STRANGER THINGS
HAPPEN AT SEA

Phytoplankton phenotypic plasticity in response to thermal predictability across different timescales

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Stranger things happen at sea:

Phytoplankton phenotypic plasticity in response to thermal predictability across different timescales

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To my grandparents, Umberto and Felicia, Toto
And to the scientific grandfather of half of Italy,
Piero Angela

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Abstract

Phytoplankton play an essential role for aquatic ecosystems, owing to their importance in the food web and in the global carbon cycle, where they account for approximately 50% of global primary production. Due to their large population size and fast growth rates, there is ample scope for evolutionary responses to occur. In natural populations, species' persistence and adaptive trajectories are already shaped by the physical conditions they have faced, e.g., within species phenotypic variation is often high and can be partially explained by the environmental variability at the sampling location. Still, the direction, speed and magnitude of phenotypic and genotypic changes are yet to be thoroughly tested in ecologically relevant scenarios, especially in an environment changing as quickly as the world does now. In particular, it remains partially unknown whether past (here, evolutionary past) and present environmental predictability may influence evolutionary trajectories and could shape adaptive potential in future scenarios.

During my PhD, I have evaluated the consequences of past evolutionary history on phytoplankton responses to increasing and fluctuating temperatures, on three different timescales: immediate (within one generation), short-term and long-term. This approach allowed me to test plastic responses on physiological, seasonal, micro-evolutionary and geological timescales.

I have collected whole phytoplankton communities and isolated single species from two unique areas of the South-western Baltic Sea: the more thermally predictable Bornholm Basin and the less predictable Kiel Area. These two regions are close enough to mitigate confounding effects, such as light and nutrients availability.

I first investigated the immediate (i.e., within one generation) metabolic responses of phytoplankton communities during seven oceanographic cruises spanning two years. I proved that communities' respiration is less sensitive to seasonal warming than photosynthesis and that communities from more variable bodies of water, such as the Kiel Area, are able to express a higher degree of phenotypic plasticity. The same result was obtained for fitness thermal tolerances curves in the short-term. These experiments also highlighted the mechanistic influence of biotic sorting and habitat filtering on plasticity in phytoplankton assemblages.

The previous results shed light onto the role of past thermal predictability, but in order to understand how present environmental predictability can play a role in enhancing or hindering the evolution of plasticity, I tested the responses of a single species, *Ostreococcus* spp. isolated from the same regions of the Baltic Sea, in the long-term (ca. 120 generations) to different levels of predictability of amplitude and frequency of the fluctuations. I found strong differences with respect to the treatments in plasticity´s evolution. Plasticity increased in strains from the completely predictable environment. Instead, I found low levels of plastic responses when frequency of the fluctuations was unpredictable, especially in samples coming from the most thermally predictable environment. Moreover, analysis of phenotypic traits beyond growth rates demonstrated that microevolution can manifest itself in changes in trait values and trait correlations that are not necessarily visible in the trait-scape (i.e., bidimensional representation of multitrait phenotypes).

This thesis represents a comprehensive analysis of the adaptive potential of phytoplankton in response to past experienced environmental conditions. The findings show that even adjacent areas within one geographical region can yield fundamentally different strategies to deal with and responses to environmental fluctuations, thus stressing the urge to monitor phytoplankton responses using a more local approach beyond global averages. However, we are still lacking the full picture of the complexity of natural systems in order to grasp the consequences of anthropogenic changes on the small, green organisms that inhabit the waters (and consequently of the whole ecosystem).

Zusammenfassung

Phytoplankton spielt aufgrund seiner Bedeutung für das Nahrungsnetz und den globalen Kohlenstoffkreislauf eine wesentliche Rolle für aquatische Ökosysteme, da es für etwa 50 % der globalen Primärproduktion verantwortlich ist. Aufgrund der großen Populationen und schnellen Wachstumsraten gibt es viel Spielraum für evolutionäre Reaktionen. In natürlichen Populationen sind das Fortbestehen und die Anpassungsfähigkeit der Arten bereits durch die physikalischen Bedingungen geprägt, mit denen sie konfrontiert waren. Zum Beispiel. ist die phänotypische Variation innerhalb einer Art oft hoch und kann teilweise durch die Umweltvariabilität am Ort der Probenahme erklärt werden. Richtung, Geschwindigkeit und Ausmaß der phänotypischen und genotypischen Veränderungen müssen jedoch noch gründlich in ökologisch relevanten Szenarien getestet werden, insbesondere in einer Umwelt, die sich so schnell verändert wie die Welt heute. Des Weiteren ist teilweise noch unbekannt, ob die Vorhersagbarkeit der Vergangenheit (hier der evolutionären Vergangenheit) und der Gegenwart der Umwelt die Evolutionspfade beeinflussen und das Anpassungspotenzial in zukünftigen Szenarien prägen könnte.

Während meiner Doktorarbeit habe ich die Auswirkungen der vergangenen Evolutionsgeschichte auf die Reaktionen des Phytoplanktons auf steigende und schwankende Temperaturen auf drei verschiedenen Zeitskalen untersucht: unmittelbar (innerhalb einer Generation), kurzfristig und langfristig. Dieser Ansatz ermöglichte es mir, plastische Reaktionen auf physiologischer, saisonaler, mikroevolutionärer und geologischer Ebene zu untersuchen.

Ich habe ganze Phytoplanktongemeinschaften und isolierte Einzelarten aus zwei unterschiedlichen Gebieten der südwestlichen Ostsee gesammelt: dem thermisch besser vorhersagbaren Bornholm-Becken und dem weniger vorhersagbaren Kieler Gebiet. Diese beiden Regionen liegen nahe genug beieinander, um störende Einflüsse wie die Verfügbarkeit von Licht und Nährstoffen abzuschwächen.

Ich untersuchte zunächst die unmittelbaren (d. h. innerhalb einer Generation) Stoffwechselreaktionen von Phytoplanktongemeinschaften während sieben

ozeanographischen Fahrten über zwei Jahre. Ich konnte nachweisen, dass die Atmung der Gemeinschaften weniger empfindlich auf die jahreszeitliche Erwärmung reagiert als die Photosynthese und dass Gemeinschaften aus Gewässern mit größerer Variabilität, wie z. B. dem Kieler Becken, ein höheres Maß an phänotypischer Plastizität aufweisen können. Das gleiche Ergebnis wurde für die Kurven der kurzfristigen Wärmetoleranz der Fitness erzielt. Diese Experimente verdeutlichten auch den mechanistischen Einfluss der biotischen Sortierung und der Habitatfilterung auf die Plastizität in Phytoplanktongemeinschaften.

Um jedoch zu verstehen, wie die gegenwärtige Vorhersagbarkeit der Umwelt die Entwicklung der Plastizität fördern oder behindern kann, habe ich die Reaktionen einer einzigen Art, Ostreococcus spp., die aus denselben Regionen der Ostsee isoliert wurde, langfristig (ca. 120 Generationen) auf verschiedene Stufen der Vorhersagbarkeit von Amplitude und Frequenz der Schwankungen getestet. Ich fand starke Unterschiede in Bezug auf die Behandlungen in der Entwicklung der Plastizität. Die Plastizität nahm bei Genotypen aus einer vollständig vorhersehbaren Umgebung zu. Stattdessen fand ich geringe plastische Reaktionen, wenn die Häufigkeit der Fluktuationen unvorhersehbar war, insbesondere bei Proben aus der thermisch am besten vorhersehbaren Umgebung. Darüber hinaus zeigte die Analyse phänotypischer Merkmale über die Wachstumsraten hinaus, dass sich Mikroevolution in Veränderungen von Merkmalswerten und Merkmalskorrelationen manifestieren kann, die nicht unbedingt in der Merkmalslandschaft (d. h. der zweidimensionalen Darstellung von Phänotypen mit mehreren Merkmalen) sichtbar sind.

Die vorliegende Arbeit stellt eine umfassende Analyse des Anpassungspotenzials von Phytoplankton als Reaktion auf die in der Vergangenheit erlebten Umweltbedingungen dar. Die Ergebnisse zeigen, dass selbst benachbarte Gebiete innerhalb einer geografischen Region grundlegend unterschiedliche Strategien im Umgang mit und Reaktionen auf Umweltschwankungen aufweisen können, was die Notwendigkeit unterstreicht, die Reaktionen des Phytoplanktons über globale Durchschnittswerte hinaus mit einem lokaleren Ansatz zu überwachen. Allerdings fehlt uns noch immer ein vollständiges Bild von der Komplexität natürlicher Systeme, um die Folgen anthropogener Veränderungen auf die kleinen grünen Organismen, die die Gewässer (und damit das gesamte Ökosystem) bewohnen, zu erfassen.

Riassunto

Il fitoplancton svolge un ruolo essenziale per gli ecosistemi acquatici, grazie alla sua importanza nella rete trofica e nel ciclo del carbonio, dove rappresenta circa il 50% della produzione primaria globale. A causa delle grandi dimensioni delle loro popolazioni e dei loro rapidi tassi di crescita, vi è un ampio margine di manovra per le risposte evolutive. Nelle popolazioni naturali, la persistenza delle species e le traiettorie adattative sono già modellate dalle condizioni fisiche che hanno affrontato, ad esempio la variazione fenotipica all'interno della specie è spesso elevata e può essere parzialmente spiegata dalla variabilità ambientale nel luogo di campionamento. Tuttavia, la direzione, la velocità e l'entità dei cambiamenti fenotipici e genotipici devono ancora essere testati a fondo in scenari ecologicamente rilevanti, soprattutto in un ambiente che cambia così rapidamente come quello attuale. In particolare, rimane ancora parzialmente sconosciuto se la prevedibilità ambientale passata (in questo caso, il passato evolutivo) e presente possano influenzare le traiettorie evolutive e possano plasmare il potenziale adattativo in scenari futuri.

Durante il mio dottorato, ho valutato le conseguenze della storia evolutiva passata sulle risposte del fitoplancton all'aumento e alla fluttuazione delle temperature, su tre diverse scale temporali: immediata (entro una generazione), a breve termine e a lungo termine. Questo approccio mi ha permesso di verificare le risposte plastiche su scale temporali fisiologiche, stagionali, microevolutive e geologiche.

Ho campionato comunità fitoplanctoniche e singole specie isolate da due aree uniche del Mar Baltico sud-occidentale: il bacino di Bornholm, più prevedibile dal punto di vista termico, e l'area di Kiel, meno prevedibile. Queste due regioni sono abbastanza vicine da attenuare fattori di disturbo che possono confondere i risultati, come la disponibilità di luce e nutrienti.

Per prima cosa ho studiato le risposte metaboliche immediate (cioè entro una generazione) delle comunità fitoplanctoniche durante sette crociere oceanografiche durate due anni. Ho dimostrato che la respirazione delle comunità è meno sensibile al riscaldamento stagionale rispetto alla fotosintesi e che le comunità provenienti da corpi idrici più variabili, come l'area di Kiel, sono in grado di esprimere un maggiore plasticità fenotipica. Lo stesso risultato è stato ottenuto per le curve di tolleranza termica nel breve termine. Questi esperimenti hanno anche evidenziato l'influenza del sorting biotico e del filtraggio ambientale sulla plasticità degli assemblaggi di fitoplancton.

I risultati precedenti hanno fatto luce sul ruolo della prevedibilità termica precedentemente sperimentata dagli organismi, ma per capire come la prevedibilità ambientale attuale possa giocare un ruolo nel potenziare o ostacolare l'evoluzione della plasticità, ho testato le risposte di una singola specie, *Ostreococcus* spp. isolata dalle stesse regioni del Mar Baltico sopra menzionate, nel lungo termine (circa 120 generazioni) a diversi livelli di prevedibilità dell'ampiezza e della frequenza delle fluttuazioni. Ho trovato forti differenze rispetto ai trattamenti circa l'evoluzione della plasticità. La plasticità è aumentata nei ceppi provenienti dall'ambiente completamente prevedibile. Ho invece riscontrato bassi livelli di risposte plastiche quando la frequenza delle fluttuazioni era imprevedibile, soprattutto nei campioni provenienti dall'ambiente termicamente più prevedibile. Inoltre, l'analisi di tratti fenotipici al di là dei tassi di crescita ha dimostrato che la microevoluzione può manifestarsi in cambiamenti nei valori e nelle correlazioni dei tratti che non sono necessariamente visibili nel trait-scape (cioè la rappresentazione bidimensionale di fenotipi).
Questa tesi rappresenta un'analisi completa del potenziale adattativo del fitoplancton in risposta alle condizioni ambientali sperimentate in passato. I risultati mostrano che anche aree adiacenti all'interno di una stessa regione geografica possono produrre strategie fondamentalmente diverse per affrontare e rispondere alle fluttuazioni ambientali, sottolineando così la necessità di monitorare le risposte del fitoplancton utilizzando un approccio più locale rispetto alle medie globali. Tuttavia, manca ancora un quadro completo della complessità dei sistemi naturali per cogliere le conseguenze dei cambiamenti antropici sui piccoli organismi verdi che popolano le acque (e di conseguenza sull'intero ecosistema).

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1 Introduction

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"Everything is everywhere
but the environment
selects"
(Baas Becking,1934)

Motivation of the thesis

This thesis aims to understand the adaptive potential of phytoplankton (communities and single species) in response to thermal unpredictability. I asked four main questions:

1) **How does a history of variability influence thermal tolerance?**
2) **From few to many: Which are the challenges upscaling single species responses?**
3) **Time goes by: can we relate immediate responses to short and long-term responses?**
4) **What are the effects of different levels of predictability of thermal fluctuations on evolution and maintenance of phenotypic plasticity and on evolutionary responses?**

The motivation of this thesis is rooted in the urge to define how phytoplankton - the dominant primary producers and the base of the food web in the ocean - will change in response to the climate emergency. We know phytoplankton have ample scope for acclimation and adaptation, but the dynamics and interactions of phenotypic plasticity and evolutionary responses in environments that vary in predictability, remain an open question.
My work combines aspects of ecology, evolutionary biology and basic phytoplankton biology. As such, it straddles multiple bodies of work with shared ideas, but disparate vocabulary. Below, I therefore provide a short glossary box on the vocabulary specific to this thesis:

Glossary Box:

Evolution: changes in genotypes frequency within a population. Evolution can be adaptive when a species or individuals improve their fitness, i.e. their ability to survive in an environment and pass on their genes.

Phenotypic plasticity: responses of organisms to environmental changes that induce a change in the phenotype with no underlying genotypic changes. Neutral, adaptive and maladaptive plasticity have been reported. Plasticity can also evolve.

Fitness: Throughout this thesis, I used growth rates as a measure of fitness, as is (mostly) the standard in experimental evolution. Growth rates are usually strongly under selection, especially in semi-continuous batch cultures, where populations are kept in exponential growth.

Strain: Distinct genotypes within a species. In Chapter 3, strains are clonal and originated from a single individual by means of dilution followed by asexual divisions. This procedure is used in order to avoid initial genotypic differences and ensure only *de novo* mutations are inducing a change in phenotype (warning note: this strategy could also impose severe bottlenecks).

Heatwave: A prolonged period of abnormally warm temperatures. Here, I used the definition given by Hobday *et al* (2016): "an anomalously warm event that lasts for five or more days with temperatures warmer than the 90th percentile based on a 30-years historical period".

An ecologist's view of climate change: phytoplankton and their responses in a warmer, more variable ocean

The ocean regulates climate on Earth through different processes: it works as a storage for the heat of the Sun, buffering the temperatures on land (Ramanathan and Feng 2009) and also transporting heat *via* currents (Trenberth and Solomon 1994). Since the development of the industrial era, the stability of the Earth system is facing what can be considered as its toughest challenge, the climate crisis. The speed of warming is unprecedented and as a result, by the end of 2100, oceans and land masses may be on average between 1.1 and 3.2°C by 2100.

Climate change affecting oceans will eventually result in a disrupted heat system of Earth, not to mention the dramatic effect on organisms, especially marine ones. The abrupt changes, superimposed to a naturally highly variable environment, will provoke an unprecedented, and especially fast, change of aquatic environments.

Alongside the abrupt warming, increase in thermal unpredictability will also increase. Frequency and intensity of extreme events (such as heatwaves) have already increased

(Thornton et al. 2014) and studies suggest that predictions on organisms´ thermal tolerances based on constant conditions could be significantly different when fluctuations and variance are considered (e.g., (Vasseur et al. 2014) (Fig. 1.1).

The immediate question arising from this situation is if organisms can respond to changes fast enough. We know that 'fast enough' almost always hinges on the length of generation time relative to the speed of change. Specifically, in this thesis I focus on fast growing organisms, phytoplankton. Their ability to quickly divide can help them keep the pace of the environment, sensing as gradual what for other organisms are abrupt changes. Phytoplankton and other marine microbes are thus likely to survive in the environment as it changes, but it remains to be tested here whether there are predictable patterns to the phenotypes they display and the communities they inhabit in an increasingly unpredictable world.

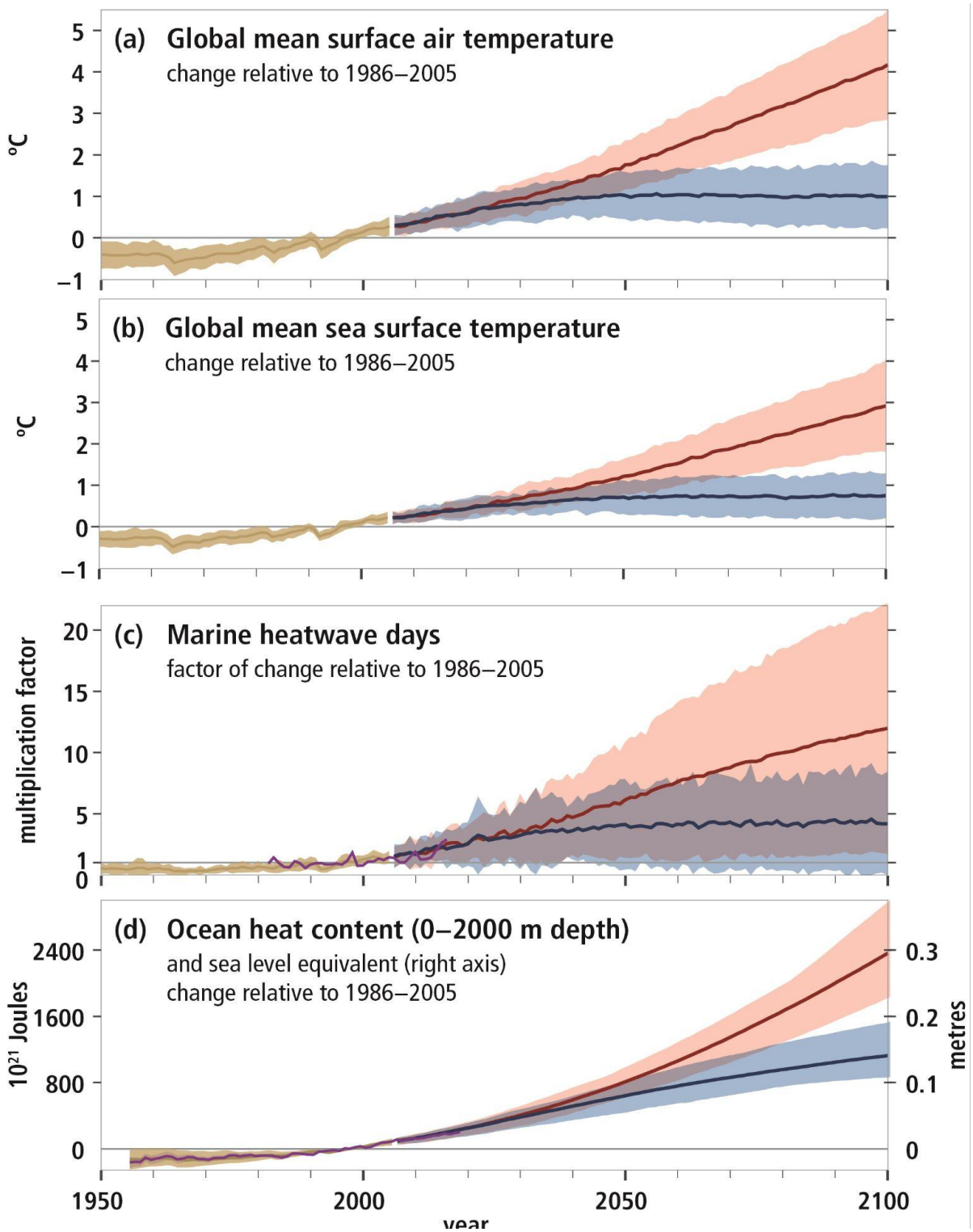


Figure 1.1. Historical (yellow) and projected (red, referring to high greenhouse gas emission and blue, referring to low emission) changes in the ocean from 1950. From up to the bottom plots are describing global mean surface air temperature change, mean sea surface temperature change, change factor in days of marine heatwave and global ocean heat content change (IPCC report, 2019).

Even if representing a small percentage (~1,966 ∓ 126 mg C m^{-2} mean values in the Open ocean (Gasol et al, 1997) in terms of biomass in the oceans, phytoplankton contribute up to 50% of net global productivity (Armbrust 2009). Photosynthesis converts CO_2 into organic carbon, storing it within the organisms before being remineralized when organisms sink or die. CO_2 is then returned to the atmosphere *via* respiration (Riebesell et al. 2007). Due to their immense role in biogeochemical cycles and food webs, it is relevant to understand how phytoplankton will respond to increased temperatures and fluctuation, spanning from purely ecological reasons (e.g., shifts in biodiversity and consequently, potential changes to ecosystem function and services) to improved predictive power for climate feedbacks.

Temperature sets the boundaries of phytoplankton distribution and productivity in the world ocean and other aquatic systems (Boyd et al. 2013). Warmer (or simply changing) temperatures could lead to a number of ecologically-relevant changes: reorganisation of microbial communities, pole-ward shifts, decline in species diversity, and reduction especially at mid-latitudes of primary production (Kling et al. 2020; Thomas et al. 2012; Rasconi et al. 2017; Roxy et al. 2016). But in nature, nothing happens in isolation and organisms do not live in a void; synergistic effects with other properties of the environment e.g., nutrient depletion, iron limitation, salinity, carbon dioxide concentration, could lead to unexpected changes in physiological responses (e.g., Fu et al. 2007). Moreover, a more holistic approach that relates changes driven by temperature with the underlying ecological drivers, such as local adaptation or complex biotic relations at the community level, is still lacking.

Phytoplankton thermal tolerances

Growth or fitness responses to temperature are usually described by thermal tolerance curves, which share a common shape in all ectotherms. The curve is unimodal and negatively skewed (Joel G. Kingsolver 2009), meaning that the curve will likely decline more rapidly above optimum temperature (T_{opt}), where growth is fastest, than below.

