (Figure 4a; assay I vs assay II) (Table S3 Analysis Assay I and II *E. huxleyi*: “Time” $F_{=1,47}=391.511$, $p<0.0001$). The effect is most prominent when growth rates were measured in an environment where the diatom was present and in the cultures that had adapted to high CO₂. None of these uniform changes were found in *C. affinis* even though we did find a significant overall decrease of growth rates over time.

Discussion

This study represents to our knowledge one of the first to successfully investigate eco-evolutionary dynamics in a competitive phytoplankton system. Our long-term experiment revealed that initial expected (or first order) interactions among a coccolithophore and a

diatom gave way to unexpected and different species interactions over time, demonstrating strong eco-evolutionary coupling. Specifically, the initially expected dominance of the diatom *C. affinis* over *E. huxleyi* weakened over time, with *E. huxleyi* attaining more abundance and almost equal biovolume compared to *C. affinis* at the end of the experiment. Additionally, the negative effect of increased CO₂ on *E. huxleyi*, which was only seen in combination with the presence of *C. affinis*, disappeared over time. It was surprising that the reciprocal adaptation assay and genotype sorting showed no direct adaptation to CO₂ or the presence of the 2nd species in *E. huxleyi* which would manifest as any interaction of selection * assay treatments. However, in *C. affinis* the second assay revealed adaptation to increased CO₂ after ca. 200 generations including costs to adaptation, the classical "smile" pattern of adaptation (Figure 5b) Assay II). This is puzzling since we found no differential genotype sorting across CO₂ or species co-occurrence treatments, but we should note that the time elapsed until the 2nd assay experiment (approximately 200 asexual generations) is sufficient to produce adaptation via novel mutations (Lakeman et al., 2009) and not just through genotype sorting.

The weakening effect of the presence of the second species in *E. huxleyi* and the disappearance of the negative CO₂-effect in the second half of the experiment could not be attributed to direct adaptation to the respective treatment environment. Assuming that the assay experiments have been performed under nearly identical, highly controlled conditions, their different results provide evidence as to what has changed across all intended selection treatments (Collins, 2011; Lakeman et al., 2009). The observed temporal improvement in growth rates over time in the coccolithophore in particular in the inter-specific treatment and the absence of any such response in the diatom suggests that the overall experimental regime represented a strong unintended selection regime which was also supported by highly reproducible and equal genotype sorting in both species. To simulate a bloom-like situation where species interactions are fully met we had to let the cultures grow into stationary phase. A prerequisite to reach the stationary phase is the limitation of at least one major nutrient (i.e. nitrate, phosphate or silicate) (Paulton, 1991). Therefore, in addition to the temperature, light and rotation regime we had strong nutrient limitation at the end of each batch cycle in all the experimental treatments.

Contrary to our experiment all other long-term experimental studies in phytoplankton were kept under exponential growth and non-limiting nutrient conditions throughout the experiment (Li et al., 2017; Lohbeck et al., 2012; Pardew et al., 2018). This implies that

resource competition for macronutrients such as P and N is negligible, while there is a premium for all populations to attain maximal growth rates. Various meta-analyses (Gao and Campbell, 2014; Rost et al., 2008) argue that varying responses of phytoplankton to increased CO₂ could be confounded by different experimental setups concerning CO₂ manipulation, light, temperature and growth conditions. Lohbeck et al. (2012) showed that in response to increased CO₂ (i.e. 1000 μ atm) there was strong differential genotype sorting compared to the ambient control treatments. These findings made us confident that we would see differential genotype sorting as well. However, one important difference between our long term study and the one conducted by Lohbeck et al. (2012) is the experimental regime. Where Lohbeck et al. (2012) transferred their cultures always during the exponential phase we waited until the cultures had reached the stationary phase.

The hidden selection via the batch cycle regime resulted in a single surviving genotype in *E. huxleyi* and *C. affinis*, respectively (genotype C41 and B57) and their reciprocal interaction played out very differently compared to the full 9 genotypes in each species initially. In *E. huxleyi* the remaining genotype apparently had an increased ability to compete against the remaining diatom genotype and an increased ability to cope with CO₂. In the beginning we did find CO₂ to have a negative effect on *E. huxleyi* by reducing its biovolume and growth rates (selection experiment and first reciprocal assay, respectively). Yet unexpectedly this was only the case when the diatom was present. As such we could not show that CO₂ and the 2nd species were classical additive negative stressors. Rather the additive negative effect of increased CO₂ and the presence of the second species was hidden. When *E. huxleyi* grew alone it was able to tolerate increased CO₂ concentrations but when another species was added, its tolerance to both enhanced CO₂ concentrations and the need to compete for resources initially exceeded its tolerance threshold. The winning genotype of *C. affinis* had the ability to sustain its competitive ability against the remaining *E. huxleyi* genotype, and was able to adapt to increased CO₂ concentrations. Even though the microsatellite analysis detected the same genotype in *C. affinis* it is likely that during the experimental selection within strain variation arose due to novel mutations (Lakeman et al., 2009) that lead to adaptive evolution to increased CO₂. While the primary goal of our experiment was not to fully characterize the underlying traits of all genotypes and both species we have some predictions on what the prerequisites for the genotype selection and change on the species level could be. High growth rates and tolerance against increased CO₂ are among the required traits and both remaining genotypes C41

and B57 in *E. huxleyi* and *C. affinis*, respectively, feature exactly these traits (Hattich et al. 2017). However, other traits that relate to strong competitive ability such as nutrient uptake related attributes (e.g. high nutrient affinities and uptake velocities) should also be of importance (Sommer, 1984).