As a result,ectotherms currently growing near or at their thermal optimum are going to be more sensitive to increase in temperature (Thomas 2014) if they are not malleable enough to modify the shape of the curve. Thermal specialisations are common, with a clear latitudinal trend in the optimal growth temperature (M. K. Thomas et al. 2012). Optimal growth rates in single species are strongly related to mean annual temperature at the site of sampling, indicating that temperature is a clear driver for local adaptation, even stronger than genetic

flow (Muir et al. 2014). In fact, growth thermal optima for polar and mid-latitude phytoplankton are often higher than the mean temperature of the environment (Thomas et al. 2012), but are closer to the environmental mean for tropical phytoplankton.

Metabolic pathways shape fitness, mortality, interspecific interactions (Dell, Pawar, and Savage 2014) and the mechanisms that set the limits of thermal tolerance in phytoplankton (Barton et al. 2018). As enzymatic activity changes with temperature, so do the metabolic pathways these enzymes are involved in. Therefore, understanding how metabolism will be affected by thermal variation in the near future, can be translated into knowledge about and if aquatic phytoplankton will mitigate the effects of anthropogenic changes. Metabolism is determined in all phytoplankton species by two main processes: photosynthesis and respiration (Raven and Geider 1988). Light-reactions convert inorganic carbon into organic carbon and provides ATP and reductants used in the Calvin cycle. Dark reactions provide carbon skeletons (Falkowski 1980) that can then be used to produce ATP through respiration. The vast majority of energy required from the cell to grow, (between 60 and 90%) is supposed to come from photosynthesis (Raven 1976). In the short-term, respiration rates are more sensitive to warming than are photosynthesis rates. Further, thermal performance curves for respiration show a higher thermal optimum temperature than photosynthesis (Yvon-Durocher et al 2017; Barton et al. 2018). All else being equal, higher respiration than photosynthesis would translate into lower growth rates, and this can be quantified by calculating Carbon Use Efficiency (CUE). CUE allows us to estimate the amount of carbon fixed during photosynthesis (P) left to be allocated for growth after losses through respiration (R) have been accounted for (1-R/P).

Of course, there is “more to it than meets the eyes" and temperature affects many different traits other than growth rate. This proves relevant especially because evolutionary studies usually focus on fitness (estimated as growth rates), while ecology often does not rely on good fitness estimates. For example, one of the main rules of temperature dependence is “hotter is smaller” (Joel G. Kingsolver and Huey 1998). Phenotypic plasticity often induces a reduction in size, or better an increase of area-to-volume ratio, at higher temperature. Absolute cell abundance of small species of phytoplankton (picophytoplankton, between 0.2 and 2 μm in diameter) is observed to increase with increasing temperature (Li, Glen Harrison, and Head 2006). This phenomenon can be explained in the context of interactions between the temperature-size rule, cross-community scaling relationship and individual size distribution (Morán et al. 2010).

An increase in mean sea surface temperature, as well as more frequent, prolonged heatwaves, and other thermal variations around the mean, will directly affect phytoplankton physiology. Physiological and individual responses (easy to observe, manipulate and measure) will , in ways that are not always intuitive or well-studied, scale up to community level responses. The latter are much more difficult to investigate, not least because ecological and evolutionary processes interact to shape characteristics of phytoplankton assemblages. Ecologically relevant processes include but are not limited to biotic sorting, when a better adapted species outcompetes the others, or abiotic filtering, when a less adapted taxonomic group is excluded from the competition.

Evolutionary processes include all processes that change the frequency of genotypes. They are, for example, driven by specialists-generalists trade-offs, the evolution of phenotypic plasticity (see also below) or simply sorting of existing standing genetic variation (HilleRisLambers et al. 2012).

Who said small?

Picophytoplankton and the model organism Ostreococcus spp.

In this thesis, I used a combination of space-for-time and experimental evolution (also known as 'forward in time') as a tool to watch evolution happening in real time on natural assemblages of picophytoplankton and single cells of *Ostreococcus* spp. Below I will elaborate on my choice of model organism.

Picophytoplankton comprise unicellular organisms with a small cell size. The usual accepted size limit of 2 µm has recently been disputed, since cell counting from Blanes Bay Microbial Observatory in the Western Mediterranean Sea revealed that assemblages of <3 µm cells display a clear seasonality (Massana 2011). In Study I and II of this thesis, I used the latter size threshold to determine picophytoplankton communities limits.

While limited to a small range in size, this group is taxonomically and physiologically diverse. Picophytoplankton are composed of both prokaryotes (i.e., cyanobacteria) and eukaryotes (e.g., green algae), and organisms can be autotrophs, heterotrophs (Li 1994) or mixotrophs (Listmann et al. 2021). Picophytoplankton are omnipresent; the average abundance is 10^3 cells mL^{-1} in oligotrophic waters (e.g., the Sargasso Sea) and up to 10^5 cells mL^{-1} in coastal regions (Sanders et al. 2000), representing about 90% of the primary

producers biomass all over the world (Worden et al 2004). They represent one of the most abundant group of photosynthetic organisms in the sea (e.g., dominating in the subtropical gyres (Rii et al 2016)) and greatly contribute to biogeochemical cycles, even if they may need three trophic links to transfer primary production to copepods (Massana 2011). Eukaryotic picoplankton´s relative abundance is relatively lower than that of prokaryotic counterparts (Marie et al. 2010). They do not form blooms, but are present all year around, significantly contributing to primary production (Worden and Not 2008).

Ostreococcus spp. (Fig. 1.2) belongs to the Prasinophyceae. The Prasinophyceae are one of the most abundant and most ancient picoeukaryotic groups (Foresi et al. 2010). *Ostreococcus* is considered the smallest known picoeukaryote, with a diameter ranging from 0.7 to 1.5 μm (Courties et al. 1998; Worden et al 2004) in the wild. Moreover, *Ostreococcus* spp. have fairly simple cellular characteristics and represent sharply the assumption that in order to reach small size and in the meantime keep an independent (i.e., non-symbiotic) life, it is vital to maintain only the minimum-required cellular components (Raven 1998). Indeed, *Ostreococcus* spp. does not have a cell wall, but only a thin membrane. The cell is roundish and presents one mitochondrion, a chloroplast reduced in size and with only three layers of stacked thylakoid membranes (Cardol et al. 2008) and a starch granule (Six et al. 2008). The simplified cellular organisation is also reflected in the substantial inability to biosynthesize certain micronutrients (i.e., thiamine and vitamin B_{12}) which must be collected from associated bacteria (Palenik et al. 2007).

The genome of *Ostreococcus tauri* (specifically the strain OTH95, retrieved from the Thau Lagoon, in France), has been sequenced by Derelle et al. (2006). The genome size has been estimated at 12.56 Mb distributed across 20 chromosomes, with a high degree of genome compaction. Furthermore, it displays some unusual characteristics, such as lacking genes encoding the light-harvesting complex proteins associated with photosystem II (LHCII) (Derelle et al. 2006). Since the genome is pretty simple, it offers the chance to investigate deeper into functional genomics and make it easier to understand phenomena regulated by multiple genetic feedbacks.

The simple organisation, makes the “unbreakable *Ostreococcus*” the perfect candidate as a model organism for physiological and evolutionary investigations: quite easy to be kept in culture in the lab, rapidly dividing, sequenced genome, anciently arisen lineage and basic pathways are just some of the amazing traits that make it the perfect life-companion of a

researcher.

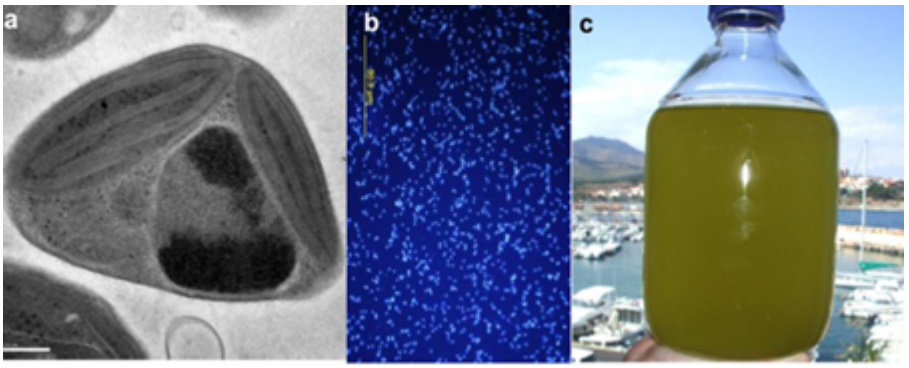


Figure 1.2. (a) image of *Ostreococcus tauri* at the TEM microscope. (b) *Ostreococcus tauri* cells stained with DAPI and (c) 2 litres of culture of *O. tauri* (Photos by Moreau lab, CNRS, Banyul sur mer, France).

In this thesis, I used samples I collected from the South-Western Baltic Sea to describe in high-resolution the contribution of local adaptation and divergent environmental histories to evolutionary and acclimative trajectories.

The Baltic Sea can be seen historically as a nordic sister of the Mediterranean Sea: a fairly young, semi-enclosed sea around which different cultures and populations were able to develop and influence human history.

The Baltic Sea is brackish owing to its history as a former glacier, with connections to the Atlantic Ocean only through the Danish straits. Therefore, water circulation is mainly driven by salinity and temperature gradients.

There is a strong gradient of salinity, temperature and variability. Surface salinity and temperature decrease northwards and eastwards, due to input of fresh water melting from the ice covering in the north and from the freshwater influxes from the eastern rivers. Most of the Baltic Sea presents a fixed halocline that makes mixing difficult.

All the basins also have a thermocline during summer (Snoeijs-Leijonmalm, Schubert, and Radziejewska 2017). Superimposed to all this, there is a thermal variability gradient running westwards.

While not a main factor under investigation in this thesis, there is also a North-South East-West natural and anthropogenic nutrients gradient present.

For our purposes, we in particular considered the Kiel Area and the Bornholm Basin (Fig.

1.3). The two areas are close enough to avoid confounding effects (e.g., through seasonality, cloud coverage, meteorological events and nutrients concentration) but different in terms of temperature regimes:. The Kiel Area is overall warmer and more thermally unpredictable than the Bornholm Basin (see Study I). In terms of biodiversity, in the Bornholm Basin the summer bloom is dominated by cyanobacteria and displays a slightly lower species richness than the Kiel Area (Zhong et al., 2020).

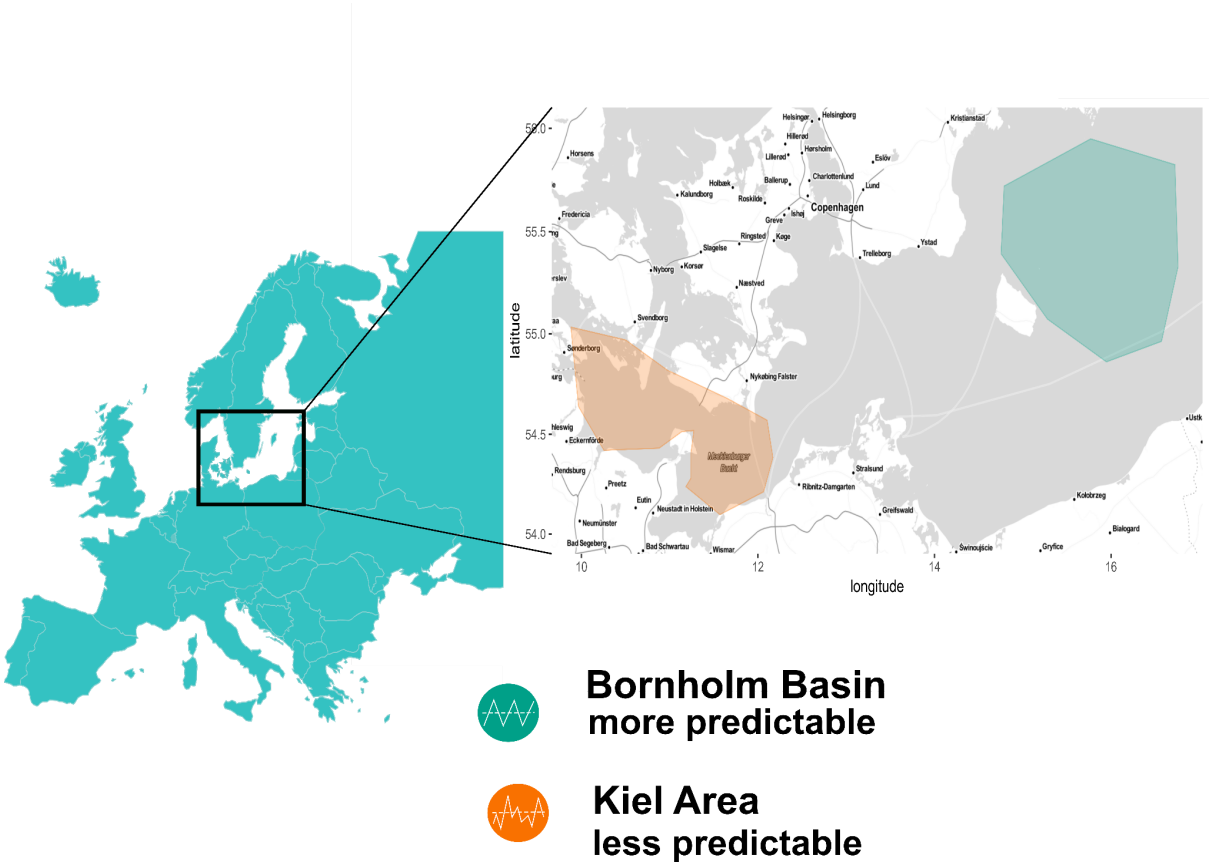


Figure 1.3. Map of the South-Western Baltic Sea. The areas of interest described in this thesis are depicted in orange (Kiel Area) and blue (Bornholm Basin).

The evolution´s guide to the ocean´s galaxy: phenotypic plasticity and evolution

Phytoplankton can divide fast and reach notably high population densities. These characteristics give them a comparatively high evolutionary potential (Bell and Collins 2008) and make them good candidates for evolutionary research on ecologically relevant organisms. Fast generation times allow phytoplankton to perceive abrupt environmental changes as gradual, leaving ample scope for evolution. A vast body of research on short and long-term responses of phytoplankton to environmental variables exists (e.g., Listmann et al. 2016; Bach et al. 2018; Burgmer and Hillebrand 2011; Low-Décarie et al. 2013; Barton et al. 2020), but little is known regarding when on what time scales short-term acclimative responses (mostly driven by phenotypic plasticity) and long-term evolution (adaptation and local adaptation) happen and the interplay between them. Therefore, the timing of environmental changes (e.g. frequencies of fluctuations, seasonal changes…) related to specific generation times, should receive attention.

Phenotypic plasticity is the ability of a genotype to generate different phenotypes (i.e., with no underlying and heritable genetic changes) when exposed to a different environment (West-Eberhard, 1989). Many traits can be considered plastic and altered by differences in environmental parameters: behavioural traits, physiology, morphology (Price et al, 2003), or growth rate as a trait that emerges from the interplay of other traits.

Adaptive plasticity is a change in the phenotype in the same direction as the optimal value in the new environment (reviewed in Ghalambor et al. 2007) resulting in an increase of fitness (usually considered as growth rates) (Fig. 1.4) . In contrast, plasticity can also result in a non-adaptive acclimation. This situation may arise from a strong environmental stressor, i.e., the occurrence of a genuinely detrimental condition outside the range of tolerance of an organism. Maintaining adaptive plasticity in this case is not an easy task; however a stress-inducing condition could also increase the variance of trait expression. The occurrence of maladaptive plasticity, is one of the main reasons why the debate under which circumstances plasticity helps or hinders evolution is still an on-going issue in evolutionary biology.

Reaction norms are described as continuous functions expressing the relationship between an environmental variable and the phenotype expressed by a genotype (Angilletta et al. 2003) and describe phenotypic plasticity (Massimo Pigliucci 2005) in ways that can be analysed and quantified mathematically. Reaction norms explain the relationship between phenotypic value

of different traits and the experienced environment**.** Plasticity can be quantified by the slope of the reaction norm (Chevin et al, 2013): within a linear relationship, the more plastic a genotype is, the steeper the reaction norm will be. Indeed, the easiest way to model plasticity is through a linear reaction norm (Callahan et al, 2008).

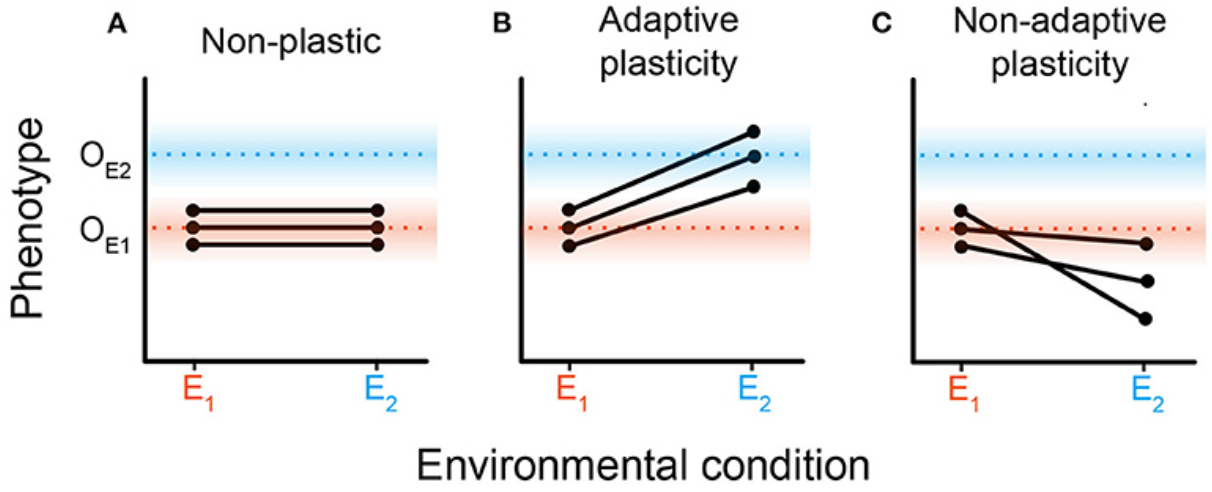


Figure 1.4. Hypothesis of reaction norms for genotypes placed in different environments, from Lalejini et al. 2022. E_1 and E_2 represent two environments, the points depict the expressed phenotypes and the connecting line the genotype.O_{E1} and O_{E2} represent the optimum phenotypes for, respectively, environment 1 and 2. (A) Phenotypes do not change through the environment; the population is not plastic. (B) Phenotypes change in a new optimum, describing adaptive plasticity. (C) Non-adaptive plasticity, where phenotypes change further away from the optimum.

Biologically, most reaction norms are not linear if tested across a sufficiently large number of environments. For example, temperature reaction norms have a unimodal shape, while nutrient reaction norms follow the Monod function (Sunda et al, 2009). Plasticity can result in not only a change in mean trait value, but also in a change in some or all of the parameters describing the reaction curve, such as the slope and intercept with the y axis. Given this, it is maybe biologically more accurate to refer to a “tolerance reaction norm”.

I have until now focused on phenotypic plasticity as a way of organisms to deal with environmental change only, but given the aforementioned fact that phytoplankton will perceive climate change as gradual, it is likely that they will respond at least in part also through evolutionary processes. There is a growing body of literature proving that evolution and plasticity are not mutually exclusive (Wund 2012; Pazzaglia et al. 2021; C. E. Schaum

and Collins 2014). Plasticity has long since been defined as a "genetic capacitor" (Rutherford and Lindquist 1998), increasing genetic variation under environmental stress (Gomez-Mestre and Jovani 2013). Also, in presence of extreme changes imposed by anthropogenic pressures (read, climate emergency), plasticity can indeed help populations´ rescue and evolutionary processes (reviewed in Pfennig 2021). In a new environment, plasticity can rapidly evolve, moving the phenotypes to a new optimum, which is then fixed by genetic assimilation (Lande 2009). The mechanisms of plasticity and the extent to which they occur in gradually changing environments are largely underdiscovered. I argued before that phytoplankton, due to their fast generation times, perceived the time of environmental change differently than slower growing organisms. Chevin et al. (2010) found, using a modelling approach, that gradual change increases plasticity but reduces rate of genetic evolution, decreasing natural selection. Microbial experimental evolution remains a powerful tool to disentangle the strategies underpinning adaptation and plasticity and clearly discriminate between evolution and plasticity, quantifying the relationships between the two.

Experimental evolution studies, especially those focusing on bacteria, were traditionally designed around one very stressful new environment that suddenly changes and is kept constant over hundreds of generations (as reviewed by Collins 2011). However, in nature, and particularly at sea, there is never only a single factor changing. More likely, many abiotic and biotic parameters change on similar time scales and influence each other. Empirical studies showed that adaptation can be altered by more complex environmental dynamics. Brennan and Collins (2015) proved that in multidriver selection environments, the numbers of factors acting on evolutionary outcome shape fitness. In particular, when one strongly stressful driver is present , the fitness will not decrease further, regardless of the different combinations.

Nevertheless, in this thesis, we manipulated only one parameter, temperature. We considered one factor in isolation to clearly disentangle the effect of fluctuations´ predictability. In addition, it is important to notice that the samples used throughout this thesis (both single species and communities), have until the point of sampling, experienced many factors changing in concert anyway. Fluctuations and their predictability can also strongly impact evolutionary and acclimative patterns. Botero et al. (2015) proposed a comprehensive and detailed model on environmentally driven fluctuating selection: in a highly unpredictable environment, with fluctuations happening at a faster timescale, plasticity may not be enough (or not favourable enough) to track the environment, thus bet-hedging strategies can be more

advantageous. Other theoretical studies predict that plasticity should evolve in heterogeneous environments with predictable cues (Chevin and Hoffmann 2017). Still, not many experimental studies focused on the relationship between environmental predictability and plastic responses. A powerful way to analyse this relationship, is to assess plastic and adaptive responses in natural populations assayed over a natural gradient of predictability. For example, Huertas et al. (2011) found evidence that temperature sensibility and adaptive potential in 12 phytoplankton species were related to the thermal windows to which the different species were used to. Thus, populations with different environmental and evolutionary histories, may display different levels of intrinsic plasticity, which may imply divergent adaptive outcomes when exposed to novel environmental conditions (Adams and Collyer 2009).