There are several possible reasons for the significant increase of the total biovolume of the coccolithophore and the almost absent overall changes in the diatom (Figure 2). Here we present one possible explanation for the different changes in absolute biovolume between the species we studied. An increase of biovolume over time is possible when there are nutrients available to use or overall the nutrients are used more efficiently within an organism (REF). In our study we found that in the beginning of the selection experiment apparently nitrate, compared to the other macronutrients phosphate and silicate, was not fully used (Figure S2 BC11) indicating that the experimental system was phosphate-limited. This is a condition that *E. huxleyi* (compared to *C. affinis*) is rather well equipped to grow in (REF P limitation Cocos, Dyna 2), such that it could possibly use the available nitrate to increase its biovolume. This use of available dissolved nitrate was reflected in general reshuffling of particulate nutrients between the first and second assay and differences between the species (Figure S3 and S4): In *E. huxleyi* the particulate organic nitrate (PON) concentrations increased between the two assays (Figure S3) whereas in *C. affinis* they stayed the same (Figure S4). Additionally, particulate organic phosphate (POP) slightly increased in *E. huxleyi* (Figure S3) and reciprocally decreased in *C. affinis* (Figure S4). It is beyond the scope of this study and the available data to infer something about the changes in nutrient use efficiencies; however it is likely that they changed as well over time. After all, the question remains though, why the diatom did not use the available nitrate to increase its biovolume as it is known that diatoms normally use available nutrients very rapidly (Sommer 1984). As a diatom *C. affinis* requires silicate to grow its silicate shells (Egge 1992) and if silicate becomes limiting diatoms cannot grow anymore (Ranueneau 2000). In order to ensure coexistence of the two species we limited the silicate concentrations to ca 5 μ M in our study (see further explanation in Listmann et al. in prep). It is possible that the low silicate concentrations constrained the growth of *C. affinis* (more so than *E. huxleyi* or another macronutrient) and as such its potential of adaptation. This again shows that the experimental regime likely played an unexpected and important role in the species' responses.

Conclusion

Over approximately 200 generations, the interactions of *C. affinis* and *E. huxleyi* changed strongly converting the winner to the loser species and vice versa. Whether this altered interaction is already an attribute of both winning genotypes, or whether additional evolutionary change contributed was beyond the scope of the study. Notwithstanding, the results demonstrate that strong eco-evolutionary coupling alters the dynamics in the simplest possible two-species phytoplankton "community". The eco-evolutionary coupling was a result of overriding selection via the batch cycle regime in combination with constrained diatom growth. Community experiments often cannot differentiate between or identify ecological and evolutionary change and usually do not run for a long enough time to investigate both processes. Therefore, our constrained laboratory "community" experiment highlights the uniqueness of the experimental selection approach followed by reciprocal adaptation assays to understand eco-evolutionary processes and that rapid experimental evolution is possible within a few 100 generations even though the stronger effects were possibly due to unintended laboratory selection. Since nutrient limitation had possibly a strong effect on our system we emphasize that future studies should focus more on the consequences of nutrient limitation. Increasing temperatures due to global change are predicted to lead to less mixing of the water column and thus more stratification. Consequently, there will be more nutrient limitation on phytoplankton in the near future (Boyd et al., 2013; Moore et al., 2013) as a relevant abiotic driver in environmental change.

Overview supplementary material

Table S1-S3: statistical analysis of the selection experiment, genotype sorting and reciprocal adaptation assays respectively

Figure S1: Semi continuous batch cycle growth curves

Figure S2: Dissolved inorganic nutrients in one batch cycle

Figure S3&4: Particulate nitrate (PON) and phosphate (POP) for *E. huxleyi* and *C. affinis*, respectively

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Additional information Chapter IV

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Supplementary material Chapter IV

Supplementary material: Statistical analysis

Table S1: Analysis report for absolute species abundance: a repeated measures ANOVA for comparison between BC8 and BC36 was used whereas factorial ANOVAS were used for BC8 and BC36 separately.

| <i>Emiliania huxleyi</i> | | | | |
|---|----------|----------------------------|---------------|-------------------|
| <i>Analysis BC8 and BC36</i> | df | MS | F-value | P-Value |
| <i>Error: replicate</i> | | | | |
| <i>CO₂</i> | 1 | 2.637e ¹² | 1.221 | 0.291 |
| <i>Culture</i> | 1 | 1.258e¹⁴ | 58.251 | <0.0001 |
| <i>CO₂ × 2nd species</i> | 1 | 4.554e ¹² | 2.108 | 0.172 |
| <i>Residual</i> | 12 | 2.160e ¹² | | |
| <i>Error: replicate:Time</i> | | | | |
| <i>Time</i> | 1 | 5.310e¹³ | 13.541 | 0.003 |
| <i>CO₂ × Time</i> | 1 | 3.499e ¹¹ | 0.089 | 0.770 |
| <i>2nd species × Time</i> | 1 | 9.340e ¹⁰ | 0.024 | 0.880 |
| <i>CO₂ × 2nd species × Time</i> | 1 | 6.276e ¹¹ | 0.160 | 0.696 |
| <i>Residual</i> | 12 | 3.921e ¹² | | |
| <i>BC8 alone</i> | | | | |
| <i>CO₂</i> | 1 | 1.86 | 13.91 | 0.003 |
| <i>2nd species</i> | 1 | 47.42 | 355.50 | <0.0001 |
| <i>CO₂ × 2nd species</i> | 1 | 4.64 | 34.78 | <0.0001 |
| <i>Residual</i> | 12 | 0.13 | | |
| <i>BC36 alone</i> | | | | |
| <i>CO₂</i> | 1 | 5.329e ¹¹ | 0.114 | 0.741 |
| <i>Culture</i> | 1 | 5.953e¹³ | 12.742 | 0.004 |
| <i>CO₂ × 2nd species</i> | 1 | 9.001e ¹¹ | 0.193 | 0.669 |
| <i>Residual</i> | 12 | 4.672e ¹² | | |
| <i>Chaetoceros affinis</i> | | | | |
| <i>Analysis BC8 and BC36</i> | | MS | F-value | P-Value |
| <i>Error: replicate</i> | 0.158 | | | |
| <i>CO₂</i> | 0.222 | 149733 | 2.240 | 0.158 |
| <i>2nd species</i> | 0.734 | 109824 | 1.643 | 0.222 |
| <i>CO₂ × 2nd species</i> | | 8064 | 0.121 | 0.734 |
| <i>Residual</i> | | 66830 | | |
| <i>Error: replicate:Time</i> | 0.083 | | | |
| <i>Time</i> | 0.056 | 208185 | 3.517 | 0.083 |
| <i>CO₂ × Time</i> | 0.348 | 261686 | 4.421 | 0.056 |
| <i>2nd species × Time</i> | 0.959 | 56042 | 0.947 | 0.348 |
| <i>CO₂ × 2nd species × Time</i> | | 160 | 0.003 | 0.959 |
| <i>Residual</i> | 12 | 59194 | | |

Table S2: Permanova results for analysis of genotype compositional change; permutations=999

| <i>Emiliania huxleyi</i> | | | | | | <i>Chaetoceros affinis</i> | | | | | |
|---|----------|--------------|----------------|----------------|--------------|------------------------------|----------|--------------|----------------|----------------|--------------|
| <i>Permanova</i> | df | MS | F Model | R ² | P-Value | <i>Permanova</i> | df | MS | F-value | R ² | P-Value |
| <i>Time</i> | 4 | 92402 | 1855273 | 0.999 | 0.001 | <i>Time</i> | 1 | 84505 | 2883621 | 0.999 | 0.001 |
| <i>CO₂</i> | 1 | 0 | 4 | 0 | 0.085 | <i>CO₂</i> | 1 | 0 | 2 | 0 | 0.180 |
| <i>2nd species</i> | 1 | 0 | 1 | 0 | 0.401 | <i>Time × CO₂</i> | 1 | 0 | 2 | 0.0001 | 0.191 |
| <i>Time × CO₂</i> | 4 | 0 | 1 | 0 | 0.324 | <i>Residual</i> | 16 | | | | |
| <i>Time × 2nd species</i> | 4 | 0 | 1 | 0 | 0.346 | | | | | | |
| <i>CO₂ × 2nd species</i> | 1 | 0 | 0 | 0 | 1 | | | | | | |
| <i>Time × CO₂ × 2nd species</i> | 4 | 0 | 1 | 0.0001 | 0.452 | | | | | | |
| <i>Residual</i> | 80 | | | | | | | | | | |

Table S3: Analysis report for reciprocal adaptation assay: a repeated measures ANOVA for comparison between Assay I and II was used whereas factorial ANOVAS were used for Assay I and II separately. Only the significant results are reported here.

| <i>Emiliania huxleyi</i> | | | | | <i>Chaetoceros affinis</i> | | | | |
|--|----|----------------------|---------|---------|---|----|--------|---------|---------|
| <i>Analysis Assay I and II</i> | df | MS | F-value | P-Value | <i>Analysis Assay I and II</i> | df | MS | F-value | P-Value |
| <u><i>Error: replicate</i></u> | | | | | <u><i>Error: replicate</i></u> | | | | |
| <i>Selection CO₂</i> | 1 | 0.0542 | 14.469 | <0.001 | none | | | | |
| <i>Assay CO₂</i> | 1 | 0.0386 | 10.311 | 0.002 | | | | | |
| <i>Assay 2nd species</i> | 1 | 0.5745 | 153.307 | <0.0001 | | | | | |
| <i>Selection CO₂ × Assay 2nd species</i> | 1 | 0.0330 | | 0.005 | | | | | |
| <i>Assay CO₂ × Assay 2nd species</i> | 1 | 0.0195 | | 0.027 | | | | | |
| <i>Residuals</i> | 47 | 0.0037 | | | <u><i>Error: replicate:Time</i></u> | | | | |
| <u><i>Error: replicate:Time</i></u> | | | | | <i>Time</i> | 1 | 0.6238 | 14.843 | <0.001 |
| <i>Time</i> | 1 | 1.4208 | 391.511 | <0.0001 | <i>Selection CO₂ × Selection 2nd species × Time</i> | 1 | 0.4607 | 10.961 | 0.002 |
| <i>Selection CO₂ × Time</i> | 1 | 0.0246 | 6.779 | 0.012 | <i>Selection CO₂ × Assay CO₂ × Time</i> | 1 | 0.2481 | 5.902 | 0.019 |
| <i>Assay 2nd species × Time</i> | 1 | 0.5125 | 141.223 | <0.0001 | <i>Selection CO₂ × Assay 2nd species × Time</i> | 1 | 0.2967 | 7.061 | 0.011 |
| <i>Residual</i> | 47 | 3.921e ¹² | | | <i>Residuals</i> | 46 | 0.0420 | | |
| | | | | | | | | | |

| Table S3 continued: | | | | | | | | | |
|--|----|----------|---------|---------|---|----|---------|-------|-------|
| Assay I | | | | | Assay I | | | | |
| <i>Selection CO₂</i> | 1 | 0.0759 | 17.807 | 0.0001 | | | | | |
| <i>Assay 2nd species</i> | 1 | 1.0861 | 254.708 | <0.0001 | | | | | |
| <i>Selection CO₂ × Assay 2nd species</i> | 1 | 0.0448 | 10.511 | 0.002 | | | | | |
| <i>Assay CO₂ × Assay 2nd species</i> | 1 | 0.0277 | 6.485 | 0.014 | <i>Selection CO₂ × Selection 2nd species × Assay 2nd species</i> | 1 | 0.19669 | 4.295 | 0.044 |
| <i>Residuals</i> | 47 | 0.0043 | | | <i>Residuals</i> | 48 | | | |
| Assay II | | | | | Assay II | | | | |
| | | | | | <i>Selection CO₂ × Selection 2nd species</i> | 1 | 0.3318 | 6.808 | 0.012 |
| <i>Assay CO₂</i> | 1 | 0.025103 | 8.066 | 0.007 | <i>Selection CO₂ × Assay CO₂</i> | 1 | 0.2844 | 5.834 | 0.020 |
| <i>Residuals</i> | 47 | 0.003112 | | | <i>Residuals</i> | 46 | 0.0487 | | |