Eco-evo coupling

Ecological and evolutionary phenomena can influence each other and can happen at the same pace (Pelletier et al. 2009; Garnier et al. 2016). Interactions among species, individuals' physiological capacities and organisms´ dominance are prime examples for ecological phenomena that might be particularly vulnerable to environmental change. Here I want to stress the fundamental coupling between ecology (here intended as interactions among species and past experienced biotic and abiotic environmental conditions) and evolutionary trajectories. Rapid evolution of one organism or group of organisms can have a large impact on the entire ecosystem and its function (Bassar et al. 2010). Anthropogenic changes in particular, can force evolutionary forces to occur at a faster timescale, so that contemporary interplay between the two compartments is starting to be frequently assessed (Strauss, Lau, and Carroll 2006). This translates into a stronger feedback of evolution on ecology (e.g., community processes and demographic effects (Carroll et al. 2007).

Eco-evo interactions (feedbacks between ecology and evolution) cannot be disconnected from the spatial dimension in which they occur; local adaptation patterns at a fine scale affect the ecosystem and therefore the adaptive potential of the organisms. In my thesis, I tried to connect eco-evo feedbacks with the locally diverse environment that shaped the already existing evolutionary trajectories, hypothesising that directional selection´s influence is driven by strong difference in the predictability of the environment.

"Ars longa, vita brevis"
Science is unlimited in its course; life is short

Thesis outline

Study I: In the first study, my co-authors and I have analysed thermal optima (T_{opt}) for metabolic processes (gross photosynthesis and respiration) occurring within one generation in natural assemblages of phytoplankton from two areas of the Baltic Sea. We hypothesised that immediate responses can be used to predict communities´ adaptive potential. The amount of plasticity in traits, such as metabolism, will ultimately determine the occurrence of community sorting. I have related changes in T_{opt} with temperatures at the site of sampling and investigated the underlying mechanisms of community sorting.

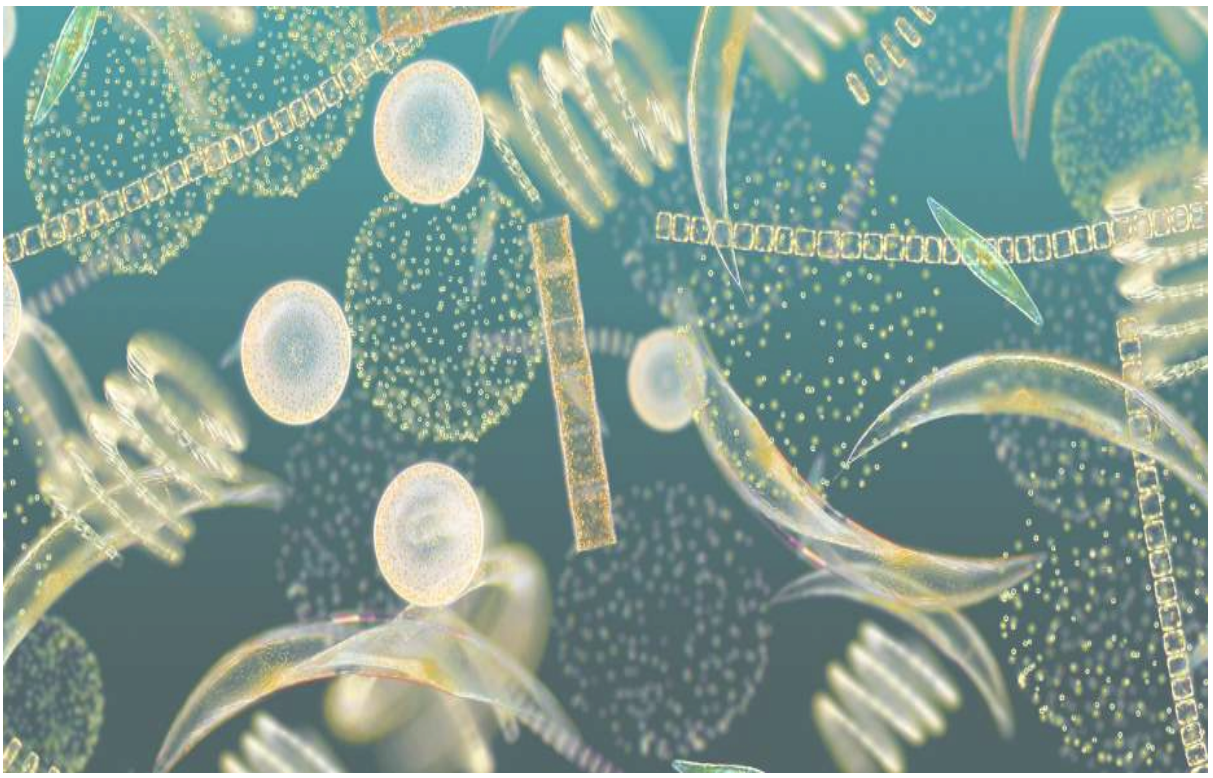
Study II: In the second study, my co-authors and I performed a short-term study (spanning ca. 20 generations) on picophytoplankton communities collected in the Baltic Sea during a summer and a winter cruise. I have analysed phenotypic plasticity *via* thermal performance curves in response to temperatures and changes in biodiversity indexes occurring both during the experiment and during seasons prior to the laboratory assays. We expected a strong influence of local adaptation on the occurrence and degree of phenotypic plasticity. Our results point toward a strong connection between local adaptation to different degrees of thermal predictability and the timing of habitat tracking, which in turn can explain the higher degree of phenotypic plasticity in the communities coming from the less predictable area .

Study III: In the third study, my co-authors and I have focused on responses of cultures grown from single cells to different predictability levels of fluctuating temperatures instead. Clonal *Ostreococcus* strains isolated during the aforementioned Baltic Sea cruises were subjected to five treatments, spanning from completely unpredictable fluctuations (in terms of amplitude and timing) to completely predictable ones. I followed growth rate trajectories over ca. 1 year and performed reciprocal transplant experiments at fixed time points in order to study the evolution of the plastic response and its relation with habitat tracking and fitness.

Study IV: In the fourth study, my co-authors and I have applied a statistical multivariate approach to determine the trait-scape of the evolved samples from the long-term experiment (Study III). We analysed different traits (e.g., photophysiological parameters, size, membrane

potential, photopigments concentration) and the differences in trait-space and in trait correlations in the treatments and over time.

2 Studies of the thesis



Phytoplankton diversity

Image: www.secchidisk.or

Contents

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2.1 *Study I: Immediate metabolic responses of phytoplankton communities*

Predicting the unpredictable: heatwaves and history of variability shape phytoplankton community thermal responses within one generation

Maria Elisabetta Santelia, Luisa Listmann, Stefanie Schnell, C-Elisa Schaum

Submitted to Nature Ecology and Evolution in 2022

Abstract

Predicting the effect of increased thermal unpredictability, for example in the shape of heatwaves on phytoplankton metabolic responses is ripe with challenges. While single genotypes in laboratory environments will respond to environmental fluctuations in predictable and repeatable ways, it is difficult to relate rapid evolutionary responses of whole communities to their ecological history. Previously experienced environments, including fluctuations therein, can shape an organism's specific niche as well as their responses to further environmental changes. This is a testable hypothesis as long as samples can be obtained where the environmental history is known, sufficiently diverse, and not obscured by confounding parameters such as day length and precipitation patterns. Here, we tested immediate (i.e., within one generation) metabolic temperature responses of natural phytoplankton assemblages from two thermally distinct regions in the Baltic Sea: the warmer and less predictable Kiel Area, and the overall colder and more predictable Bornholm Basin. Our approach allows us to investigate effects on immediate physiological time scales (response curves), ecological and evolutionary processes on longer time scales (seasonal differences between basins) as well as mid-term responses during a natural occurring heatwave. We found evidence for a higher degree of phenotypic plasticity in samples from unpredictable environments (Kiel Area).

Introduction

Rising temperatures, more frequent heatwaves and increased unpredictability in sea surface temperature all have the potential to change the way phytoplankton communities contribute to whether coastal waters act as carbon sources, or carbon sinks. Here, we investigate how immediate (i.e., within one generation) metabolic responses in phytoplankton communities (whole community and pico-phytoplankton fraction) are shaped by a history of biotic relationships and physical conditions.

Warming strongly affects phytoplankton populations: for example, geographical distribution of phytoplankton communities may shift (M. K. Thomas et al. 2012) in addition to changes in community composition, e.g., higher occurrence of cyanobacteria blooms (Visser et al. 2016) and altered phenotypic trait expression. Photosynthesis (P) and respiration (R) in particular, are labile phenotypic traits, i.e., they exhibit high phenotypic plasticity (C.-Elisa Schaum et al. 2017), where traits can change without there being underlying heritable changes to the genome. Phytoplankton are crucial components of the carbon cycle and determine whether surface waters act primarily as CO_2 sources or sinks (Field et al. 1998). Therefore, it is crucial that we understand how rapidly photosynthesis and respiration can change in response to the projected temperature variations of the next decades. Metabolic rates dictate the amount of carbon that can be allocated for growth and basal cellular maintenance (Brown et al. 2004) and shape other responses related to fitness (e.g., mortality, abundance (e.g., (Dell et al. 2011)), and competitive ability (Bestion et al. 2018). Numerous short-term studies over one or a few generations and usually carried out on single species have shown that respiration is overall more sensitive to warming than photosynthesis (López-Urrutia et al. 2006; Regaudie-de-Gioux et al. 2014; Laufkötter et al. 2015; Barton et al. 2020). Therefore, the amount of carbon available to cells for growth and other processes declines as temperature rises. On the ecosystem level, models predict that this may lead to a sharp decline in primary productivity (up to 20%) in the next decades (Boyce et al. 2010; Bopp et al. 2013). However, for more accurate predictions, models need multi-species data on evolutionary time scales.

The effect of temperature on traits is described by thermal reaction norms (Joel G. Kingsolver 2009). In ectotherms, they show a common pattern (Eppley 1972; Kingsolver 2009): rates increase exponentially with rising temperature up to an optimum, after which they decline abruptly. On a cellular level, this pattern is mainly driven by enzymatic constraints (Schoolfield et al. 1981; Daniel et al. 2008). On an ecological level, the shape of thermal

reaction norms is driven by thermal tolerance and the interplay of acclimation and local adaptation (Padfield et al. 2016a).

Thermal optima (T_{opt}) are usually correlated with environmental mean temperature (Pawar et al. 2015; Schaum et al. 2017). This relationship determines the degree of local adaptation (Mitchell and Lampert 2000; Souther and McGraw 2011), while other parameters describing the shape of thermal reaction norms give indication about thermal tolerance. It describes the range of temperatures at which an organism or a population can survive, and can change and adapt as the environment changes (e.g., Bennett and Lenski 2007; Lande 2014; Thomas et al. 2016)). Information on temperatures that phytoplankton communities can tolerate is crucial for a better understanding about the extent to which reaction norms of a trait can stay the same across environments (Magozzi and Calosi 2015; Pacifici et al. 2015).

Sea surface temperature is naturally variable and largely depends on geographical and seasonal patterns (Karl et al. 2003). In the future, unpredictability is expected to increase, with a rise in frequency and severity of anomalous events such as heatwaves (Karl and Trenberth 2003). Theory predicts that frequency and predictability of the environmental fluctuations will have an impact on the shapes of thermal tolerance curves in the short-term and long-term and thereby determines which strategies allow populations to persist in the environment. This can range from phenotypic plasticity to bet hedging (Botero et al. 2015). Ultimately, either strategy serves to avoid extinction and to increase the variance and mean in fitness (Starrfelt and Kokko 2012).

Theoretical predictions are based on models and yet to be thoroughly tested in ecologically relevant scenarios. In natural populations, species persistence and adaptive trajectories are already shaped by the physical conditions they have faced, e.g., within species phenotypic variation is often high and can be partially explained by the environmental variability at the sampling location (Godhe and Rynearson 2017). Theory and observational studies agree that local adaptation contributes to shaping adaptive and plastic trajectories organisms would potentially follow if exposed to new conditions (e.g., Pörtner 2002; Boyd et al. 2016; Thomas et al. 2016)). For phytoplankton, a strong correlation between mean temperature of the environment and thermal niches has been clearly demonstrated, showing a general large scale pattern of local adaptation, with tropical species tending to have thermal optima closer to the mean experienced temperature, probably due to thermal constraints at higher temperatures (Thomas et al. 2012).

We use the term eco-evolutionary history to describe the combination of biotic and abiotic factors that an organism has previously experienced and that have likely influenced the organisms' adaptive trajectories. Specifically, past eco-evolutionary history can either influence the speed at which organisms adapt to novel and changing environments (Andrade‐Restrepo et al. 2019) or, if ecosystems change on the same time scale as evolution occurs, it can be translated into changing community composition or interaction between species (Post and Palkovacs 2009; Padfield et al. 2017).

Upscaling single species´ responses to the population level may not be a good predictor for population responses (Wolf et al. 2018). Community responses are often more than the sum of their parts and whole assemblages may respond in different ways to environmental parameters: trait-expression on the community level may stay unaltered e.g., via shifts at species or functional group level, or when individuals show a high degree of phenotypic plasticity (Godhe and Rynearson 2017; Hoppe et al. 2018). Shifts in community composition are one of the main consequences of climate change. Evidence suggests warming is likely to increase the relative proportion of pico-phytoplankton, the smallest fraction of aquatic autotrophs (Morán et al. 2010). This shift toward environments dominated by smaller cells could potentially disrupt aquatic food webs and alter carbon export fluxes (Falkowski et al. 1998).

Understanding how the composition of communities and metabolic rates of phytoplankton change globally is a complicated task. Specifically, global scale studies face confounding factors, e.g., different light intensity and nutrient availability in spatially distant environments. Here, to minimise these confounding variables, we obtained natural phytoplankton communities from two well-characterised areas of the South-Western Baltic Sea: the Kiel Area and the Bornholm Basin. The two regions are close enough to be connected by currents, but at the same time, offer the rare chance to explore how a history of thermal variability may influence metabolic responses. Specifically, the Kiel Area is on average warmer and more thermally unpredictable than the more predictable and colder Bornholm Basin (Snoeijs-Leijonmalm et al. 2017). We leveraged a series of cruises spanning two years, including a summer heatwave, to test how phytoplankton communities´ (whole community and pico- phytoplankton fraction) immediate metabolic responses to temperature are shaped by a history of biotic relationships and physical conditions. Responses within one generation - often before cells are fully acclimated to novel conditions, are important in order to avoid

acclimation steps that can potentially alter the physiological responses of organisms to the considered variables (Munguia and Alenius 2013).

Materials and Methods

Sampling areas

We chose two areas in the South-Western Baltic Sea, (Kiel/Mecklenburg Area and Bornholm Basin, abbreviated to KA and BB throughout) as sampling sites (Fig.2.1.1 A). We consider areas corresponding geographically to the Kiel Basin and the Mecklenburg Bight to belong to the same region (KA, Kiel Area) as we found them to be statistically indiscernible in their physical and chemical (e.g., salinities, nutrient concentrations) characteristics (see Table S2.1.1).

Over a period of two years, we sampled during the following cruises on RV ALKOR: March 2018 (AL505); July 2018 (AL513); March 2019 (AL520); April 2019 (AL521); May 2019 (AL522); July 2019 (AL524); October 2019 (AL530).

We took samples from at least 3 stations from each area on each cruise.

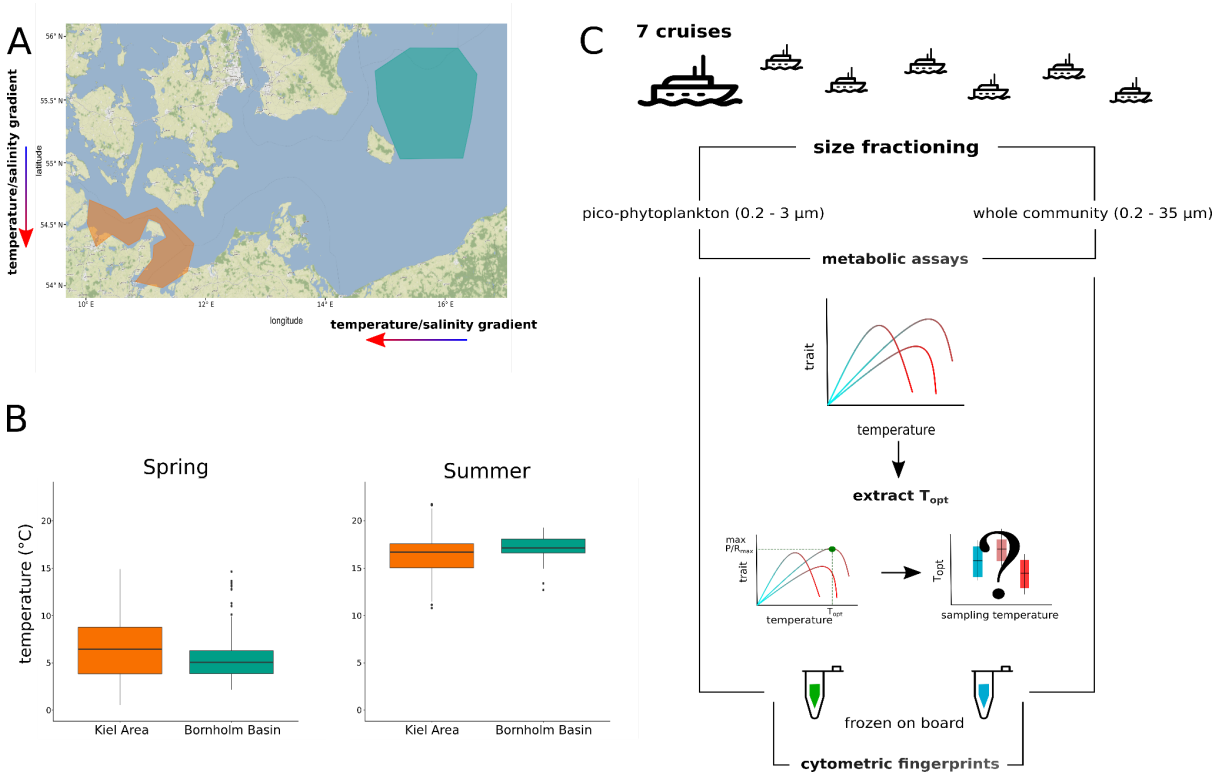


Figure 2.1.1. Sampling sites and experimental protocol flow chart (**A**) Colour-coded polygons describe the sampling areas (orange: Kiel Basin and Mecklenburg Bight, blue: Bornholm Basin). Arrows show direction of naturally occurring temperature and salinity gradients with red for warmer temperature and higher salinities, blue for colder temperatures and lower salinities. (**B**) Boxplots of surface temperatures (~ 8 m) of the last 5 years for the Kiel Area, in orange, and the Bornholm Basin, in blue, for spring and summer. As data indicate sea surface temperature (SST) to be significantly more variable in the Kiel Area than in the Bornholm Basin, these two regions were chosen as sampling areas. **(C)** Infographic describing the sampling and consequent analyses on board and in the lab.

Abiotic environment

We determined abiotic environmental conditions via i) decomposition analysis and ii) water sampling and nutrient measurements during the cruises prior to sample collection. We carried out a decomposition analysis using the function decompose of the *anomalize* package (0.2.0) in R version 4.0.2 to test for the differences in trend, seasonality and random impacts of temperature for the two chosen areas. Both additive (seasonal + trend + random) and multiplicative (seasonal * trend * random) approaches were used to analyse the residuals and anomalies after accounting for trend and seasonality. The additive approach assumes a quarterly seasonality (frequency = 4), whereas the multiplicative approach assumes a monthly seasonality (frequency = 12). Data used for the decomposition analysis contained monitoring temperature data of the last 5 years collected during ALKOR and POSEIDON´s

Oceanographic cruises (data courtesy of the “GEOMAR – Helmholtz Centre for Ocean Research” of Kiel and “BSH - Bundesamt für Seeschifffahrt und Hydrographie” Centre).

Before the water sample collection, physical and biological parameters of the water column during the cruises (position, temperature, salinity, chlorophyll a), were determined with a CTD (Conductivity, Temperature, Depth Water Sampler) probe (Table 2.1.1). Water samples for measuring dissolved inorganic nutrient concentrations were collected at 5 metres (crane-controlled Niskin Bottle or CTD Rosette) for each cruise and station. Samples were passed through an 0.2µm pore size filter and immediately stored frozen at −20 °C for subsequent colorimetric determination of nitrate, nitrite, ammonia, silicate and soluble reactive phosphorus (SRP) using a segmented flow auto-analyser (SEAL Analytics AA3, UK), following the methods of Murphy and Riley (1962), Kirkwood, D. (1996), Grasshoff et al. (2009).

Table 2.1.1 Overview of water column parameters during the cruises. Geographical areas refer to the two considered regions (KA: Kiel and Mecklenburg Bight; BB: Bornholm Basin). Data were retrieved from a CTD run at each station at the sampling depth (5-10 m). Each station was sampled only once as the water column up to sampling depth was fully mixed. Data were then pooled by sampling area

Cruise data	Geographical Area	Salinity (PSU)	Water Temperature (°C)
AL505 (March 2018)	KA	9.5 ± 4.64	1.34 ± 0.199
AL505 (March 2018)	BB	7.43 ± 0.09	2.32 ± 0.29
AL513 (July 2018)	KA	14.44 ± 0.43	21.03 ± 2.39
AL513 (July 2018)	BB	7.24 ± 0.43	20.88 ± 4.74
AL520 (March 2019)	KA	15.57 ± 2.09	4 ± 0
AL521 (April 2019)	KA	14.1 ± 4.19	6.26 ± 0.34
AL521 (April 2019)	BB	8.04 ± 0.09	6.4 ± 0.31
AL522 (May 2019)	KA	14.37 ± 1.89	11.22 ± 0.52
AL522 (May 2019)	BB	7.98 ± 0.04	9.36 ± 0.43
AL524 (July 2019)	KA	12.67 ± 1.53	18.4 ± 0.69
AL524 (July 2019)	BB	7.18 ± 1.06	17.17 ± 0.31
AL530 (October 2019)	KA	16.82 ± 0.96	12.92 ± 0.52

Biological Sample collection

Water samples for measuring metabolic responses were collected with a Multi-Niskin bottle rosette sampler. Sampling depth was adjusted according to CTD profiles to make sure samples were taken above the thermocline and halocline if necessary but remained within the first 5-10 metres of the water column. Water samples were immediately passed through a 35 µm mesh to remove large grazers and particles. We then prepared two size fractions: one, containing all phytoplankton smaller than 35 µm, and another one, containing only pico-phytoplankton smaller than 3 µm. The former was passed through the 35 µm mesh and then concentrated on a 0.2 µm filter to increase biomass. The latter, pico-phytoplankton community sample, was filtered through 3 µm filters (Worden and Not 2008), and the filtrate then concentrated on a 0.2 µm filter. We used at least 2 litres of water for each size fraction.