Supplementary figures Chapter IV

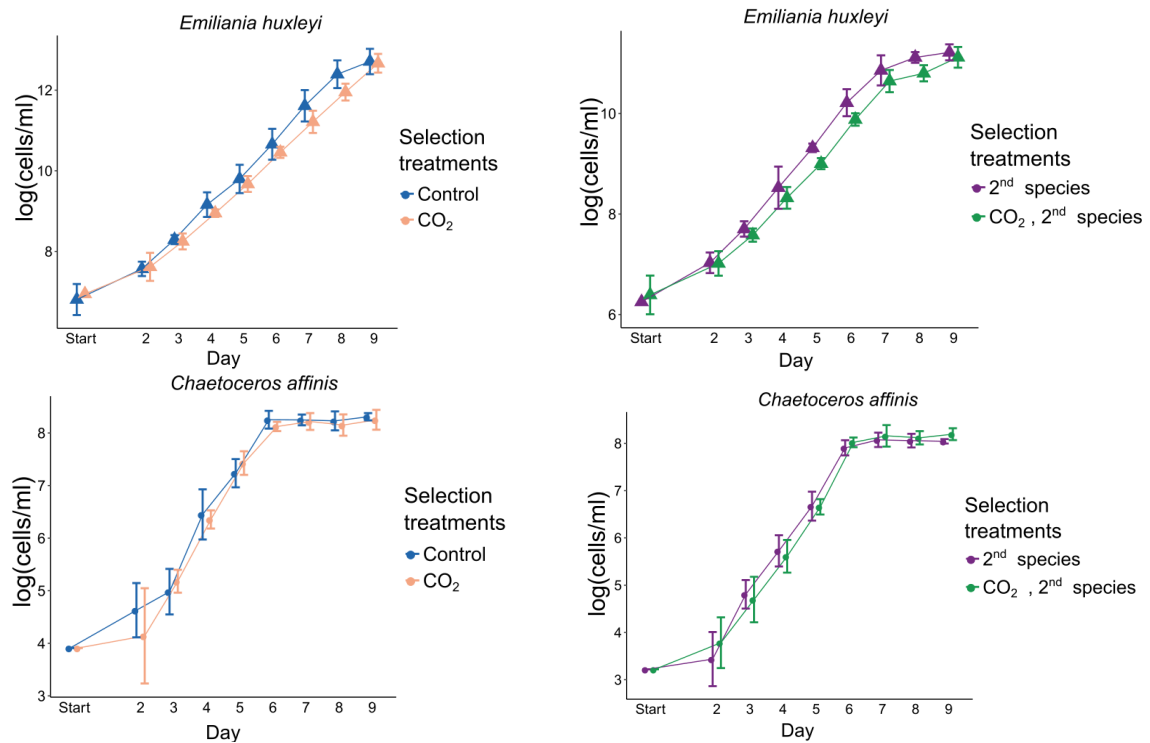


Figure S1: Here the growth within one batch cycle (beginning of the experiment) is shown for each species and all four treatments used in the selection experiment. Log cell numbers highlight the exponential phase (linear increase) and stationary phase (flattening of the curve towards day 7-9). Each dot represents the mean \pm SE, N=5.

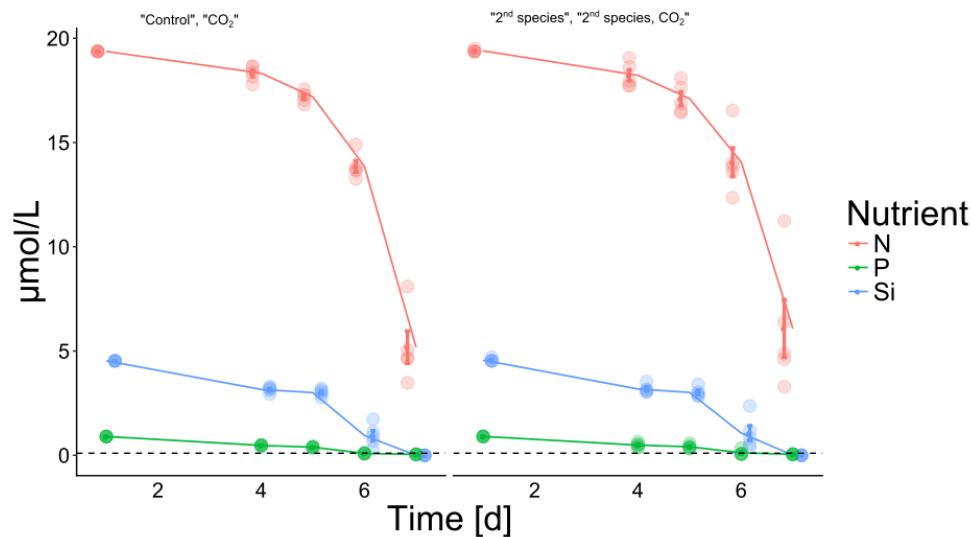


Figure S2: Dissolved inorganic nutrients measured over one batch cycles (BC11) (mean, N=4). The dotted line represents the detection limit of the measurement.