From here onwards we will refer to the concentrated water samples containing either the 0.2 – 3 µm sized community or 0.2 – 35 µm sized community as "samples".

Metabolic Activity

To assess whether the organisms in the samples from different regions and cruises differed in their immediate (i.e., within one generation) metabolic responses to warming, we measured the rates of oxygen evolution in the light (Photosynthesis, P) and oxygen consumption in the dark (Respiration, R), using a Clark-type electrode (Oxytherm, HansaTech, UK). Gross photosynthesis (GP) was calculated as P + |R|, accounting for oxygen consumption both in the light and in the dark. For further analysis, we only used data, where photosynthesis was more positive than respiration, since when R exceeds P, the system points toward heterotrophy as a source of CO_2 (del Giorgio et al. 1997). We used an aliquot of each sample to measure a Photosynthesis - Irradiance (PI) curve, to identify the optimal irradiance (I_{opt}) at which to carry out measurements across a temperature gradient. Based on research carried out on phytoplankton communities, we expected I_{opt} to not change during immediate response measurements (Schaum et al. 2017).

Photosynthesis was measured at light intensities that spanned from 50 to 1500 µmol m^{-2} s^{-1} (with unequal increments, see supporting information) over 20 minutes (one minute at each light intensity) and followed by a 3 minute measurement of respiration in the dark. PI curves were then analysed in the R environment (v 3.5.3) using a modified Eilers´ photoinhibition model (Eilers and Peeters 1988) (eq. 1),

$$np\,(I) = \frac{np_{max} I}{(np_{max}/\alpha I_{opt}^2) I^2 + (1 - (\frac{2np_{max}}{\alpha I_{opt}})) I + \frac{np_{max}}{\alpha}} - r$$

(eq. 1)

where *np*(*I*) is the rate of gross photosynthesis at the specific irradiance, *npmax* is the photosynthetic activity at the optimal light intensity (*Iopt*), α is the initial slope and *r* is the respiration when light intensity is 0.

Data were fitted using the package "*TeamPhytoplankton*", based on non-linear least square regression, and the best fits were determined based on AIC scores running 1000 different combinations of initial parameters.

To ensure that the full breadth of the unimodal curve was captured, the range of assay temperatures for each cruise was based on trials carried out in the beginning of a cruise, as we did not expect parameters to drastically change over the sampling period. The temperature range included the lowest and the highest temperature at which a physiological response was reliably measurable. This resulted in ranges spanning from 3° up to 40° C in 1, 2 or 3° C increments (smaller increments near the thermal optimum, see Table S2.1.2 for details). No further measurements were carried out after temperatures at which oxygen consumption in the dark exceeded oxygen production in the light. Prior to the measurements, samples were given time to adjust to the assay temperature and conditions in the Oxytherm chamber until the respiration signal in the dark stabilised (max. 10 minutes). P and R were measured for 5 minutes each. A new aliquot was used for each assay temperature, to avoid stress responses or hysteresis induced by samples' being subjected to multiple temperatures.

Analysis of thermal reaction norms

Thermal reaction norms of metabolic rates of GP and R obtained on the oxygen electrode were subsequently analysed in the R environment (v 3.5.3), via non-linear least squares regression as stated above, using a modified Sharpe-Schoolfield equation (Schoolfield et al. 1980) (eq. 2),

$$ln(b(T_c)) = E_a(\frac{1}{kT_c} - \frac{1}{kT}) + ln(b(T_c)) + \alpha ln(M_i) - ln\left(1 + e^{E_h(\frac{1}{kT_h} - \frac{1}{kT})}\right)$$

(eq. 2)

where k is the Boltzmann's constant (8.62 x 10-5 eV/k), E_a is the activation energy (how $ln(b(T_c))$ increases below the optimal temperature), E_h is the high-temperature induced inactivation of enzyme kinetics and $b(T)$ the metabolic trait at the assay temperature (either GP or R). The optimum temperature was identified solving the following equation (eq. 3)

$$T_{opt} = \frac{E_h T_h}{E_h + kT_h ln(\frac{E_h}{E_a - 1})}$$

(eq. 3)

E_a,E_h and T_{opt} represent biologically relevant features characterising thermal reaction norms. We extracted the T_{opt} values of GP and R for each samples and cruises thermal response curve and plotted them against *in situ* temperatures at the time of sampling.

Flow cytometry

We collected a 200 µL aliquot of each sample processed at each tested temperature and froze it in sorbitol after measuring the metabolic rates (10 µL of a 1% sorbitol solution per sample). To ensure a gentle freezing process, samples were stored immediately at +4° C in the dark after adding sorbitol and then frozen at -80° C. Storage at +4° C depended on how fast samples could be processed on board, but never exceeded 48 hours. Cell numbers for biomass correction and phenotypic diversity were determined on a flow cytometer (Accuri C6, BD Biosciences, USA) upon returning to Hamburg. In order to avoid background noise and counting of heterotrophs or debris, we applied thresholds for both side scatter (measure of cellular size) and on the FL3 (chlorophyll) fluorescence channel.

The flow cytometry data were also used to establish flow cytometric fingerprints after (Carr et al. 2003). Even though analytical flow cytometry does not allow taxonomically relevant discrimination, the high functional (reflected in photopigment diversity) and morphological (both in terms of size and intracellular composition) diversity, allows distinction of phytoplankton clusters (for an overview of nomenclature used, see Table 2.1.2).

We also froze samples for bacteria quantification. In order to exclude geographically and/or seasonally driven differences in the bacteria concentration that could have shaped respiration patterns, we measured bacterial content of the aforementioned samples, upon returning to Hamburg. Prior to the cytometry analysis, samples were quickly thawed at 37° C and stained with SYBR Green I nucleic acid (Molecular Probes Inc., USA). Purchased dye has a 10000 fold concentration. SYBR Gold was diluted to a final concentration of 10^{-4} in TE buffer filtered on a 0.02 µm pore size filter prior to use, according to the method of Brussaard et al. (2000). Then the samples were first incubated at 80° C for 10 minutes and then cooled down in the dark before adding reference beads (1 µm diameter BD Biosciences, USA). Samples were then checked using a BD Accuri C6 flow cytometer, correcting for a blank with TE buffer and SYBR Green I prepared as the analysed samples. Bacteria concentration was established using scatter plots of green fluorescence of the staining (FL1 channel) versus side scatter as a measure of cells size (FSC channel).

Table 2.1.2. Nomenclature used for flow cytometry parameters used as proxies for phenotypic characteristics of the phytoplankton communities.

Name displayed in Accuri C6 data and figures	Proxy for
SSC	Internal cell complexity
FSC	Diameter of the cell
FL2	Phycoerythrin; Phycocyanin
FL3	Chlorophyll a
FL4	Allophycocyanin; Other Chlorophylls

Data analysis and statistics

Data were processed in the R environment (version 4.0.2). Figures were made using *ggplot2* (3.2.1) and maps using *ggmap (3.0.0)*. We compared the extracted Topt values for GP and R using a mixed effects linear model using the package *nlme.* We fitted a global model including mean temperature at sampling time, geographical areas and size fraction as fixed and interacting (geo * fraction) effects and considered sampling stations as nested random effects. The model was subsequently simplified and models compared considering AIC scores and delta AIC values using the package *MuMIn* (1.43.6). In particular, we discarded models with $\Delta i > 2$. Δi was calculated as the difference between *AICi* and *AICmin*, where *AICi* is the AIC score for the *i*th model and *AICmin* is the minimum of AIC among all models

Pairwise comparisons of slopes of linear regression between Topt and mean sampling temperature, were analysed examining the ANOVA p-values from interaction between mean sampling temperature, geographical areas and size fraction, then slopes were compared using the *lstrends* function. We used the *pairs* function for running a Tukey post-hoc comparison on the family of estimates. Both functions are built in the package *emmeans* (1.4.8).

To address whether samples from different cruises varied significantly in terms of microbial community composition, we conducted a Bayesian Principal Component Analysis (PCA) in the package *FactoMineR* (2.0) on the cytometric fingerprints (SSC, FSC, FL1, FL2; FL3; FL4). To test the differences of the community composition between the two geographical

areas for both size fractions, a permutational Multivariate Analysis of Variance (PERMANOVA) was done using function *adonis* in the package *vegan* (2.5-6) using Bray-Curtis dissimilarity with 999 permutations on Euclidean matrix distances.

Results

Long-term monitoring data and sampling conditions

To choose sampling locations, we tested the difference in sea surface temperature variability of the two areas prior to the cruises and consequently chose sampling locations, and analysed environmental monitoring data. We separated spring and summer temperature data of the last 5 years (Fig. 2.1.1 B). We found that the chosen areas (Kiel Area and Bornholm Basin) indeed showed different patterns in variability (Levene test: spring values, $F_{1,475}$= 12.96, p=0.00035; summer values, $F_{1,383}$= 4.21, p=0.04), with the Kiel Area´s standard deviation being overall higher than the standard deviation found in the Bornholm Basin (spring: Kiel Area ±3.36, Bornholm Basin ±2.86; summer: Kiel Area: ±2.07, Bornholm Basin: ±1.49). A decomposition analysis also confirmed the expected differences in predictability of temperature variations (Fig. S2.1.1): When decomposing the time series, the function was not able to predict a clear seasonal pattern for the Kiel Area. The random component was substantial for the Kiel Area as well, but this was not the case for data from the Bornholm Basin (One-Way ANOVA comparing random components outcomes comparing the two geographical areas: $F_{1, 2851}$=128.9, p<2e-16; see Table S2.1.4 for statistics and random components).

The abiotic parameters (temperature, salinity, nutrients) measured during the cruises in the Kiel Area and Bornholm Basin followed a characteristic seasonal pattern: dissolved nitrogen, phosphate and silicate were replenished pre-bloom (March 2018/2019) (N: 73.92 µg L^{-1} ± 26.7; P: 16.12 ± 4.21 µg L^{-1}; Si: 12.05 µM L^{-1} ± 1.14). As the spring and successive summer blooms established, all nutrient concentrations were reduced (July 2018/2019) (N: 14.96 µg L^{-1} ± 4.45; P: 2.65 µg L^{-1} ± 0.51; Si: 4.54 µM L^{-1} ± 0.63) (Fig S2.1.2). There were no significant differences for temperature, or in major nutrient content, between the sampling regions during spring and summer (one-way ANOVA type III; temperature: $F_{1, 17}$ = 0.02 p = 0.90; nitrate $F_{1,15}$: 1.19, p = 0.29; phosphate $F_{1,15}$: 0.11, p=0.74). Salinity was consistently lower in the Bornholm Basin than in the Kiel Area (Table 2.1.1; $F_{1,17}$ = 24.87, p = 0.0001).

There was a strong signal for abnormally long, abnormally warm periods during the summer cruise of 2018 (KA: 21.03 °C ± 2.18; BB: 20.88 °C ± 4.24) and to a smaller extend, during the summer cruise of 2019, while salinity and nutrient composition were comparable to previous years and long-term monitoring data (Snoeijs-Leijonmalm et al. 2017). The summer of 2018 falls into the definition of heat wave given by Hobday et al. (2016), with mean temperatures for both areas above the 90th percentile for a period longer than 5 successive days.

Mean in situ temperatures influence gross photosynthesis but not respiration thermal optima under average thermal conditions

In order to evaluate conditions reflecting the 90th percentile of the past five years' temperature average, we excluded the heat wave data from summer 2018 from our first round of analysis.

Under conditions excluding the heatwave, the shapes of acute thermal response curves of gross photosynthesis were highly malleable with regards to their optimum temperature (see Fig 2.1.2 A and B for thermal optima of gross photosynthesis ($T_{opt}GP$) patterns over *in situ* temperatures). This reflects an ability of phytoplankton communities to alter the shape of the curve according to the environmental conditions on time scales of a single growing season, with higher T_{opt} (highest T_{opt} measured: 36.96 and 35.64 °C, respectively for Kiel Area and Bornholm Basin) at higher sea surface temperatures (highest sea surface temperature 21.03 and 20.09 °C for Kiel and Bornholm).

Specifically, samples from both size fractions from the Bornholm Basin and the whole community fraction from Kiel Area, showed an increase in thermal optima with increasing temperature whereas the pico-phytoplankton fraction from the Kiel Area showed a stable trend in thermal optima (Fig. 2.1.2 A and B). Thus, we found a significant effect of temperature and size fraction on the $T_{opt}GP$ especially regarding the whole phytoplankton community (mean sampling temperature: $F_{1,31}$=5.65 p=0.024; size fraction: $F_{1,9}$=11.64 p=0.008). In the pair-wise comparison of the slopes of the described trends between the different size fractions and geographical areas, we found that the pattern in the pico-phytoplankton Kiel Area fraction was significantly different from the other depicted trend for the pico-phytoplankton fraction in Bornholm Basin (Table 2.1.3).

In contrast to the responses of GP, thermal optima for respiration (T_{opt}RESP) did not change with increasing sea surface temperature in any of the considered areas and size spectra (Table S2.1.4 B for model comparison and ANOVA´s outputs for the most parsimonious model). Further, there were also no differences in the pairwise comparison of the slopes.

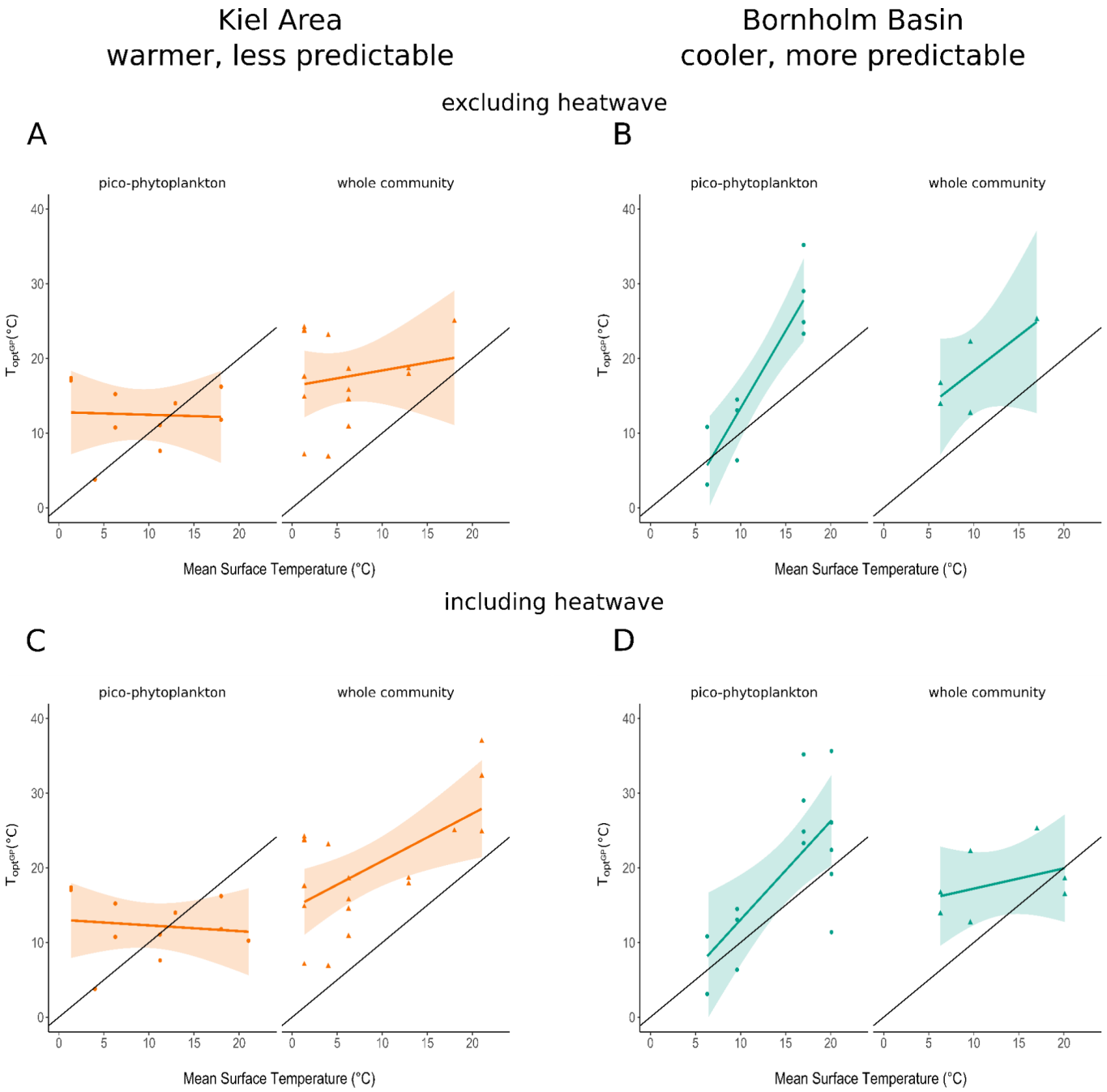


Figure 2.1.2. Scatter plot referring to the thermal optima of gross photosynthesis for the pico-phytoplankton and whole community in the two sampling areas (Kiel Area in orange and Bornholm Basin in blue). $T_{opt}GP$ was calculated according to eq. 3. The X-axis represents the mean surface water temperature at the time of the sampling in ºC. Coloured lines represent the output of linear regression with the corresponding confidence interval and the solid black line corresponds to the 1:1 regression between mean environmental temperature and thermal optima. Shaded areas are 95% confidence intervals automatically calculated in R. The first row (panel A and B) shows data excluding the 2018 summer heatwave. Second row (panel C and D) refers to the complete dataset, spanning the seven cruises of 2018 and 2019.

Table 2.1.3. Pairwise comparisons of slopes of regression for $T_{opt}GP$ (thermal optima for gross photosynthesis) and mean sampling temperature, divided per geographical area of origin and size fraction. Only significant values are stated here. (SE: standard error; df: degree of freedom; p: p- value).

Variable	*SE*	df	*t.ratio*	*p*	*estimate*
Excluding heatwave: pico-phytoplankton BB Vs. pico-phytoplankton KA	0.399	38	3.810	0.0027	1.521
Including heatwave: pico-phytoplankton BB Vs. pico-phytoplankton KA	0.43	42	3.28	0.011	1.4

Community respiration changes greatly during a heatwave

To evaluate whether the thermal optima substantially changed during a heatwave, we subsequently analysed the entire dataset including the extreme event of July 2018. Extreme events overall reinforced the relationship between $T_{opt}GP$ and seasonal changes in sea surface temperature (Fig. 2.1.2 C and D) ($F_{1,39} = 13.83$, $p = 0.0006$). Moreover, pairwise comparisons of the regression slopes additionally showed significant differences within the geographical areas and the size fraction. Specifically, we found differences between the pico-phytoplankton fraction from the two different Basins (Table 2.1.3). While inclusion of extreme events only had a small effect on responses of $T_{opt}GP$, we found that $T_{opt}RESP$ was more responsive to extreme scenarios than during an average seasonal scenario (i.e., excluding the heatwave event), i.e., $T_{opt}RESP$ was strongly influenced by mean surface temperature (Fig. 2.1.3 C and D) ($F_{1,34} = 21.97$ $p < 0.001$). This response was conserved across all samples, regardless of size fraction or region of origin.

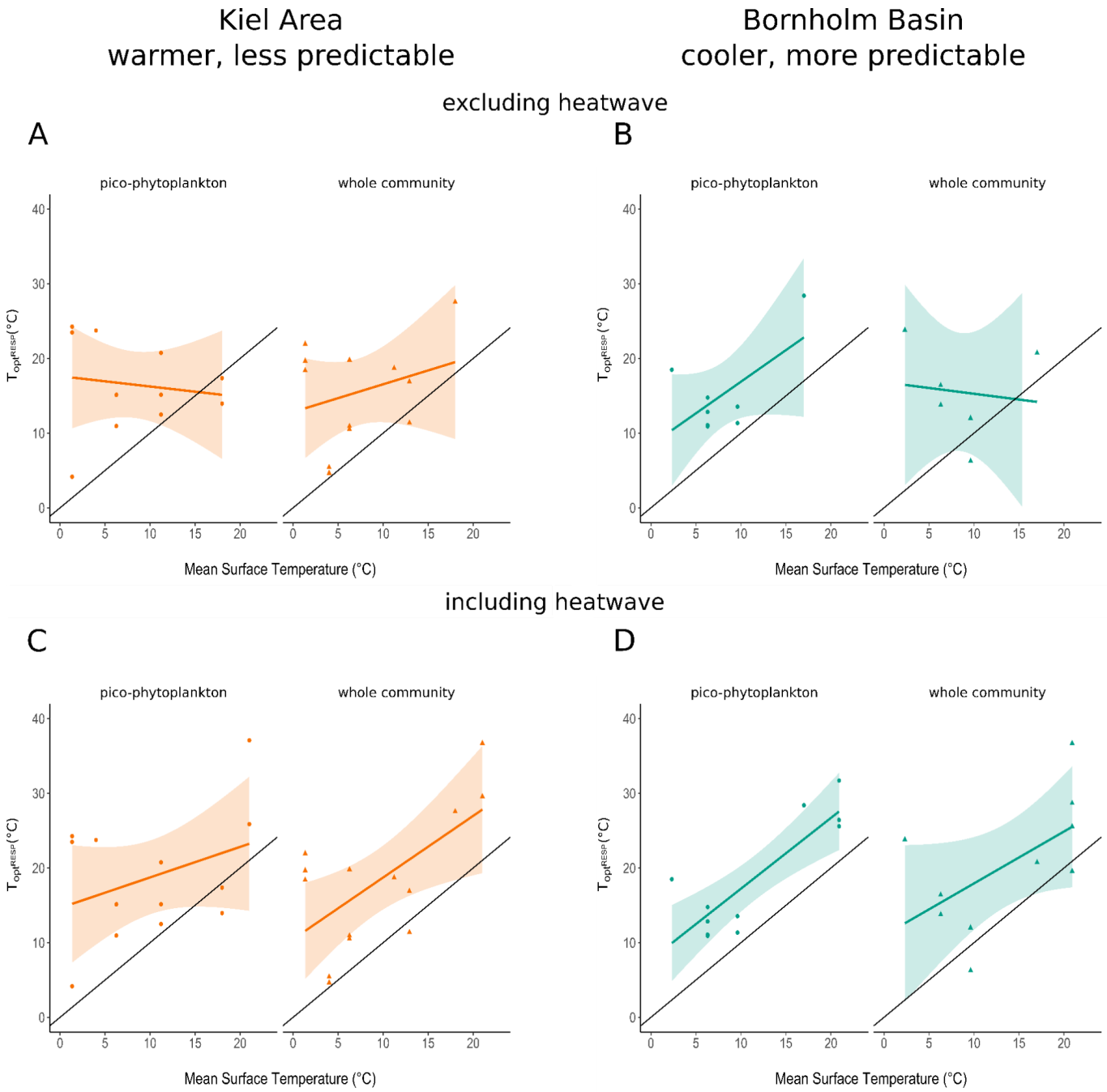


Figure 2.1.3. Scatter plot referring to the thermal optima of respiration (T_{opt}RESP) for the pico-phytoplankton and whole community in the two different areas (Kiel Area in orange and Bornholm Basin in blue). The X-axis represents the mean surface water temperature at the time of the sampling in ºC. Coloured lines represent the output of linear regression with the corresponding confidence interval and solid black line corresponds to the 1:1 regression between mean environmental temperature and thermal optima. Shaded areas are 95% confidence intervals automatically calculated in R. The first row (panel A and B) shows data excluding the 2018 summer heatwave. Second row (panel C and D) refers to the complete dataset, spanning the seven cruises of 2018 and 2019.

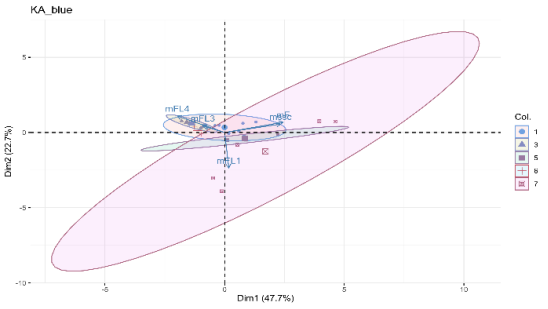
Changes in community composition offer an explanation for the changes of thermal optima responses in predictable regions but not for variable environments.

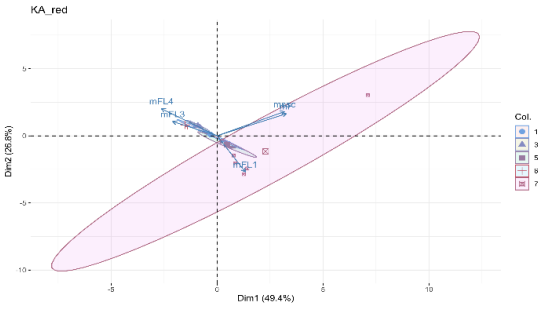
In the absence of genetic community data (but see Zhong et al. (2020) for a rough estimate of 2018 pico-phytoplankton community composition through meta-barcoding), we tracked the gross community composition on the functional group level throughout the cruises quantifying the covariance of phenotypic characteristics defined across cruises. In order to depict phenotypic features, we analysed the cytometric fingerprints, which describe the size and photopigments` characteristics of the cells, for all the cruises and both size fractions. Each cruise is here throughout described as mean *in situ* temperature at the time of sampling to reflect environmental conditions.

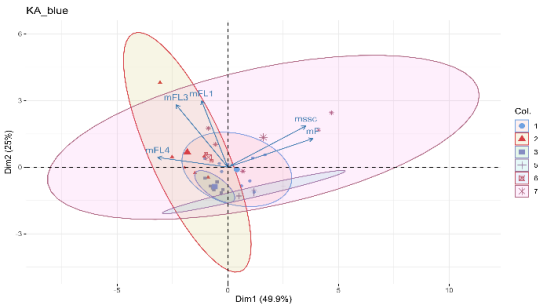
We detected two trends: one, organisms sampled in the Kiel Area were phenotypically – at least in terms of size, granularity and photopigments – similar across seasons (and thereby temperatures). This is evidenced graphically by the clusterization of cytometric fingerprints of all the cruises (PERMANOVA; for the pico-phytoplankton and whole community, $p>0.1$). Two, in the Bornholm Basin the clusters corresponding to the cruises were significantly different from each other, indicating that gross community composition changed ($p<0.005$, see also Table S2.1.5 for statistics). In both size fractions and geographical areas, the main drivers of community distinction were not due to the changes in SSC (granularity) and FCS (size), but rather photopigment characteristics. The trend was preserved regardless of whether data obtained during the heatwave was included in the dataset (Fig 2.1.4 E -H).

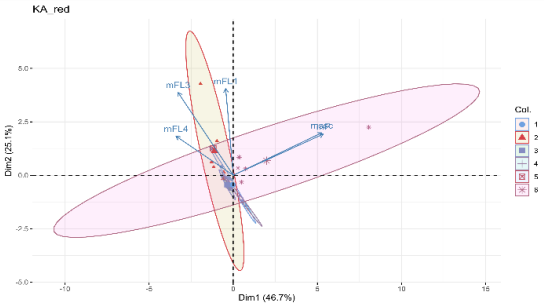
Seasonal differences in the communities´ flow cytometric data were largely driven by changes in size and internal granularity of the cells (FSC and SSC respectively; Fig 2.1.4 D G H). Only when excluding the heatwave event, FL4 (phycocyanin content, a prominent photopigment in cyanobacteria) influenced the maximum variance direction in the data in some cases (i.e., Kiel Area whole community and pico-phytoplankton; Bornholm Basin pico-phytoplankton).

Kiel Area
warmer, less predictable

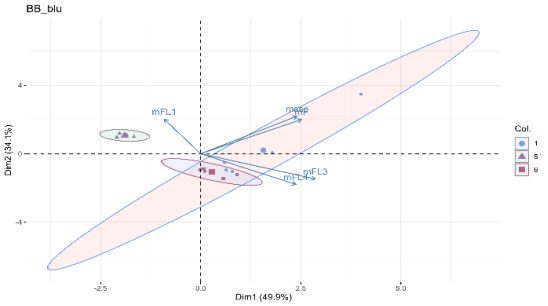


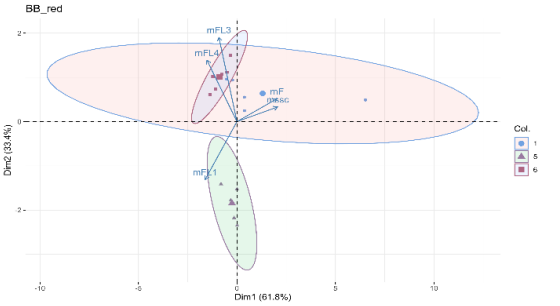


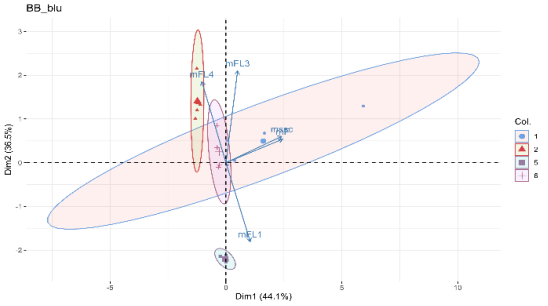




Bornholm Basin
cooler, more predictable







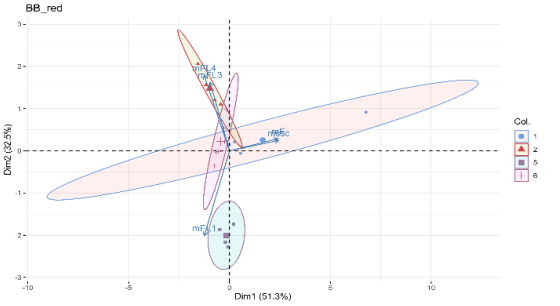


Figure 2.1.4 PCA of cytometric fingerprint covariance. Loadings represent phenotypic characteristics of functional groups derived from flow cytometry. The first row (A; B; C; D) corresponds to all events excluding the summer 2018 heatwave. The second row (E;F;G;H) instead, analysed the entire dataset. The first two principal components account for approximately 70-80% of the variance. Ellipses represent different sampling cruises. Other abbreviations: SSC, side scatter; FSC, forward scatter; FL2, FL3, FL4, photopigments composition and quantity (respectively detect: phycoerythrin, chlorophyll a and b, phycocyanin, see Table 2.1.1 for the list of parameters and abbreviations used). Arrows indicate the covariance between cruises and main physiological features describing community composition. The colour gradient reports the sampling temperature at sea in the respective cruises (blue to red from colder to warmer temperatures).

Discussion

We investigated the direct effect of temperature on phytoplankton metabolic rates on physiological time scales (response curves) as well as ecological and evolutionary processes on longer timescales (seasonal differences, differences between basins and interactions thereof). We used a local approach of comparing adjacent but characteristically different sea surface areas and thereby minimised the confounding effects that arise when comparing regions across global scales.

We show that overall T_{opt}GP in phytoplankton communities <35 μm strongly increases with sea surface temperatures, in contrast to T_{opt}RESP, which is less sensitive to warming. Regions differed strongly in their sensitivity to heatwaves and in how community composition changed throughout the seasons. While in line with rapid thermal evolution in phytoplankton, this indicates differences in the underlying adaptive patterns due to variation with overarching fundamental and realised niches (i.e., the physical-chemical conditions and the biological interactions respectively (Soberón and Arroyo-Peña 2017). Similarly, in the absence of heatwaves, seasonality was the main driver for changes in T_{opt}GP, indicating that thermal performances co-vary systematically with environmental parameters (Padfield et al. 2017). T_{opt} was overall the most reactive parameter in our dataset compared to the other parameters of the curve (i.e., E_a, E_h, lnc). This is in strong contrast with results from single species acclimated to changes in mean temperature under laboratory conditions, or model predictions. There, the shape of the curve changes rapidly, and parameters that change most with warming tend to be the elevation and steepness (E_a and E_h, respectively) of the thermal response curve (Barton et al. 2018), and, on short time scales, respiration is more sensitive than photosynthesis.

As expected (e.g., Thomas et al. 2017) we found that in all samples from the more predictable, cooler Bornholm Basin, and in community samples <35µm from the warmer, more unpredictable Kiel Area, T_{opt}GP was higher than the mean environmental temperature. More unexpectedly, in the pico-phytoplankton samples from the Kiel Area, sampling temperatures regularly exceeded T_{opt}GP. There, T_{opt}GP did not change with seasonal temperature, but remained stable at ca 16 °C throughout the year (for comparison, the five-year average temperature in the Kiel Area is 7.92 ºC), as depicted by 1:1 regression line in Fig. 2.1.2 and 2.1.3 and pairwise comparisons. Growth rates of small phytoplankton are often positively correlated with temperature (Kulk et al. 2011), so it would stand to reason that the photosynthetic traits that commonly underpin fitness (Cullen 1990) behave in a similar fashion. Differences in responsiveness of T_{opt}GP between the two size classes may be due to a buffering effect caused by a higher biodiversity, which is intuitively higher in the whole community fraction due to the contribution of cells larger than 3 µm, and in line with theoretical and empirical frameworks (e.g., García et al. 2018). Moreover, cyanobacteria tolerate high temperature well, but they are more common in the Bornholm Basin rather than in the Kiel Area (Öberg 2016).

Phytoplankton growth is a balance between GP and RESP, since a large part of the carbon produced by photosynthesis is then remineralised by respiration (Falkowski et al. 1998). Surprisingly, T_{opt} for respiration did not follow the seasonally increasing sea surface temperature at all. The lack of responsiveness of T_{opt}RESP is unexpected (Staehr and Birkeland 2006) and suggests phytoplankton communities could actively counterbalance seasonal increasing temperatures through adjusting photosynthetic activity alone. As on-board incubations to test whether this was reflected in growth rates across the same temperatures were not feasible, we cannot postulate direct effects on fitness based on this data set alone. While measures of Carbon Use Efficiency (CUE) could serve as an indirect measure of the amount of carbon available for growth (Gifford 2003), we cannot with certainty estimate the contributions of heterotrophic bacterial respiration here, making calculations for CUE or NPP not completely reliable.

Under the influence of heatwaves (i.e., August 2018 cruise), the relationship between T_{opt}RESP and sea surface temperatures greatly changed, creating a relationship that more closely aligns with model predictions concerning the slopes of thermal reaction norms, i.e., a higher reactivity of respiration than photosynthesis. When respiration rises with increasing

temperatures, phytoplankton growth can be limited if respiration rises to a point where it exceeds photosynthesis. Hence, heatwaves have the potential to drastically alter phytoplankton primary production, especially in regions that are cooler and less variable, such as the Bornholm Basin.

Most recent studies on heat wave scenarios focused on their effect on community composition (e.g., Striebel et al. 2016). Several observations indicate that biodiversity is consistently lower during heatwaves (especially true for nutrient-poor regions (Hayashida et al. 2020) resulting in drastically changed community structures. Based on flow cytometric data, we found no evidence of the heatwave significantly reducing phenotypic diversity although the capacity of the communities to metabolically compensate for extreme and sudden events seemed to decline.

Crucially, our study shows that changes in thermal reaction norms can be rapid (on a seasonal time scale) enough to adjust to seasonal variability even when the selection environment is highly complex, but not to sudden events like heatwaves. Models assume that the degree to which plasticity evolves in a fluctuating environment is ultimately limited by the cost of plasticity, though the cost has remained elusive in experiments (Murren et al. 2015). If plasticity has an intrinsic cost, heterogeneous conditions might avoid a perfect match between phenotype and environment (i.e., mean phenotype is the optimum phenotype in all scenarios) and instead force a generalist/specialist coupling. In the latter case, organisms will perform quite well in a variety of environments, but not quite as well as in the specialist specific niche (Angilletta et al. 2003). We found that regardless of the variability and fluctuations´ complexity of the previously experienced environment, i.e., the geographical region, all the analysed samples reacted the same way and we detected neither specialistic nor generalistic trends. Even if we had conducted our investigation under more natural conditions (i.e., minimising acclimation bias and forced biotic relationships), we still investigated only one environmentally induced pair of traits. Ideally, more traits (e.g., growth rates, oxidative stress) should have been examined since plasticity likely affected different traits with, in theory, different costs (e.g., Walworth et al. 2021). In our case, in the Kiel Area, populations´ reaction norms evolved closer to the optimum even in extreme conditions like during a heatwave. We can argue that the more unpredictable Kiel Area represents a not canalised environment, without decreased genetic and phenotypic variance. Therefore, maintenance costs, needed to track environmental conditions and changes, were here minimized (DeWitt 1998).

On seasonal timescales, various mechanisms, such as phenotypic plasticity or biotic filtering (i.e., species less adapted to the conditions they face are outcompeted by more adapted ones (Thomas et al. 2016), can act on and determine the way communities respond to environmental changes. In our framework, changes on the functional group level are not an underlying reason for changes in thermal tolerance in the more variable Kiel Area. It is likely that communities adjust through plasticity or rapid evolutionary responses (including sorting of standing genetic variation of different genotypes within the same species). Populations in the Kiel Area were rather similar throughout cruises demonstrating that peculiar sensitivities of communities rather than functional traits, lead to adjustments of thermal tolerances. In the more predictable Bornholm Basin, the opposite situation occurred, with a strong shift on the functional group level. Ecological implications are grand and intuitive: our findings point out that, moving toward a generally warmer and more unpredictable future, changes of the communities´ composition in formerly predictable areas, might disrupt ecosystem functions. Populations in the Kiel Area had centuries to evolve to variable conditions, whereas current changes are happening on a much faster timescale.

Here we show that even in a complex natural environment, the ways in which major metabolic pathways react to changes in temperature are at least partially predictable (i.e., as temperature rises, so does photosynthetic T_{opt}) and repeatable (our findings hold across size classes and regions). Although we currently lack information on the mechanistic processes involved in the maintenance of these patterns on acclimation timescales, our results provide essential information (e.g., timing of adaptive patterns, differential responses of natural assemblages) on adaptive dynamics of phytoplankton for ecosystem models. A better understanding of the timescales at which fast reproducing organisms react to changes could indeed make the coupling between human-induced changes and natural adaptive patterns more precisely predictable

2.2 Study II: Short-term fitness responses of picophytoplankton communities

Seasonal and evolutionary timescales shape thermal tolerance profiles of picophytoplankton

Maria Elisabetta Santelia, Luisa Listmann, C-Elisa Schaum

Abstract

In the near future, phytoplankton will exist in a warmer, more thermally variable ocean. It is intuitively important to understand how biomass, fitness and species composition will change in natural communities, locally adapted to differently predictable environments. This is challenging because in marine microbes, ecology and evolution interact on similar timescales. Habitat filtering and phenotypic plasticity can happen simultaneously and may both drive evolutionary responses. However, the timing at which they happen, can lead to modified biotic interactions and evolutionary trajectories. Here, we examined how ecology (i.e., biodiversity indexes) and evolutionary history (ultimately determined by biogeography) shape the timing of growth rates responses in pico-phytoplankton communities. Natural assemblages were isolated from the South-western Baltic Sea, along two areas with different thermal predictability. We found that growth rates´ thermal responses curves in samples collected during winter were similar for both areas and characterised by the usual left-skewed unimodal shape. In contrast, samples isolated during summer, including a heatwave, behaved differently; communities already accustomed to thermal unpredictability showed a flat reaction norm, accompanied by rapid community changes during the experiment (about 2 weeks). Samples coming from predictable environments went through a habitat filtering process on longer (seasonal) timescales. Our results point toward a strong connection between local adaptation to different degrees of thermal predictability and the timing of habitat tracking.

Introduction

In the near future, phytoplankton will have to exist in a warmer, more variable ocean (IPCC report, 2014). Despite their important role in biogeochemical cycles and foodwebs (Falkowski 1980), the basic ecology of these organisms remains poorly understood. For example, in a warmer ocean also characterised by a more pronounced variance around the mean temperature and extreme events, it is pertinent that we better understand how community interactions contribute to changes in phytoplankton biomass, species composition, and diversity. Evolution is proven to happen also at a timescale comparable to ecological processes (i.e., over a few generations), making particularly challenging to gather this type of information.

Given their fast growth rates, phytoplankton have ample scope to react quickly to environmental change through a combination of phenotypic plasticity (adjusting the phenotypes in response to environmental changes without underlying heritable genetic changes (West-Eberhard, 1989)), and habitat filtering (ecological filtering selecting traits-suited individual species within a pool (Diaz et al, 1998)). Both of these mechanisms may eventually lead to heritable changes in gene frequencies and favour fast evolutionary responses, but the degree to which they each contribute to phytoplankton responses in a changing ocean is difficult to predict as neither of them act in isolation.

Habitat filtering and evolutionary responses are interconnected (Keddy, 1992). Phenotypic plasticity indirectly affects habitat filtering, because it can act as a buffer to environmental changes (Garnier et al, 2016). As a result, diversity can be maintained in communities with high phenotypic plasticity even when habitats change rapidly, resulting in potential knock-on effects for evolutionary responses, for example, leading to slower evolutionary responses (De Mazancourt at al, 2008). Habitat filtering can also alter evolutionary trajectories directly, by driving rapid changes in community structure in response to environmental selection pressure, resulting in communities with different structure and tolerances to environmental variables than the starting community. If habitat filtering is sufficiently fast, organisms remaining in the population will not be under high selective pressure. This in turn could mean that the evolutionary response is limited to sorting on the genotype level, or that micro-evolutionary responses of the remaining genotypes will take a longer time to establish. It stands to reason that the degree to which phytoplankton communities deal with warming

through plasticity will hinge on whether or not there is a mismatch between plastic responses, filtering processes, and environmental variability.

Temperature and variability therein greatly influence phytoplankton fitness and traits (Leung et al. 2020) and can therefore impose a strong selective pressure on communities. Numerous studies have investigated the short-term responses of phytoplankton to temperature increase, usually showing a strong sensitivity of respiration, photosynthesis (Barton et al. 2020) and increased growth rates (Barton and Yvon‐Durocher 2019). Temperature dependency of traits is described by reaction norms. The shape and features are shared by all ectotherms: curves are unimodal and left-skewed. Rates increase as temperature rises until an optimum and then abruptly decrease. Reaction norms can fail to predict the fate of a population because they assume a perfect match between plastic responses and the environment (Kingsolver and Woods 2016). Thermal plastic responses can vary widely depending on species-specific degrees of local adaptation (Bennett et al. 2019). In particular, if communities are already adapted to predictable enough environmental conditions, plasticity can be gradual (i.e., occurring at the same pace as environmental changes) and a mismatch between plasticity and environmental variations can happen (Fey et al. 2021). If limits of plasticity are reached, poorly adapted species can be replaced with fitter ones (i.e., species sorting) (Ackerly 2003). In this sense, species sorting can be seen as a habitat tracking process, where evolution and community composition can interfere with each other and affect local distribution, in particular when environmental fluctuations are frequent (Loeuille and Leibold 2008).

Connecting temperature responses and complex ecological consequences at the community level is one of the largest missing pieces in order to predict the consequences of future warming scenarios. While the mechanisms and timing of phenotypic plasticity in single species or assemblages with known environmental histories (e.g., samples from culture collections) are well described (Bestion et al. 2021), the interplay of plastic responses and local adaptation in natural communities remains less well understood. It remains crucial to understand if local adaptation can influence the mechanisms by which organisms can react to environmental changes.

Here, we hypothesise that having a micro-evolutionary history of coming from a more thermally variable area lead to communities that deal with changing environmental conditions on a seasonal time scale primarily through phenotypic plasticity. Further, we hypothesise that the mechanisms explaining the degree to which plasticity helps shape

thermal tolerances also depend on the organisms´ evolutionary history (i.e., geographical origin).

In order to address these hypotheses, we used natural assemblages of pico-phytoplankton (0.2 – 3 µm in diameter) isolated from two distinct regions in the Southwestern Baltic Sea. The two regions are characterised by distinct thermal variability patterns, with the Kiel Area being overall warmer and less predictable and the Bornholm Basin being cooler and more predictable (see Study I & Santelia et al, preprint). We analysed fitness (growth rates), metabolism (gross photosynthesis and respiration) and biodiversity (proxies for species richness) over 20 generations of acclimation in response to a temperature gradient (from 15 to 30°C).

Materials and methods

Isolation of natural assemblages and maintenance of cultures

We collected natural pico-phytoplankton assemblages during 2 R/V Alkor cruises (AL505 and AL513) conducted in the Southwestern Baltic Sea during the months of March and July of 2018 (throughout referred to as winter and summer cruise). During July 2018, we encountered a heatwave (see Santelia et al, preprint for abiotic conditions during the cruises). 5 to 10 L of surface waters (min. 5 m depth, according to chlorophyll a profiles) were filtered directly on board. Specifically, water was pre-filtered through a 35 µm mesh to remove larger debris and grazers, then filtered using a vacuum pump through a 3 µm filter and finally concentrated on a 0.2 µm filter. We collected approximately 40 mL of the concentrated water in plastic flasks, added 10 µL of Bold's (Bischoff & Bold 1963) nutrients mix to not give cyanobacteria a disproportionate advantage and kept the samples at a 12:12h light:dark cycle and natural irradiance for the remainder of the cruises (maximum 14 days).