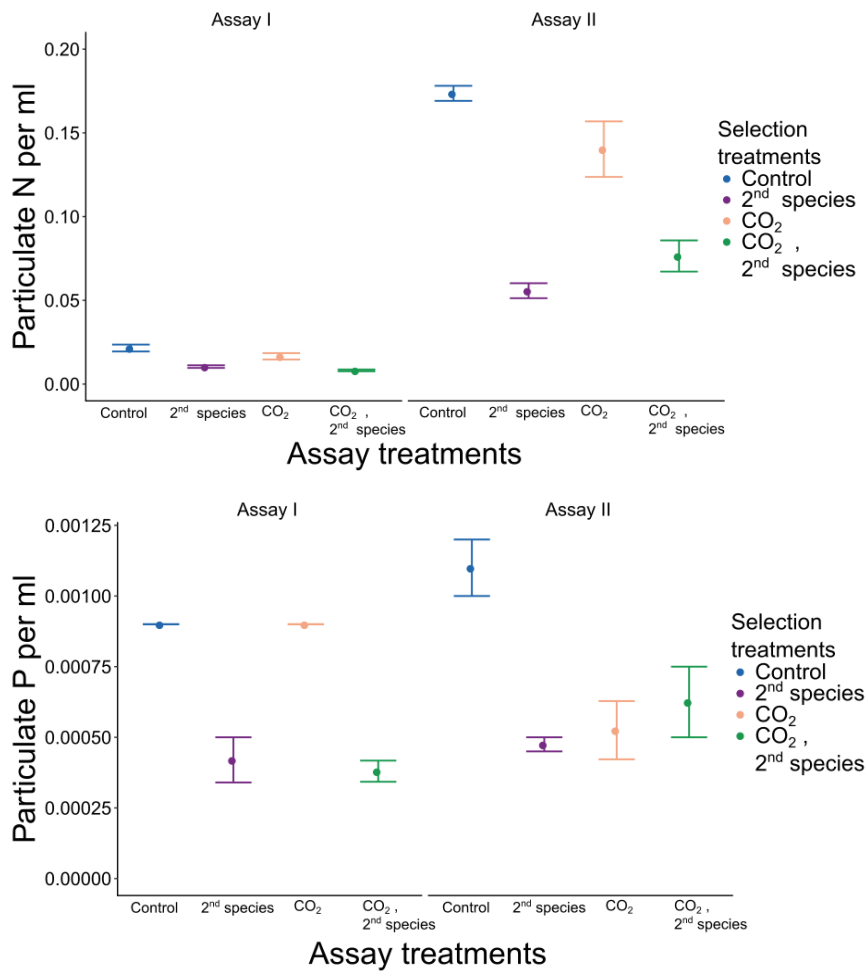


Figure S3: Particulate nitrate (PON) and phosphate (POP) concentration per mL in *E. huxleyi* (mean \pm SE, N=4) in both reciprocal assay experiments. Only the controls are shown here.

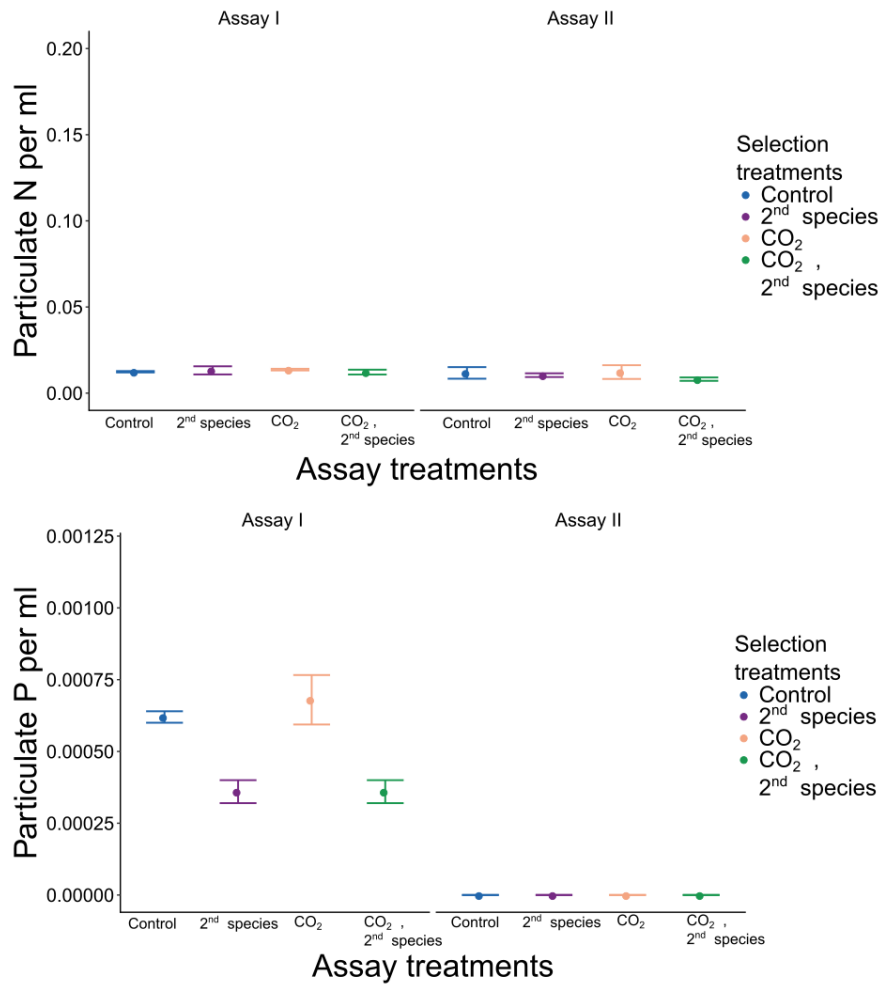


Figure S4: Particulate nitrate (PON) and phosphate (POP) concentration per mL in *C. affinis* (mean \pm SE, N=4) in both reciprocal assay experiments. Only the controls are shown here.