Upon returning to the laboratory in Hamburg, the cultures were kept in incubators (Multitron, Infors HT) at 15 ºC for March and 22 ºC for July cruises. The temperatures were chosen as a compromise between conditions at sea and logistical capacity of the laboratory.

In order to perform a pilot study on single species´ responses, we then performed serial dilutions for species isolation on aliquots of the samples (see supplementary information for isolation procedure of *Ostreococcus* spp. and main results on the isolated strains). After 2

months, we transferred the cultures into common garden conditions, in a culture chamber at 18°C, 100 µmol photons mol^{-1} sec^{-1} of irradiance and 12:12h light:dark cycle. Cultures were always nutrient repleted and were transferred every two weeks in f/2 culture (Guillard & Ryther 1962) media.

Experimental design

To test short-term responses of the community assemblages to warming, we used an aluminium temperature gradient table with two hollow compartments inside (Fig. S2.2.1). Water of two different temperatures is pumped at both ends to obtain the desired temperature gradient. The gradient consists of ten different temperatures (one temperature for each column). The table was completely isolated with Styrofoam and temperatures were checked daily during the experiment. A light system was mounted on top of the table and light irradiance was checked at the beginning and at the end of the experiment (~ 100 μmol^{-1} m^{-2} s^{-1} ± 14). Samples were grown in replicates in multiwell plates and the position of which was swapped randomly every day to avoid confounding factors due to slight differences in light intensities. Communities cultures were pre-acclimated to temperature conditions on the table for 10 generations. We then transferred 3000 cells mL^{-1} and then used those samples for another 10 generations of acclimation assays (Fig. 2.2.1).

Assay temperatures ranged from 15 to 30°C with uneven increments of max. 2°C. The experiment was run in two batches to cover all the desired temperatures. An overlapping condition (20°C) was run twice for comparison between batches, and no significant differences were found ($F_{1,363}$=1.49, p=0.22). We used 8 communities, with 3 biological replicates each, sampled from the two locations in the Baltic Sea (Kiel Area and Bornholm Basin, Table 2.2.1). Cultures were grown in f/2 media. At the higher temperatures, we faced evaporation inside the multiwells. To overcome this potential bias, wells were replenished regularly with sterile water of the correct salinity (no added nutrients) and dilution factors were calculated accordingly.

Table 2.2.1. List of samples with sampling area, cruise ID and time and coordinates of the sampling station. In orange samples from the Kiel Area (light, winter cruise; dark, summer cruise), in blues samples collected from the Bornholm Basin (light, winter cruise; dark summer cruise).

Samples id	Sampling Area	Cruise ID	Coordinates
St04.1	Kiel Area (warmer, less predictable)	AL505(March 2018)	54°34´30``N 10°30´15´´E
St21.1	Kiel Area (warmer, less predictable)	AL505(March 2018)	54°31´27``N 11°19´36´´E
St04.2	Kiel Area (warmer, less predictable)	AL513(July 2018)	54°27´87``N 11°32´43´´E
St15.2	Kiel Area (warmer, less predictable)	AL513(July 2018)	54°53´61``N 10°06´02´´E
St16.1	Bornholm Basin (cooler, more predictable)	AL505(March 2018)	55°12´08``N 15°52´28´´E
St19.1	Bornholm Basin (cooler, more predictable)	AL505(March 2018)	55°36´43``N 15°17´74´´E
St09.2	Bornholm Basin (cooler, more predictable)	AL513(July 2018)	55°47´52``N 16°29´94´´E
St10.2	Bornholm Basin (cooler, more predictable)	AL513(July 2018)	55°07´46``N 16°14´98´´E

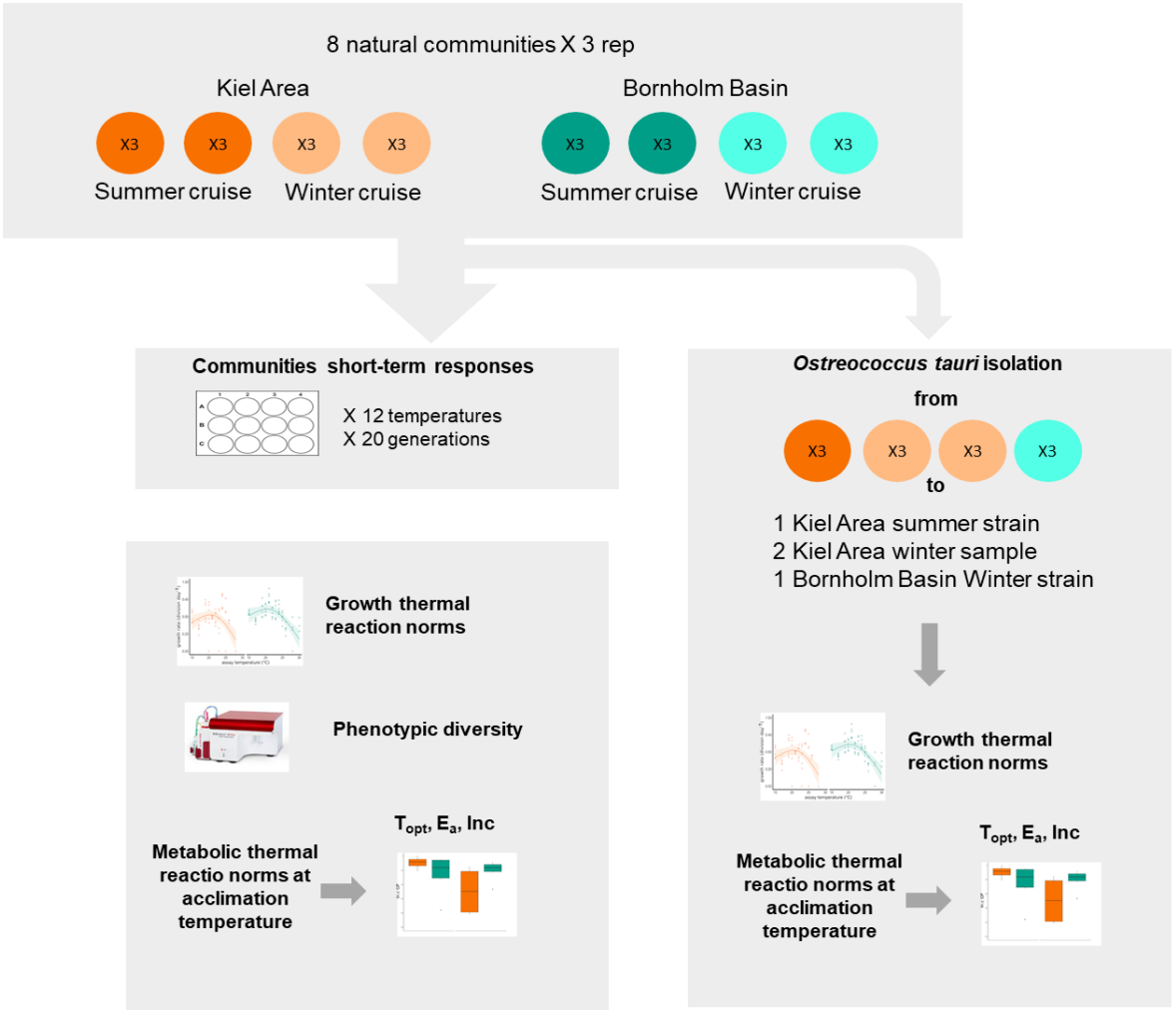


Figure 2.2.1. Graphical representation of the experimental design

Fitness responses and cytometric fingerprints

During the acclimation phase (final 10 generations), we collected 100 µL of sample each day to track growth rates. Cells numbers were determined on a flow cytometer (Accuri C6, BD Biosciences). In order to avoid background noise, we applied a threshold on the FL3 (chlorophyll) fluorescence channel (Fig. S2.2.2 for threshold references). Growth rates (µ) were calculated using the formula (Equ. 1):

$$\mu\ (divisions\ day^{-1}) = \frac{(log(N_f/N_0))}{(t_f - t_0)}$$

Equ. 1

Where N_f are the cell counts (cell/mL) at time f and N_0 are the cell counts at time t_0.

To assess the growth thermal response curve, we fitted generalised additive models (GAMs) to growth rates with temperature as a smoother term and geographical area as fixed effect (see SI for the formula used). We used the fits to estimate optimal temperature for growth (where μ was maximal). GAMs were performed using the mgcv package (1.8.31) in R.

The cytometry data were also used to assess α and β diversity indexes. The cytometric fingerprints used were relevant for functional and morphological discriminations, using size, intracellular composition and photopigment diversity.

Diversity indexes

We performed assessments of phytoplankton diversity indexes (alpha and beta diversity) using flow cytometric fingerprints (Table 2.2.2). Cytometric data were extracted using the flowcore package and vital organisms were selected applying gatings on the FL3 channel (chlorophyll a). Measures were scaled in logarithmic values and the pipeline developed in the package Phenoflow was applied. See Props et al. (2016) for detailed information about the pipeline.

We performed a GAM model on alpha diversity indexes, using geographical area as fixed effect and temperature as smoother term. To assess statistically differences in community compositions between geographical area within seasons (***β*** diversity), we performed permutational Multivariate Analysis of Variance (PERMANOVA, within vegan package::adonis, 999 permutations) using Bray-Curtis dissimilarities calculated through the Phenoflow package (2.5.7).

Table 2.2.2. Nomenclature used for flow cytometry parameters used as proxies for phenotypic characteristics of the phytoplankton communities.

Names displayed in Accuri **C6 data and figures**	**Proxy for**
SSC	Intracellular complexity
FSC	Diameter of the cell
FL2	Phycoerythrin; Phycocyanin
FL3	Chlorophyll a
FL4	Allophycocyanin; Chlorophylls

Metabolic responses (oxygen production and consumption rates)

At the end of the acclimation phase (i.e., after a total of 20 generations at assay conditions) we measured community metabolic responses (gross photosynthesis and respiration) at acclimation temperature. 2 mL of cultures in exponential phase were acclimated in the dark for 20 minutes. After that, we measured photosynthetic activity and respiration as oxygen evolution in the light and in the dark via a Clark-type electrode (Hansatech Ltd., Oxytherm) equipped with a temperature controlled chamber to ensure measurements at respective growth temperatures. Gross photosynthesis was measured at increased light intensity with uneven intervals from 50 up to 1500 µmol^{-1} m^{-2} s^{-1} to obtain a photosynthesis-irradiance curve (PI curve). Respiration was measured as oxygen consumption in the dark for three minutes. Each PI curve was fitted using Eiler´s curve for photoinhibition (equ.2)

$$NPP(I) = \frac{NPP_{max} I}{\left(\frac{NPP_{max}}{\alpha I_{opt}^2}\right) I^2 + \left(1 - \left(\frac{2NPP_{max}}{\alpha I_{opt}}\right)\right) I + \frac{NPP_{max}}{\alpha}} - CR$$

Equ. 2

Where NPP(I) is the rate of net primary production at the irradiance (I), NPPmax is the maximum rate of NPP at optimum light (I_{opt}) and CR is community respiration. Data were normalised using cells counts obtained via flow cytometry.

Only maximum photosynthetic rates at optimum light intensity (P_{max}) for each growth temperature was extracted and used as a parameter for further analysis. We fitted unimodal thermal response curves using a modified Sharpe-Schoolfield equation (equ. 3) (Schoolfield et al, 1981) via non-linear least squares regression using the nlsLoop package (version 1.0.0; Padfield, 2016):

$$\ln ln\left(b\left(T_c\right)\right) = E_a\left(\frac{1}{kT_c} - \frac{1}{kT}\right) + \ln ln\left(b\left(T_c\right)\right) - ln(1 + e^{E_h\left(\frac{1}{kT_h} - \frac{1}{kT}\right)})$$

Equ. 3

Where *k* is the Boltzmann's constant (8.62x10-5) eV/k), E_a is the activation energy, E_h is the high-temperature induced inactivation of enzyme kinetics and *b(T)* is the metabolic trait at the assay temperature of either gross photosynthesis (GP) or respiration (R). E_a and E_h are

biologically relevant features that establish, respectively, how the metabolic traits increase below the optimal temperature or decrease above.

Results and discussion

Seasons and geographical area select for different thermal response curves

We measured thermal tolerance profiles of growth as a fitness proxy for community samples from the winter and summer cruise in the Kiel Area and Bornholm Basin (respectively, average sea surface temperatures were: 1.34 and 2.32°C during the winter cruise and 21.03 and 20.09°C during the summer one). We found striking differences in thermal reaction norms driven by season and sampling location (Figure 2.2.2, where data are grouped by geographical area, i.e., Kiel Area and Bornholm Basin, and season in which they were sampled, i.e., March and July 2018, throughout respectively referred to as winter and summer cruise; model summary Table S2.2.1). Seasonal timescales are here considered as different months in the same year, while regional effects are assumed to be taking place over longer periods and thus shape communities over hundreds of generations.

In samples collected from the overall cooler and more predictable Bornholm Basin, the shape of the curve changed slightly but significantly across seasons (GAM, effect of seasons in the Bornholm Basin, p=1.25e-09, deviance explained = 30.1%), and generally followed the expected hump-shaped pattern across the temperatures tested here. There were no significant differences regarding the widths and intercepts of the thermal reaction norm curves. s. However, there was a pronounced change in the growth rate at the thermal optimum (T_{opt}^{growth} for winter cruise was 20°C, and for summer cruise, 22°C), which increased on average 1.15 fold going from winter to summer conditions. Flow cytometric fingerprints, in line with previous studies (Zhong et al. 2020), suggests that this is likely due to the different starting composition of the community, with an increased proportion of cyanobacteria in the summer months. Cyanobacteria in particular are known to be able to tolerate temperatures exceeding 25°C well (Visser et al. 2016).

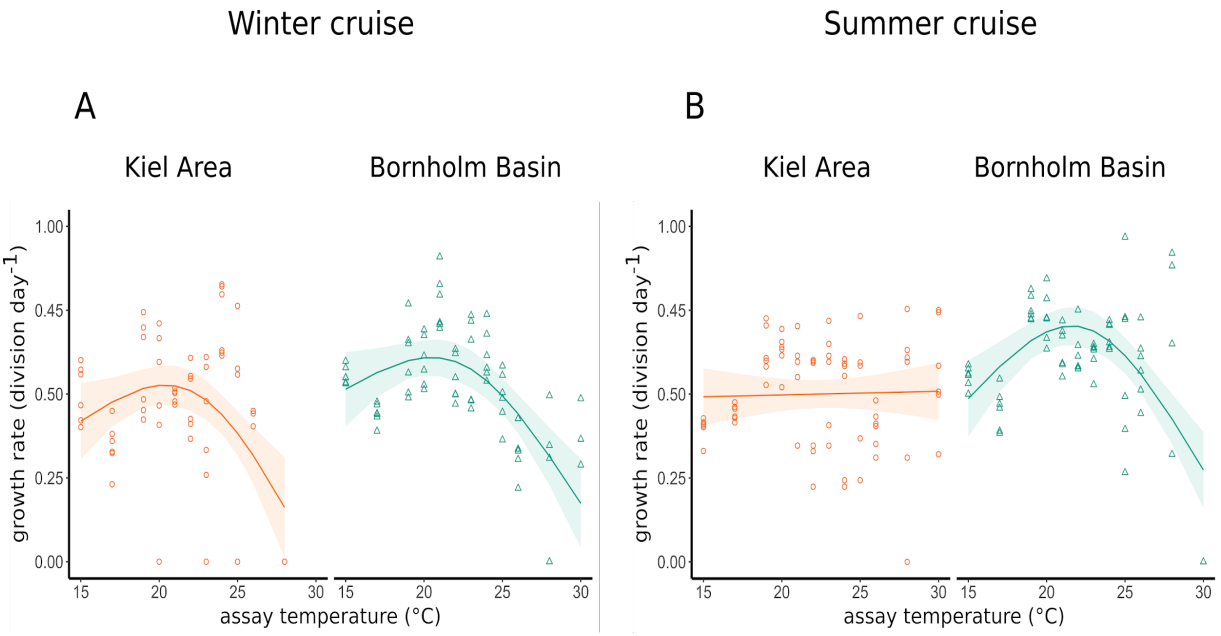


Figure 2.2.2. Growth rates over assayed temperatures for samples collected during winter (A) and summer (B) cruises (in the Northern hemisphere). The two geographical areas are depicted in orange (Kiel Area, warmer, less predictable) and blue (Bornholm Basin, cooler, more predictable). Individual points are single replicates of different samples pooled by geographical area and cruise. We fitted a GAM (generalised additive model) model including geographical area and assayed temperatures as fixed effects. The shaded areas depict the 95% confidence intervals, based on standard error fits.

In the thermally less predictable Kiel Area, the thermal response curve shape changed dramatically during the hotter summer season (Fig. 2.2.2 B). While growth rates communities sampled in spring followed the expected hump shaped curve within the chosen temperature gradient, this was not the case for summer communities. There, growth rates were on average higher than in the spring communities, and did not change significantly across the range of temperatures from 15 up to 30°C. Different mechanisms can explain why the fitness reaction norm remains flat. Community compositions might change in response to temperature, but when phenotypic plasticity in a few species is sufficiently high, growth rate will remain unchanged. Alternatively, in populations with lower phenotypic plasticity, community composition might change such that functionally redundant species with the same growth rates replace one another. The reduced thermal sensitivity in summer samples of the growth responses in the Kiel Area may be also due to an higher presence of generalists individuals in the communities, an expected scenario in fluctuating environmental conditions (Haaland et al. 2019).

Mechanisms underlying fitness responses: timing of habitat filtering

In order to test the mechanisms explaining the differences between changes in thermal tolerance profiles in communities from the two regions, we analysed flow cytometry data (e.g., size, intracellular complexity of the cells, photopigment composition) and established phenotypic fingerprints that can be used as proxy for phenotypic diversity (Zhong et al, 2020). We focused on alpha and beta diversity in order to test i) changes in community composition at each assay temperature during the final ten generations of the experiment and ii) differences in community composition due to changes in environmental parameters at a seasonal time scale.

During the final 10 generations of the laboratory based assays, we analysed alpha diversity within communities at each assayed temperature, to specifically assess how diversity within the communities changed in response to assay temperature (Fig. 2.2.3). In the winter cruise samples, alpha diversity changed slightly in response to temperature as expected: diversity decreased with increasing temperature, and did so in a similar fashion in both geographical areas (Fig. 2.2.3 A, effect of region p=0.27; model summary Table S2.2.2 A).

In the samples from the summer cruise, communities started out with similar diversity indexes at 15 ºC, but only for samples from the Kiel Area this was followed by a steep decrease with increasing temperature (Fig. 2.2.3 B, effect of region, p=2.69e-08; model summary Table S2.2.2 B). Lower phenotypic diversity can indicate a fairly simple community structure made up of just a few specialists at individual temperatures. Here, these specialist communities each have similar total growth rates. While we cannot know for sure the identity and number of taxonomic species in each community, we can assume that if there had been a stark decline in taxonomic identities, we would have been able to trace it using the flow cytometric output (Zhong et al, 2020), and that the communities show very little phenotypic variance. It is hence likely that we are observing a real phenomenon of samples from an environment with a strong history of environmental unpredictability being able to tolerate increased temperature conditions during the assays on fast time scales of 10 generations (approx. 1 month).

We analysed beta diversity indexes (Fig. 2.2.4) to account for differences in initial biodiversity between geographical areas. The winter cruises samples from both regions were heterogeneous, with no clear between-region differences (PERMANOVA, p=0.18, R^2=0.01),

indicating that starting diversity does not explain differences in alpha diversity observed throughout the assay period (Fig. 2.2.4 A).

Beta diversity in communities sampled in the summer differed significantly between sampling regions (PERMANOVA, $p=0.001$, $R^2=0.44$): in line with a higher prevalence of cyanobacteria in the Bornholm Basin, community diversity in samples from the Bornholm Basin was lower than in samples from the Kiel Area (Fig. 2.2.4 B). This can help us explain why thermal tolerance profiles reacted differently to seasonal warming in the two regions. In the Kiel Area, habitat filtering did not take place before we carried out the assays. Therefore the community reacted to warming on short time scales largely through phenotypic plasticity or species or genotype sorting. In the Bornholm Basin samples, biodiversity was already low as a result of seasonal warming before the assays started, so that the community responded to further warming through habitat filtering on longer time scales (seasonal, prior to the experimental assays). The same strategy (i.e., habitat filtering through seasons) is applied by the communities in the Bornholm Basin across different time scales, both during our short-term experiment and within single generations during an extreme heat wave (Santelia et al, preprint). This suggests that in this more predictable area, selection for a few specialists represents the preferred strategy to respond to increased temperatures and hence the more unimodal community tolerance curve. This is in line with previous studies reporting that habitat filtering in response to environmental restrictions, can cause trait convergence and a reduction in biodiversity (Gianoli and Escobedo 2021) as we found in the Bornholm Basin.

Nevertheless, when considering community studies and especially natural phytoplankton community dynamics, it is difficult to understand if habitat filtering is truly excluding plasticity. Filtering in phytoplankton communities commonly takes place naturally (i.e., seasonal succession), as in the case of the Bornholm Basin, where the timing of the shift is consistent with evolutionary timescales (i.e., over season instead of a few generations). We do assume that plasticity plays a role in the responses of the Kiel Area because the timing at which the habitat filtering happens is fast (probably faster than under natural conditions) and at the community level, traits are unchanged, guaranteeing the survival of the community itself. For both evolutionary and acclimative responses, the timing of habitat filtering or species sorting matters.

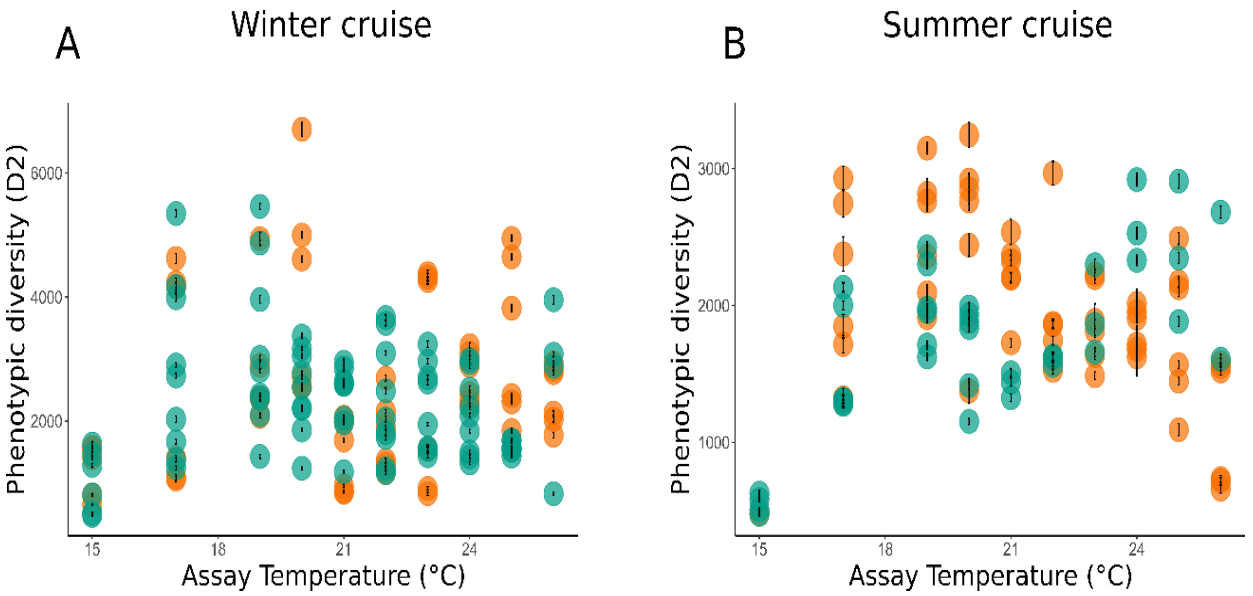


Figure 2.2.3. Alpha diversity of samples from two geographical areas (Kiel Area in orange and Bornholm Basin in blue) collected over winter (A) and summer (B) cruises (March and July 2018) over the assayed temperature range (15 to 30°C). Black lines indicate standard deviation. Diversity was inferred using flow cytometric data (size, granularity, and pigment composition).

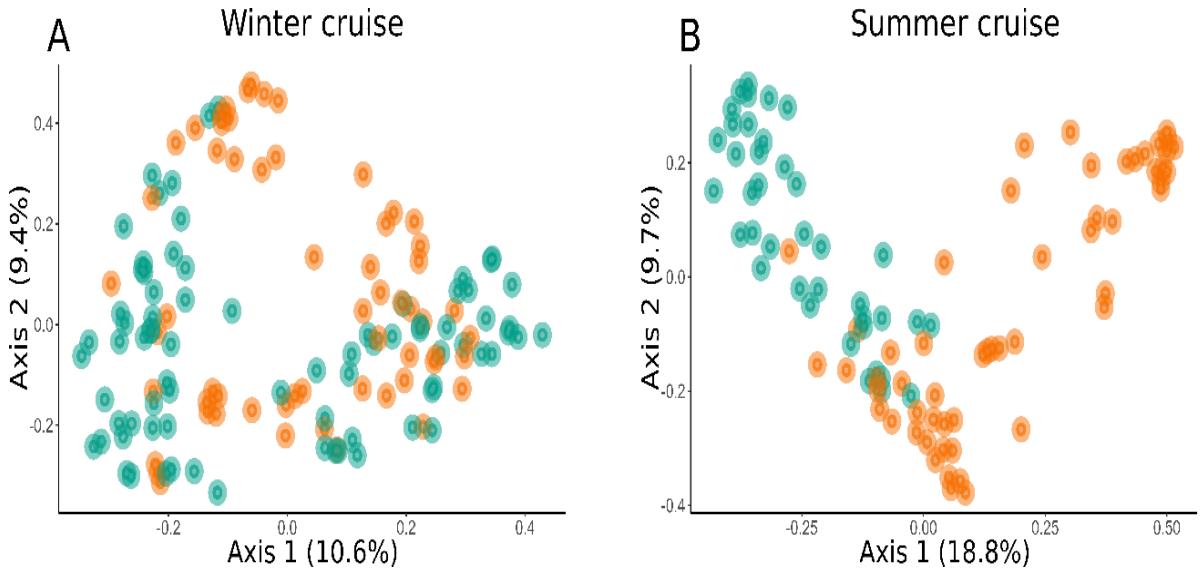


Figure 2.2.4. Beta diversity inferred from Principal Coordinate Analysis (PCoA) based on Bray-Curtis distance. Results are based on flow cytometric data. The percentage of explained variation by each axis is shown within parenthesis in the label. Data were collected on samples coming from a winter (A) and a summer (B) cruise, conducted respectively in March and July 2018. The different geographical areas are depicted in orange (Kiel Area) and blue (Bornholm Basin).

Fitness is not explained by metabolism in complex communities

Here, changes in biodiversity on the level of photopigments and size classes can explain seasonal differences between thermal tolerance curves from communities sampled in warmer, less predictable, and cooler, more predictable regions.

In addition, it is likely that variations in metabolic phenotypic traits on the community level explain variance in fitness (Pettersen, White, and Marshall 2016). Here, we focused on gross photosynthesis and respiration since data on single species cultures would suggest that there is a direct and strong link between metabolism and fitness (Padfield et al. 2016a). Further, photosynthesis and respiration directly tie phytoplankton to the carbon cycle and thereby to ecosystem functioning (Falkowski 1994).

We did not find any relevant differences in the two areas for all the other parameters describing the thermal response curve (E_a, E_h and T_{opt}). We observed a sharp decline in the intercept of the thermal response curves (which indicates the metabolic rates at the lowest temperature tested) for gross photosynthesis (ln.cGP) in the Kiel Area across seasons (from winter to summer) (Fig. 2.2.5 A and B, ANOVA p=0.0031 $F_{1,13}$=13.14). In the Bornholm Basin, the values remained stable (Fig. 2.2.4 A and B, ANOVA p=0.701 $F_{1,10}$=3.67). This decline in ln.cGP, is counterbalanced by a decrease in ln.c for respiration rates as well.

It is not possible to infer from our data that the metabolic responses measurements are showing the same pattern as the fitness responses. In particular, the decrease in metabolic rates does not match the horizontal fitness reaction norm of the communities in the Kiel Area in summer, in other words, fitness remained stable despite a decline in the amount of carbon from photosynthesis available for growth. This is in contrast with previous studies, both on single species (Padfield et al. 2016) and on phytoplankton assemblages in mesocosms (Schaum et al. 2017).

To test whether this discrepancy owed to the particularities of the community, or to strategies from phytoplankton from this region in general, we isolated single cells of *Ostreococcus spp.* from a subset of isolates from the Bornholm Basin and the Kiel Area (see SI for details about the isolates) and tested their responses to the same temperature degree used for the communities (15 to 30°C). When looking at single species responses, we detected an increase in fitness thermal tolerance at high temperatures in the stable Bornholm Basin (Fig. S2.2.3). Both GP and R T_{opt} for the Bornholm Basin are higher than those in the Kiel Area, denoting a rapid adaptation to warming (Fig. S2.2.4). Results gathered point to a clearer match between

metabolism and fitness when considering single species responses for phytoplankton from these regions. At the community level, undetectable trade-off between other relevant traits (e.g., nutrient uptake (Ward et al. 2017)) could probably alter the expected results. Especially in areas such as the Kiel Basin, where a complex environment and biotic interactions may mask links between fitness and metabolism.

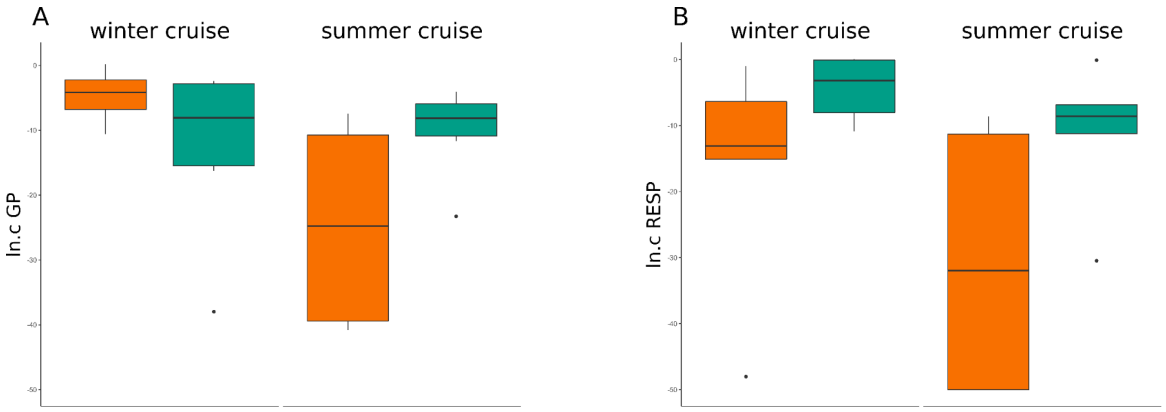


Figure 2.2.5. intercept of the thermal response curves (indicating the metabolic rates at the lowest temperature tested) for gross photosynthesis (ln.cGP, A) and respiration (ln.cRESP, B). The geographical areas are depicted in orange (Kiel Area) and blue (Bornholm Basin). Boxplots are displayed as standard: bold lines represent the media, whiskers the highest and lowest values in the lower and upper quartile and boxes the ends of the lower and upper quartile.

Conclusions

In this study, temperature sensitivities of phytoplankton communities seem to be ultimately determined by local predictability patterns and starting composition of the communities at time of sampling. Predictions about evolutionary potential in future scenarios should take into account that community level responses might not always be well represented by single species studies, but that knowing the geography, and therefore local adaptation, can give us useful hints about community responses. In fact, our results regarding previous local adaptation (eco-evo history) as a major driver of the strategies used by picophytoplankton to grow across a wide range of temperatures, are consistent with our previous study conducted directly on board during the cruises and within only one generation. We suggest that the timing of community sorting can indicate the relative importance of phenotypic plasticity when comparing between communities. If this pattern is truly generalisable, it could mean that areas with a lesser degree of thermal unpredictability, will face a sudden shift in the

communities in response to extreme events. To better understand the impact of thermal variability on acclimative and adaptive responses, we recommend to perform similar studies in other areas of the oceans with different thermal characteristics (e.g., tropical or arctic regions) and experimental evolution approaches to clearly identify the effects of variability patterns on traits and in complex conditions where more than one driver is present (e.g., temperature and nutrients concentration).

2.3 Study III: Long-term responses of *Ostreococcus* spp to thermal fluctuations

The road goes ever on and on: Predictability of thermal fluctuations affects evolution of phenotypic plasticity in a cosmopolitan phytoplankton species.

Maria Elisabetta Santelia, Carina Peters, Luisa Listmann, C-Elisa Schaum

Abstract

Phenotypic plasticity is a key mechanism for coping with changing environments. As environmental variability is expected to increase even further in the near future, the role of stochastic environments in shaping the interactions between plasticity and evolution has received great attention. The predictability of environmental drivers influences the evolution of plasticity, with more plastic responses in more predictable environments. However, we still need to shed light onto the role that differences in predictability of the amplitude and frequency of the fluctuations in past (here, predictability of fluctuations and ecology in geographical origins) and present (here, predictability of fluctuations in the selection treatments) environments play in enhancing or hindering the evolution of plastic responses. Here, I tested how these factors affect the magnitude of evolutionary responses and the strength of evolved plastic responses in two species of the genus *Ostreococcus*. I isolated 5 strains with different evolutionary histories and exposed them to 4 thermally fluctuating treatments and a control treatment for 120 generations. The selection environments differed in the levels of predictability of frequency and amplitude. I found that organisms evolved slow growth rates regardless of the evolutionary past, ultimately determined by the geographical origin: here, a more thermally predictable area, the Kiel Area and the more predictable Bornholm Basin. I nevertheless detected effects of past evolution and ecological constraints in the evolution of growth rate plasticity. Plasticity increased in strains from the completely predictable environment, while I found low levels of plasticity when the frequency of fluctuations was unpredictable, especially in samples coming from the most thermally predictable environment. My study highlights that the predictability of thermal fluctuations is an important factor affecting both ancestral and evolved plastic phytoplankton responses.

Introduction

Phenotypic plasticity is the ability of a genotype to produce different phenotypes in response to changes in the environment (West-Eberhard 1989). Plasticity plays a major role in determining evolutionary responses (Lande 2009; Pigliucci 2005; Price et al. 2003). Numerous theoretical and experimental studies have shown a positive correlation between plasticity and evolution (Chevin et al. 2013; Lande 2014; Schaum and Collins 2014). Still, the details of this relationship are controversial and different theories exist, many of them mutually exclusive: on the one hand, a more plastic population could be less likely to be under selection by shielding genotypes and therefore less likely to deal with a changing environment primarily through evolution (Merilä and Hendry 2014; Ghalambor et al. 2007). On the other hand, when an increase in fitness due to a strong plastic response leads to large population size, the chances of fixing beneficial alleles may increase (Lande 2009). Thereby, a strong plastic response to an environmental change may lead to a stronger evolutionary response.

The nature of the relationship between plasticity (also sometimes referred to as acclimation, especially in literature with a more ecological focus) and evolutionary or adaptive responses also hinges on environmental stability. As environmental variability is expected to increase even further in the near future (Thornton et al. 2014), the role of stochastic environments in shaping the interactions between plasticity and evolution has received great attention. Several studies have thoroughly analysed phytoplankton and microbial responses to thermal fluctuations in the short (e.g. Gill et al. 2022; Fu et al. 2022) and long-term (e.g. Leung et al. 2020; Schaum et al. 2022) , proving that the strength and the direction of responses, regardless of the timing of selection, largely depend on the frequencies and amplitude of the fluctuations. Density-dependent dynamics can also - by their very nature- explain responses in microbes in fluctuating environments (Chevin et al. 2017). However, phytoplankton under laboratory conditions reach high concentrations (10^4 cells ml^{-1}), minimising demographic effects in all but the most detrimental environments.

Phytoplankton in today's oceans already experience a thermally variable environment, for example due to drifting, ocean circulation and seasonal variations (e.g. Doblin and van Sebille 2016; Zaiss et al. 2021). These fluctuations span timescales from short, diurnal fluctuations within one generation, to longer, seasonal fluctuations across several generations. Whether or not fluctuations or an increase therein leads to a lower quality environment depends strongly

on the speed, amplitude, and predictability of the fluctuations, and of how often and how long for organisms are experiencing undesirable *vs* ameliorated conditions. Fast growing phytoplankton can actively counteract potentially stressful effects of fluctuating conditions in an efficient way. Fast generation times allow them to experience even rapid changes as gradual and therefore evolutionary responses are more likely. Negative demographic effects, while probably rare even in nature, can thus be effectively minimised (Kremer et al. 2018).

Ectotherms in general respond to thermal variability following a phenomenon known as Jensen's inequality. There, the response of a system (e.g. individuals, communities) to constant conditions is different from its mean response to variable conditions (Vasseur et al. 2014). Environmental fluctuations affect plasticity, the effect of plasticity on evolutionary dynamics, and the way plasticity itself evolves (King and Hadfield 2019): in predictably, slowly changing environments and in constant conditions, plasticity is low, while plasticity evolves more and is advantageous in rapidly fluctuating environments (Lande 2009). Most theoretical models infer a cost of plasticity (Chevin and Lande 2010; Siljestam and Östman 2017), as it is likely that plasticity cannot increase infinitely. Costs of plasticity as measurably lower fitness or similar trade-offs in highly plastic individuals could limit benefits in biologically more realistic settings (Auld et al. 2010; Leung et al. 2020; Botero et al. 2015).

As any other trait, phenotypic plasticity can evolve in response to natural selection (Massimo Pigliucci 2005). Evolution of plasticity has been studied with experimental and modelling approaches in relation to environmental predictability (Lande 2014; Hallsson and Björklund 2012). However, there is to date no explicit test of the role of differences in the predictability of frequency and amplitude of environmental fluctuations on phytoplankton fitness and phenotypes. In addition, the evolution of plasticity, and therefore evolutionary trajectories affected by selection acting on plasticity, can be strongly impacted by previously experienced environmental conditions (Leung et al. 2020). This raises the question: when populations have already experienced a certain degree of environmental unpredictability, will the strength and direction of evolution, including the evolution of plasticity, be determined by external forcing (here, temperature fluctuations) or ecological feedback (here, evolutionary past)? To address these questions, I established a long-term selection experiment with a full factorial design of predictable or unpredictable amplitudes and frequencies of thermal fluctuations, using the model picoplankton: *Ostreococcus spp*. I used two species of *Ostreococcus* spp., *O. tauri* and *O. mediterraneus,* both isolated from the Southwestern Baltic Sea. Our isolates of *Ostreococcus* spp. were collected in two thermally distinct regions in the Southwestern Baltic, with naturally different levels of thermal predictability (Santelia et al. 2022, preprint). I tested

the contributions of past (predictability of fluctuations and ecology in geographical origins) and recent (predictability of fluctuations in the selection experiment) environmental changes on i) speed and magnitude of evolutionary trajectories, ii) strength of ancestral and evolved plastic responses, iii) changes to phytoplankton on short, metabolic timescales.

Methods

Experimental design and strains culturing

5 surface water strains, 3 from the Kiel Area and 2 from the Bornholm Basin, (derived from single cells isolated from samples collected at different sampling locations) of *Ostreococcus* spp. (both *O. tauri* and *O. mediteranneus*, see Table 2.3.1) were isolated from two regions of the Baltic Sea (i.e., 3 strains from the Kiel Area, warmer and less predictable and 2 from the Bornholm Basin, cooler and more predictable (Zhong et al. 2020) from 2 successive cruises in these areas (see Supplementary Information Study II for a detailed description of the isolation procedures).

O. mediterraneus was described as a new species by Subirana et al. (2013), noting it was morphologically identical to *O. tauri* but showed differences at the level of the karyotype. The two species have been proven to have similar physiological responses in terms of growth rates in response to light exposure (Six et al. 2008). Here, cultures were kept in common garden conditions (at 18°C and 100 µmol photons in a culture chamber, monitored externally) between 6 months and 1 year, depending on the day cultures were isolated and established as clonal cultures prior to the start of the experiment. To identify upper and lower limits for the fluctuations, and to make sure that differences in time spent in the common garden had not systematically elicited differences in phenotype, I carried out a pilot study investigating thermal performance curves for all samples used in this study.

To test the impact of the predictability of frequency and amplitude of thermal fluctuations) on evolutionary trajectories and the evolution (or loss) of plasticity, I established 5 selection environments (Fig. 2.3.1; see table S2.3.1 for detailed description of the conditions during the experiment):

1. A control treatment (CC) with constant temperature conditions of 22°C (an on average beneficial condition, as determined by pilot studies)

2. A completely unpredictable treatment (RR), where temperature changed unpredictably with regards to amplitude frequency. The amplitude stayed within a range between 15 and 26°C and frequency (i.e. time of exposure to each unique fluctuation, between 4 and 11 generations (frequency calculated as generation time to improve comparison between differently growing strains and replicates).
3. A completely predictable treatment (PP), where the frequency and the amplitude were kept constant. Cultures were grown for 10 generations at different temperature steps (15-22-24-26°C). The set-up was repeated through the entire selection experiment.
4. A treatment, where the frequency was kept unpredictable, but the amplitude, predictable (RP). As a result, samples spent a random number of generations spent in each (predictable) temperature
5. A treatment, where the amplitude was kept unpredictable (but again stayed between 15 and 26ºC), but cultures spent the same number of generations (10) at those temperatures (PR)

I kept the cultures in exponential growth by transferring them every 14 days in new f/2 medium and with a starting concentration of approx. 3000 cell/mL (diluted enough to avoid stationary phase; Fig. S2.3.1). All cultures were kept at the same light irradiance of 100 µmol photons m^{-2} s^{-1} on a 12:12 light:dark cycle with constant shaking (60 rpm). Temperatures were also monitored externally . Below, I describe the experimental procedures associated with each research question. For each method, I provide information on how the respective statistical tests were carried out in that same section.

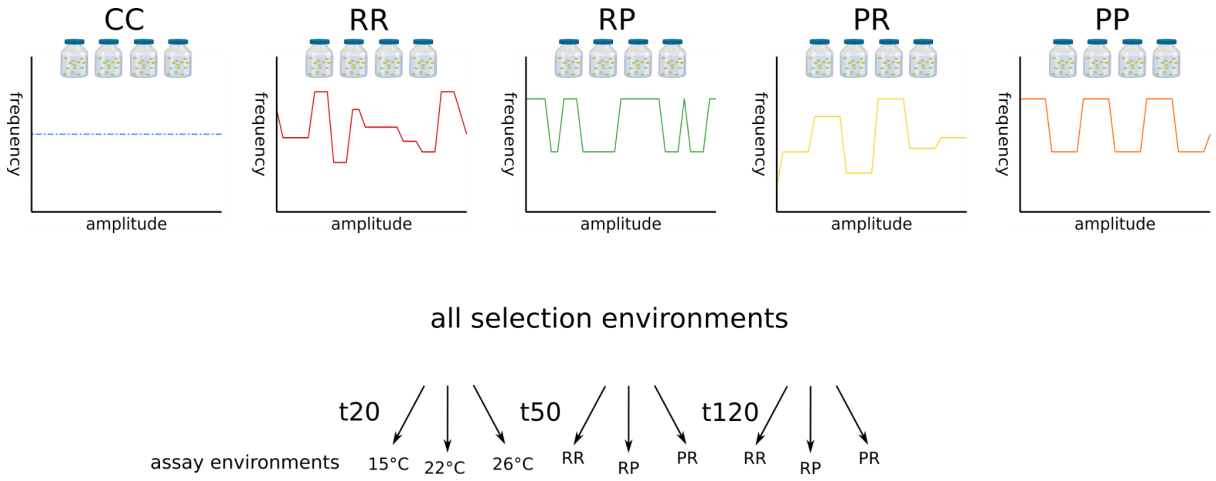


Figure 2.3.1. Experimental set-up. 4 biological replicates of 5 strains were grown in 5 different treatments. Growth rates were assessed during all the experiment, while metabolic measurements were taken after 20 (t20), 50 (t50) and 120 (t120) generations. At t20 direct plastic responses were assayed at 15,22 and 26°C. At t50 and t120 short-term plastic responses for all treatments were assayed in the RR, RP and PR treatments.

Table 2.3.1. Summary table with information regarding the strains used during the experiment.

Internal UHH Strain ID	***Species***	***Geographical region of isolation***	***Cruise ID / Date***
19	*O. tauri*	Bornholm Basin	AL505 / March 2018
12	*O. mediterraneus*	Bornholm Basin	AL524 / July 2019
13	*O. mediterraneus*	Kiel Area	AL524 / July 2019
21	*O. tauri*	Kiel Area	AL505 / March 2018
30	*O. mediterraneus*	Kiel Area	AL505 / March 2018

Growth rate and metabolism measurements

I took cell counts to measure growth rates (and generation times) every three days. A 100µL aliquot of each sample was taken and cells counted using a flow cytometer (Accuri C6, BD). Growth rates were calculated as running mean over three days using the following formula:

$$\mu = \frac{lnN_{tf} - lnN_{ti}}{\Delta t}$$

Where N_{tf} is the population density at the end time point, N_{ti} is the population density at the initial time point and Δt is the time in days between the two time points.