DNA extraction

DNA from two strains of *C. affinis* was extracted using a modified CTAB extraction method (Gagnon et al. 1980). The samples were frozen in liquid nitrogen and crushed with a cold adjusted pestle. Then CTAB extraction buffer as well as 100 μ L of RNase were added followed by an incubation for 15 minutes at 60°C and 15 minutes at room temperature. Two steps of elution with a chloroform-isoamyl alcohol mixture (24:1 vol:vol) were done. The DNA was precipitated from the water phase with ice cold isopropanol for 30 minutes. The DNA was additionally cleaned with 70% EtOH. With this method high quality DNA containing long strands can be extracted. The yields were 19.1 ng/ μ L for G05786 (strain B81) and 52.2 ng/ μ L for G05787 (strain B64).

DNA sequencing and primer search and testing

Sequencing libraries with 100 bp insert size were generated from genomic DNA using the TruSeq DNA Nano protocol. The libraries were sequenced on an Illumina NextSeq500, resulting in 33.5 and 17.5 million read pairs of 150 bp length for the samples G05786 and G05787, respectively. Raw reads were quality trimmed to an average quality of 10 and adapters clipped using BBDuk from the BBTools suite (Bushnell, n.d.) and then assembled into one draft genome per strain using the Platanus software with default parameters (Kajitani et al. 2014). Suitable microsatellite loci were searched in the resulting scaffolds using Tandem Repeats Finder (Benson 1999), and primers for amplification were designed with the help of the Primer3 software (Untergasser et al. 2012, 3). Microsatellite loci with different alleles between the two strains of each species were selected for analysis.

The primers (Table S1) were tested on the nine genotypes used in the experiment and yielded all positive microsatellite amplification in most strains. The primers were tested in the following reaction: 2.5 μ L multiplex mastermix (Qiagen), 1 μ L forward and reverse primer pair, 2 μ L Q solution (Qiagen), 1 μ L H₂O and 1 μ L of DNA template. The PCR reaction run for microsatellite amplification was set up as follows: an initial phase of 15min at 95°C, 30 cycles of 30sec at 94°C, 90sec at 57°C and 1min at 72°C and final step of 30 min at 60°C. 1 μ L PCR product was then added to a mix of ROX and Hidi (Qiagen) of 0.25 μ L and 8.75 μ L, respectively and incubated for 3 min at 94°C to denature double stranded products. We employed capillary electrophoresis coupled to fragment analysis

on an ABI 3130xl genetic analyzer to score the microsatellite composition using the software GeneMarker software.

Table: Primers for microsatellite analysis in *C. affinis*

| Primer Name | Forward seq. | Access No. | Reverse seq. | Access No. |
|-------------------|-------------------------------|--------------|---------------------------------|--------------|
| C.a._LL_1 | ACTTACCATGACAACAGCAATG | t.b.a. | ACCCATTGAGATTTGAGTTCATG | t.b.a |
| C.a._LL_3 | TGGTCATATGCCTCTTTCCGG | t.b.a | GGCAAACACAAACACAAACAC | t.b.a |
| C.a._LL_5 | CACTTTTGAAGGTACACAGTGG | t.b.a | ACGTTGGGAGAGCTATTGAG | t.b.a |
| <i>C.a._LL_8</i> | <i>GACGCTGGTAGTTTCGTTTG</i> | <i>t.b.a</i> | <i>AGTCCCTTGGAAATGGACTG</i> | <i>t.b.a</i> |
| C.a._LL_9 | GTGTAAGCGTAAAAGAATGCATG | t.b.a | AGTTCCTGCCTTCAGTCTTC | t.b.a |
| C.a._LL_11 | TGCATCTTGCTTGAGGAGG | t.b.a | AGTGCAAGGTTTACTCAATTCTG | t.b.a |
| C.a._LL_14 | TGAACCTGTGGATATGACGG | t.b.a | CACCCTCTTTATATGATGAGCG | t.b.a |
| C.a._LL_15 | TCTGCTCTGCGTTGTAGTTC | t.b.a | CTTTGTGTTTGCCTCCTTCC | t.b.a |
| <i>C.a._LL_17</i> | <i>GGGGTAATGAAATCTTTGGTGC</i> | <i>t.b.a</i> | <i>GTA CTGATTATCAACAGGTGCTC</i> | <i>t.b.a</i> |
| C.a._LL_19 | TAGGAATCATCGGCATCTGC | t.b.a | ATACGAAGGCCTTTCTGGAG | t.b.a |

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Synthesis

Community response to environmental change

In my dissertation I have for the first time demonstrated a feasible approach to simultaneously investigate both ecological and evolutionary processes in marine phytoplankton that include changes on the species and genotype level, respectively. My findings on single species showed weak but varying physiological responses to increased CO₂ concentrations and shifts in temperature reaction norms. However, when species were allowed to interact the response to increased CO₂ was almost negligible while the species interactions changed a lot over time. These findings show that understanding eco-evolutionary changes to climate change are likely more complex than previously considered. To further discuss and understand the implications of my findings, I briefly discuss the different chapters of my thesis.

With respect to phenotypic plasticity, I have shown that despite strongly varying response the coccolithophore species unexpectedly mostly buffered increasing CO₂ concentrations and that the diatom showed no significant fertilization response. A striking result was the difference between the inter- and intraspecific plasticity of a species when testing a mixture of genotypes versus the mean response of all genotypes tested separately. The plasticity response of a mixture of genotypes did not represent the full range of species plasticity which can only be identified when all genotypes are tested separately. Knowing the intraspecific variation is likely very important when making predictions for long term changes under climate change scenarios (Des Roches et al., 2018). As predicted, the adaptation over 2000 generations to new environments changed the physiological response of a species (i.e. phenotypic plasticity). Specifically, I could demonstrate that the optimal growth temperature of *E. huxleyi* shifted due to warming and ocean acidification (Chapter III) through a change in the whole temperature reaction norm. This is interesting since the adaptation was only to a single level of increased temperature but highlight the potential of adaptation which could positively influence adaptation to warming in the future.

The findings of these two chapters again only include the responses of one species or single genotypes and as insightful as they may be by showing different responses to environmental change, I was also interested to observe responses simultaneously on the genotype and species level as well and how these changes would be affected by increased CO₂. Unlike my expectations, as soon as species and genotypes were allowed to interact

and as such compete with each other, the responses to the environmental driver CO₂ was negligible.

Precisely, I found this in two chapters of my thesis where I addressed how adaptation to ocean acidification played out in a two-species phytoplankton community that was established and coexisted for up to 220 generations. Importantly, it included a controlled assembly of nine genotypes within each species that could be followed owing to their multi-locus microsatellite genotyping. Initially, I found that increased CO₂ concentrations affected the species composition enhancing the expected dominance of the diatom in the community but not the genotype composition. However, on the long term the effect of increased CO₂ concentrations disappeared. These findings are interestingly in line with weak community responses on the short term to increased CO₂ (Sommer et al., 2015) where many species and definitely many genotypes are interacting in a complex way. Therefore, it might be necessary to consider what direct or indirect effects ocean acidification has compared to other unknown and less well studied effects such as competition resulting from species interactions. My application of a Lotka-Volterra modeling approach highlighted that competitive interferences between and within species are indeed essential for ecological and evolutionary short-term sorting (Chapter II). However, changes in competitive interferences due to adaptation could not be captured by this approach. Rather, I demonstrated experimentally that species interactions could change over ca. 220 generations. Interestingly, these changes were not driven by any adaptation to intended experimental treatments (i.e. increased CO₂) but likely due to adaptation to the overall experimental regime underlined by swift genotype frequency changes in both *E. huxleyi* and *C. affinis* (Chapter II, Chapter IV). This swift selection was then associated with an increase in competitive ability and ability to cope with high CO₂ in *E. huxleyi*. As such, there were strong changes of the species interactions (Chapter IV), implying that in this constrained laboratory experiment, eco-evolutionary coupling happened. Changes in species interactions could alter relative species composition of a community which in turn could disturb community functioning if different major groups of species are affected. Consequently, future (long-term) experiments should include more complexity (i.e. species interactions) to understand how they may change in the long term. However, including complexity and as such “realism” in experimental studies imposes some difficulties.

Realism in experimental studies

There exists a trade-off between control/reproducibility and realism when doing experiments in the laboratory (Garland, 2009) and investigating how environmental change affects natural systems (Riebesell et al., 2013). In laboratory (evolution) experiments on microorganisms the environmental conditions can be controlled and experimental units replicated giving these studies high power to uncover basic (evolutionary) mechanisms (Elena and Lenski, 2003). These mechanisms include understanding the dynamics or genetic basis of adaptation or discovering trade-offs and specificity of adaptation (Merilä and Hendry, 2014). Nevertheless, currently we are still trying to fully understand how species can adapt and trying to associate the affected traits to the findings in community experiments (e.g. mesocosm experiments). One of the first approaches to look at the potential of adaptation to increased CO₂ in a natural system was done by Scheinin et al. (2015) where they found rapid adaptation of a diatom *Skeletonema marionori* to enriched pCO₂ in large (20 m³) pelagic mesocosms via sorting of naturally occurring genotypic variation. Predominantly those genotypes seemed to prevail that were able to take best advantage of enhanced inorganic carbon availability, as such their reaction norms was steeper than within the non-CO₂ enriched controls. In my dissertation my intention was to come from highly controlled experimentation in the laboratory and include more complexity into experimental evolution while keeping a tightly controlled environment with many replicates. My objective was to investigate the adaptation to an environmental driver (e.g. CO₂) in a system where two species (e.g. coccolithophore and diatom) compete (Chapter II, IV). Contrary to our expectations we could only show adaptation to increased CO₂ in the diatom and not the coccolithophore. More importantly though, we could monitor eco-evolutionary coupling over time as a consequence of very strong hidden selection by the experimental set-up. The hidden selection could stem from nutrient limitation as a consequence of the required semi-continuous batch cycle regime and the fact that the cultures were not kept in exponential growth. These findings highlight that experimental studies with more complexity are valuable, but it is important to understand the consequences of the experimental conditions.

Future research perspectives

Nutrient limitation

The experimental conditions I used in my studies are – compared to other studies – more realistic in relation to the nutrient concentrations and growth cycles that the organisms experienced but they lead to nutrient limitation. Since this ‘nutrient limitation’ possibly had a strong effect on our system future studies should focus more on the consequences of nutrient limitation, a common condition experienced by marine phytoplankton except in bloom conditions. Increasing temperatures due to global change are predicted to lead to less mixing of the surface waters and thus more stratification (Behrenfeld et al., 2006). Consequently, phytoplankton will increasingly grow under nutrient limitation in the future ocean (Boyd et al., 2013; Moore et al., 2013). Phytoplankton species require different macronutrients to grow including nitrate, phosphate and silicate (mostly diatoms) Therefore, ‘nutrient limitation’ should be studied considering the depletion of different macronutrients. Depending on the requirements and environmentally imposed changes in nutrient composition species and genotypes and as such communities may be altered in different ways (Boyd 2011). In relation to my findings I would re-evaluate the potential of eco-evolutionary coupling in the two-species community under phosphate and nitrate limitation. It is likely that the evolutionary change will vary with the consequence of differential adaptation and possibly different changes of the species interactions. Additionally, I would try to include more than just two species in future experiments. In order to understand the eco-evolutionary changes when more species are included, I think it is important to control for changes when the species are growing alone as well as in the different combinations.

Gene expression

When adapting to new environmental conditions an interesting question is the genetic basis of such evolutionary change. As a first step, assessing changes in gene expression or protein synthesis may help to understand how organisms cope with different environments (Becks et al., 2012; Ottesen et al.), although the transcription level of genes is strictly speaking only a molecular phenotype. Nevertheless, it brings us as close as it gets to the genomic basis of physiological adaptation. As a case in point, Lohbeck et al 2014 have shown that the expression levels of putative carbon transport and pH regulating genes were upregulated as an adaptive response to increased CO₂ concentrations which

could help maintain adequate ion concentrations in the cell compared to the surrounding environment. Similarly, to cope with limiting nutrient conditions, increasing the expression of nutrient transporters on the cell surface would help acquire nutrients more efficiently (Kang et al., 2009, 2015). Such changes have both been shown at least on the short term in *C. affinis* and *E. huxleyi* where under nitrate and phosphate (only *E. huxleyi*) limitation, there is strong reshuffling of the necessary nutrient acquisition pathways (Rokitta et al., 2014, 2016; Shih et al., 2015). However, it is unclear how these changes would play out in the long term. I would use the available samples I collected at the end of the long-term experiment as well as during a short-term “ancestor” test of the remaining genotypes C41 and B57 and assess potential transcriptomic changes. It would be very helpful to characterize molecular changes that explain for example how the competitive ability of *E. huxleyi* could increase or how it was able to better cope with increased CO₂. If it is possible to adequately characterize nutrient uptake related activity by molecular approaches, it could be feasible to apply such molecular approaches to experimental communities to help understand underlying mechanisms of community change.

Modeling and experiments

The combination of experimental studies and modeling approaches have often been used and helped to understand processes and mechanisms that are associated with ecological and evolutionary change (Govaert et al., 2016; Yoshida et al., 2003) (Chapter II). It is worthwhile and important to consider modeling approaches in future experimental studies because they may help fill knowledge gaps that cannot be explained by observed experimental responses. The Lotka-Volterra model I used, includes competitive parameters that reflect in a phenomenological sense an interactive depression among competitors, which are not the performance of K (carrying capacity) and r (growth rates). Growth rates and carrying capacities are relatively easy to determine in species or genotypes which makes the use of this model relatively easy to understand what intra- and interspecific interactions play a role. Nevertheless, the competitive parameters include any processes (predation, competition, allelopathy) related to inter- and intraspecific interactions. Another way to model competitive outcomes among primary producers would be to use a Monod model approach (Tilman, 1977). This approach relates to different nutrient (or generally resource) requirements and uptake related strategies of the focal organisms (Chesson, 2000; Sommer, 1984; Tilman, 1977). However, in my studies I did not have the required parameters (i.e. maximal growth rates related to different nutrient concentrations) of a

Monod modelling approach. A possibility would be to determine the required parameters of the study species for a Monod modelling approach and in retrospect compare modelling outcomes with the experimental data that I have found in my studies. The comparison of both modelling approaches could help understand whether species and genotype interactions are mainly determined by resource competition or if other interactive processes play a role that cannot be captured by the Monod modelling approach but possibly by the Lotka-Volterra modelling approach.

Concluding remarks

Here I have shown that to address simultaneous long-term changes on the species and genotype level and thus investigate potential eco-evolutionary coupling in laboratory experiments is indeed possible. However, the 'community' I investigated is completely underestimating the complexity of natural systems. Thus, one of the big questions that remain, is how to bridge the gap between findings from simple laboratory experiments to findings in natural communities (e.g. mesocosm or field studies). One idea could be an interdisciplinary investigation on how a community adapts to environmental change: evolutionary biologists could investigate eco-evolutionary changes in laboratory communities that consist of dominant species and genotypes isolated from a natural community whereas community ecologists could characterize how a natural community changes (i.e. in a mesocosm experiment) on the same time scale in response to environmental change. Additionally, modeling approaches could possibly help associate the findings from the different approaches. I conclude that environmental change will affect our ecosystems and it is essential to study changes in complex natural communities as well as constrained laboratory conditions. However, to do so fully and in an integrative way will be challenging.

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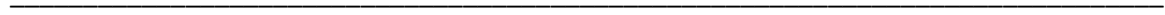
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Description of Author contributions

Chapter I

Inter- and intra-specific phenotypic plasticity of three phytoplankton species in response to ocean acidification

Giannina S. I. Hattich*, Luisa Listmann*, Julia Raab, Dorthe Ozod-Seradj, Thorsten B. H. Reusch⁺, Birte Matthiessen⁺

*shared 1st authorship, ⁺ shared last-authorship

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Study designed by GH, LL, TBHR, BM. Lab work carried out by GH, LL, JR, DO; data analysis by GH, LL. All authors drafted or revised the manuscript, gave final approval and agree to be held accountable for the content.

Chapter II

Competition drives eco-evolutionary sorting among competing marine phytoplankton species

Luisa Listmann¹, Giannina S. I. Hattich², Birte Matthiessen², Thorsten B.H. Reusch¹

This chapter will be submitted to Ecology and Evolution

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Chapter III

Swift thermal reaction norm evolution in a key marine phytoplankton species

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Chapter IV

Rapid experimental selection changes species interactions and the CO₂ response in a two-species phytoplankton community through eco-evolutionary coupling

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The decision of submission of this chapter is ongoing

Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation mit dem Titel:

“Assessing marine phytoplankton eco-evolutionary dynamics and physiological responses to environmental change”

selbstständig, mit der Beratung meiner Betreuer, verfasst habe. Die Dissertation ist in Form und Inhalt meine eigene Arbeit und es wurden keine anderen als die angegebenen Hilfsmittel und Quellen verwendet. Die Arbeit ist unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft entstanden. Diese Arbeit wurde an keiner anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegt und ist mein erstes und einziges Promotionsverfahren.

Teile dieser Arbeit wurden in wissenschaftlichen Fachzeitschriften publiziert: Kapitel I wurde in *Biology Letters* 2017 publiziert und Kapitel III wurde in *Evolutionary Applications* 2016 publiziert. Die Koautoren aller Kapitel befinden sich zu Beginn des jeweiligen Kapitels in der Autorenliste. Der Anteil der Autoren an den Manuskripten wird im Abschnitt „Author contributions“ erläutert.

Kiel, den 07. Mai 2018