Generation time was calculated as:

$$number\ of\ generations = \frac{\Delta t}{(\frac{0.6931}{\mu})}$$

Where Δt is the time in days between the two time points and 0.6931/µ gives an estimation of the doubling time based on the growth rate and assuming mortality is 0.

A Generalised Additive Model (GAMM) was fitted to the growth rate trajectories due to the different shape of the trajectories across treatments. The model was fitted using the "gamm4" package in R (v. 0.2.6). I treated the 4 biological replicates as random effects on the intercept and treatment, geographical area of sampling and species as fixed effects on both intercepts and the smoother term. The best model was selected using Akaike Information Criterion Score (AICc), with the best model having the lowest AICc score and more than 2 ΔAICc score. Model comparison was performed using the package "MuMIn" (v. 1.43.17).

I used growth rates of single isolates from the predictable treatment (PP) over each week of the experiment to perform a segmented regression analysis. This allows us to assess, in statistical terms, the dependence of growth rate change on temperature when fluctuations are constant. The analysis was performed with the "segmented" package (v. 1.3.4) in R.

At fixed generation steps (after 20, 50 and 120 generations) I measured metabolic responses. I chose to account for generations rather than time to ensure comparable responses within the samples that showed different growth rates. Metabolic measurements (net photosynthesis, P and respiration, R) were taken using a PreSens optode (SDR SensorDish Reader, PreSens). I transferred 2 mL of each sample into a glass cuvette, provided with a sensor spot applied at the bottom. The cuvettes were tightly sealed with parafilm and placed in incubators set to the

assay temperatures of 15, 22, 24 and 26°C. After samples had reached the assay temperature (approx. 10 minutes), the cuvettes were placed in the dark for 20 minutes. After this time, photosynthetic measurements took place for 25 minutes and respiration was measured in the dark for 25 minutes. The cuvettes were gently shaken in between all the steps to ensure no sedimentation occurred. Responses were recorded using the SDR software (v. 4.0.0) as µmol/L. We previously found that salinity affects measurements. To ensure standardisation between our measurements, I used salinity controls with only media each time, and used the obtained values in the light and in the dark for normalisation.

Reciprocal transplants

After 20 generations of selection, I exposed the samples to constant temperatures framing the fluctuations (15, 22 and 26°C) for 20 generations. This allows us to test whether, after 20 generations under fluctuations, samples from different selection regimes differ in the plasticity of growth in response to temperature.

After 50 and 120 generations, samples from each treatment were exposed for 20 generations to three different treatments (RR, RP and PR) where at least one of the components of fluctuations was kept unpredictable. Thereby, I can test whether the samples show signs of local adaptation to (un) predictability in and on itself.

All transplants were conducted in plastic flasks and samples were transferred every 14 days into fresh f/2 medium. Growth rates were measured every three days as described before. I measured metabolic responses after 20 generations following the protocol explained earlier. I calculated short-term plastic responses as (Equ.1) according to Schaum and Collins (2014):

$$short - term\ plastic\ responses = \frac{\mu\ selected\ responses\ in\ assayed\ environment - \mu\ evolved\ responses}{\mu\ evolved\ responses}$$

Equ. 1

Data were statistically analysed using linear mixed effect models in the R environment with the lme4 package. The model was built considering selection treatments, geographical area and species as fixed effects. Strain, biological replicates and selection treatments were considered as random effects (nested).

Results

Evolution but not adaptation: growth rate trajectories

Growth rate (μ day^{-1}), as a proxy for fitness, showed a weak increase over the duration of the experiment, and this trend was shared by all treatments, geographical regions and species (Fig. 2.3.2) except for the control treatment, which showed a weak decrease. Regression slopes from models analysing the slope from the beginning (t0) to the end point (t120) of the trajectories, showed the strongest positive trend for *O. mediterraneus* samples from the Bornholm Basin (Fig. 2.3.3; Table S2.3.2). There, the variance of the growth rates in a week-by-week comparison was however larger than the increase in growth from beginning to end.

In addition to comparing the ancestral fitness (growth at t0) to the fitness of evolved samples in the selection environment, I also tested for differences in the shapes of the growth rate trajectories. To do so, I fitted a Generalised Additive Model (GAMM), and found a significant effect of species, treatments and geographical area of origin on both the intercept and the shape of the growth rate trajectories (Table S2.3.3). This means that while all samples evolved to similar endpoints, they got to these endpoints *via* different strategies. In the control treatment, apart from a 1.13 fold increase in growth rates at the beginning (i.e. within the first 20 generations) in *O. tauri*, I detected no significant changes in growth. In the fluctuating treatments, growth rate trajectories were similar to each other, with the only substantial difference in the *Ostreococcus mediterraneus* strains of the Bornholm Basin, which showed a clear evolutionary rescue pattern in the Random frequency/Predictable amplitude treatment. There, about 10 weeks (approximately 20 generations) into the experiment, growth rates abruptly collapsed down to 0.03 divisions day^{-1} ∓ 0.12, and were restored to 0.47 divisions day^{-1} ∓ 0.02 after approximately one month. This was not an isolated case, with growth rates of maximum 2 out of 3 biological replicates of each strain in all treatments, approaching zero but for a shorter period of time.

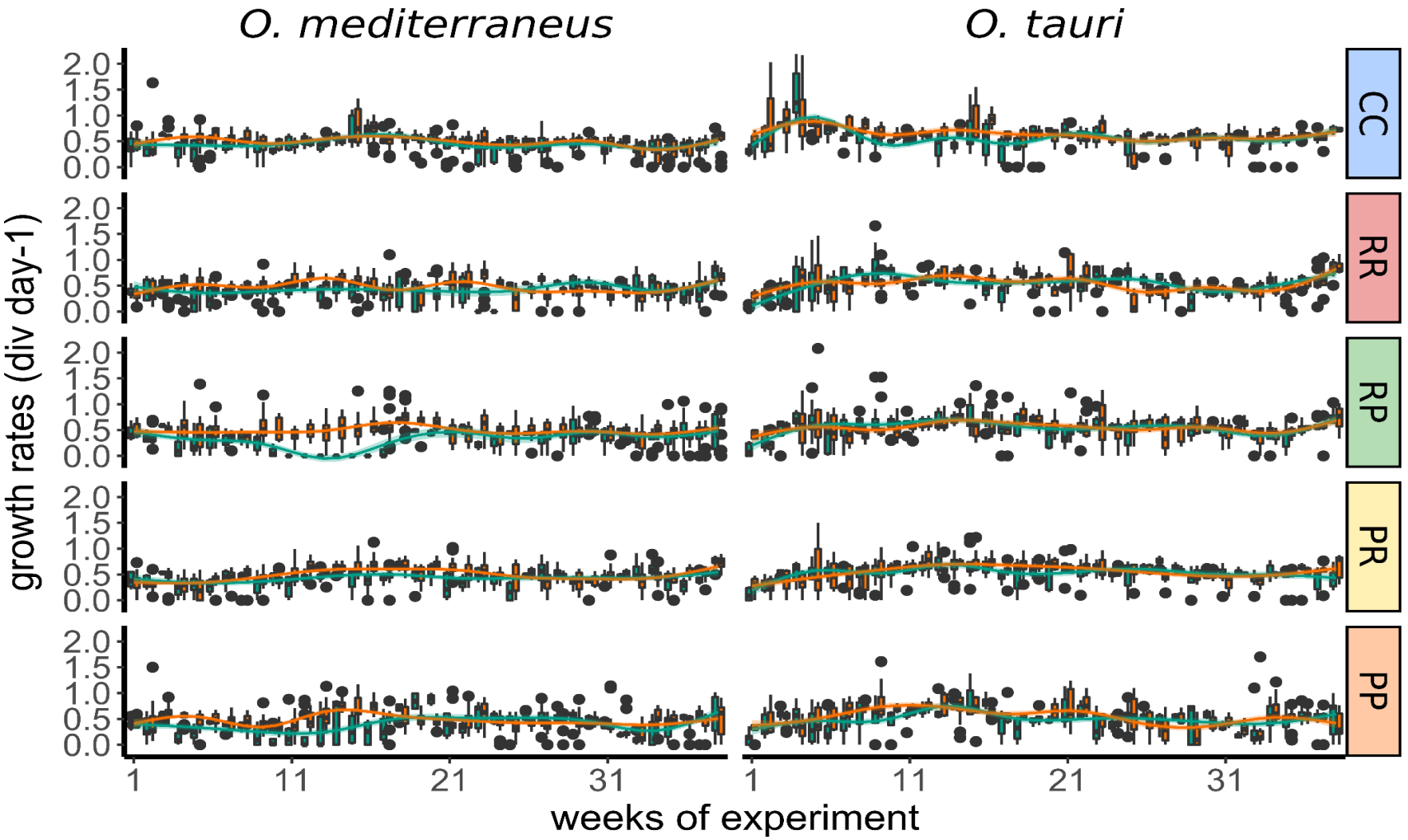


Figure 2.3.2. growth rate trajectories. Time series (over weeks of experiment) of growth rates for each of the treatments with different levels of predictability of fluctuations and for both tested species. Abbreviations stand for: Control (**CC**), unpredictable frequency and amplitude (**RR**), unpredictable frequency predictable amplitude (**RP**), predictable frequency unpredictable amplitude (**PR**) and predictable frequency predictable amplitude (**PP**). Boxplots are displayed as is standard, and were created pooling growth rates across replicates (n=4) and strains. Fitted lines are from a GAMM model with shaded areas indicating the 95% confidence intervals. Colours of the boxplots and fitted lines refer to the two geographical areas considered (orange for Kiel Area and blue for Bornholm Basin).

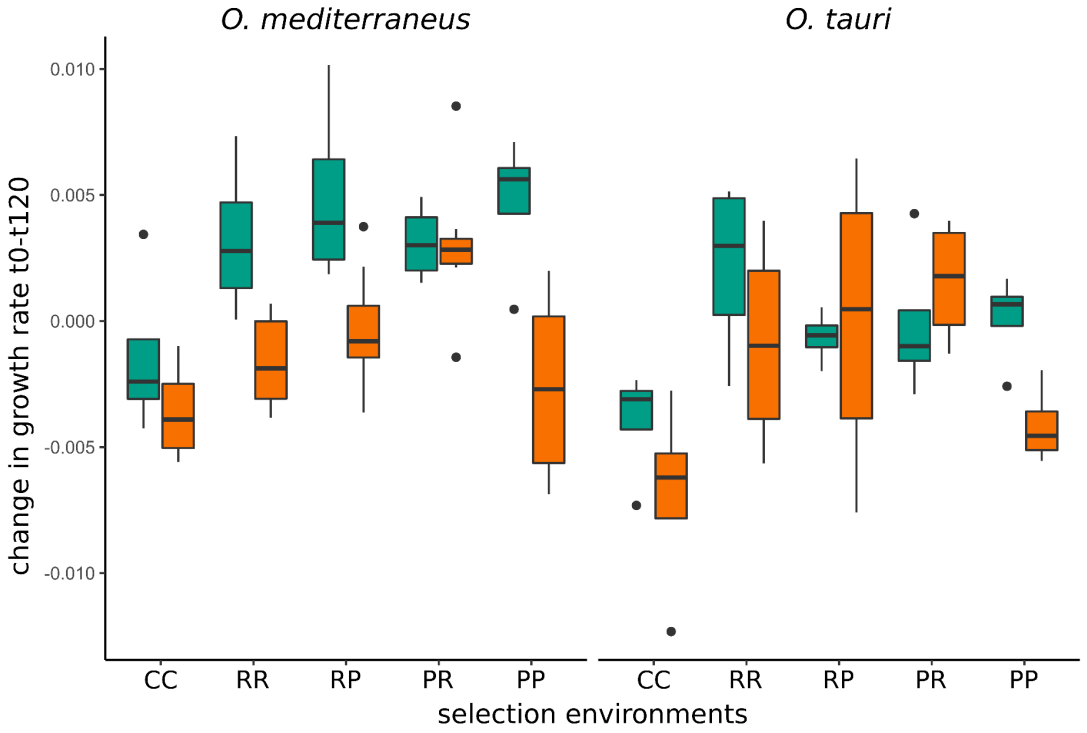


Figure 2.3.3. Regression slopes estimated from a linear model (formula here) fitted across growth rate trajectories (see Fig. 2.3.2, from t0 to t120) and pooling replicates (n=4) and strains (n=5 in total). Different treatments are represented on the x-axis and colours represent the geographical area of sampling (orange for Kiel Area and blue for Bornholm Basin). Abbreviations stand for: Control (**CC**), unpredictable frequency and amplitude (**RR**), unpredictable frequency predictable amplitude (**RP**), predictable frequency unpredictable amplitude (**PR**) and predictable frequency predictable amplitude (**PP**).

Evolution but not adaptation: metabolism

To test whether the small overall increase in fitness, and particularly, significant differences between species and regions could be explained by differences in the plasticity of metabolic rates, I measured net photosynthesis (NP) at four different assay temperatures (15, 20, 22 and 26°C) after 20, 50 and 120 generations of selection. The assay temperatures covered the full range of temperatures of the fluctuating selection environments. For all samples from all selection regimes at t20, and for Bornholm Basin strains at t50, metabolic responses were following the expected hump-shaped trend. After 120 generations of selection instead, significant differences in metabolic rates attributable to selection regimes emerged, regardless of geographical area of origin and species: In the RR (completely unpredictable) treatment,

rates of NP were noticeably stable across all the analysed temperatures and rates were on average similar to rates of NP at t20. The RP treatment showed similar trends to the RR treatment (see Table S2.3.4 for the summary of the GAMM model) indicating that random frequencies are more important for metabolic responses than random amplitudes.

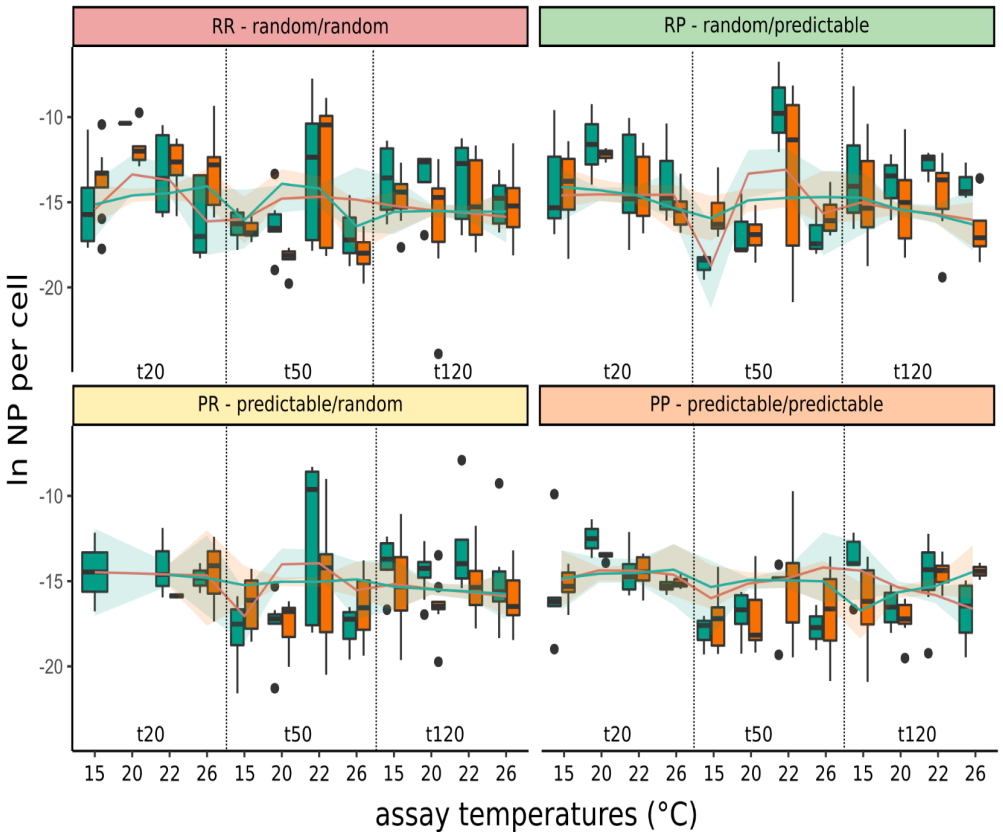


Figure 2.3.4. Logarithmic values of NP (net photosynthesis) over assayed temperatures (15,20,22 and 26°C) for the different treatments (see methods for detailed explanation of the acronyms). Colours indicate the different geographical areas (orange for Kiel Area and blue for Bornholm Basin). The dashed lines delimit the three different time points when the data were collected (t20, t50 and t120). Fitted lines are from a GAMM model with shaded areas indicating the 95% confidence intervals.

Evolution of plasticity: reciprocal transplants

I assayed growth responses of the evolved samples after 20 generations of selection at constant temperatures framing the fluctuations (15 ºC, 22 ºC, and 26°C) for 20 generations. This allows us to assess the magnitude of plastic responses in the short-term and to test whether short-term exposure to environments that differ in the predictability of their fluctuations has an impact on thermal tolerance in general. This is important to test because it gives us an indication regarding thermal constraints, i.e. adaptation to past selection regimes. After approximately 50 and 120 generations, I ran a reciprocal 20-generation assay, where the

evolved samples from all selection regimes were grown in fluctuating conditions with at least one unpredictable component (RR,RP and PR) and calculated growth rate responses. This yielded information on whether evolution in an environment with a specific fluctuation pattern can affect the way phytoplankton behave when the type of fluctuation changes. This set-up also allowed us to test how plasticity changes over time, because the 20 generations of exposure of the reciprocals to the respective assay conditions allowed us to infer plastic responses at different time points of the selection regime (t50, t120). I expected a higher degree of plastic responses at t50 than at t120, when evolution should have a stronger effect.

Thermal tolerances of growth after 20 generations of selection, t20, showed significant differences at the treatments level (ANOVA $p = 0.01$, $F_{4,172} = 3.26$). In the RR and PR-evolved strains, growth rates at 22°C, which represented the baseline control temperature, were 1.29 ∓ 0.07 higher than ones measured at the other two temperatures. Instead, in the RP and PP-evolved populations, growth rates were similar through all the assayed temperatures (Fig. S2.3.2 C and E).

In the samples evolved in the completely unpredictable scenario (RR), plastic responses at t20 at 22°C were 1.29 ∓ 0.07 higher than the ones measured at the other two temperatures. 22°C represented the average growth temperature for RR evolved samples before the assays were conducted (approx. maximum treatment, 4th) (Fig. S2.3.2 B).

In the RR-evolved populations at t50, only *O. mediterraneus* from the Bornholm Basin showed a surge in growth rates when exposed to PR and RP treatments ($p = 0.008$, t value = -2.81; Fig. 2.3.5 A). For the RP-evolved *O. mediterraneus*, I found reduced plastic responses already at t50, with most samples from the Bornholm Basin already collapsing when exposed to differently predictable environments (Fig. 2.3.6 A).

Repeating the assay after 120 generations of selection, yielded different results. In the RR-evolved populations, 2 of 4 replicates of *O. mediterraneus* from Bornholm died in the assayd conditions after approx. 10 generations (Fig. 2.3.5 A). The other populations did not show any significant change of responses over time. The RP treatments proved to be the most detrimental in terms of loss of short-term plastic responses and exacerbated the situation displayed at t50. There, most of the samples from the Bornholm Basin (regardless of the species) collapsed and did not reach the 20 generations threshold (Fig. 2.3.6). In the PP-evolved, plastic responses increased over time ($p = 0.05$, t value = 2.03) (Fig. 2.3.7).

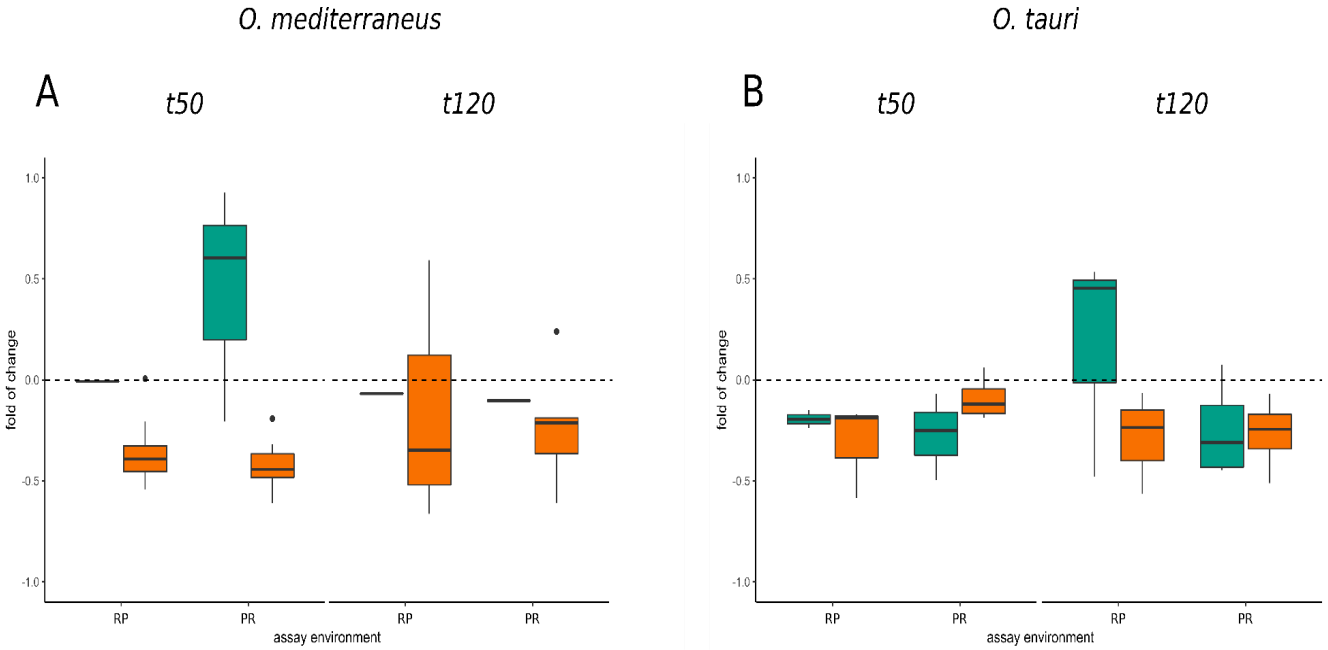
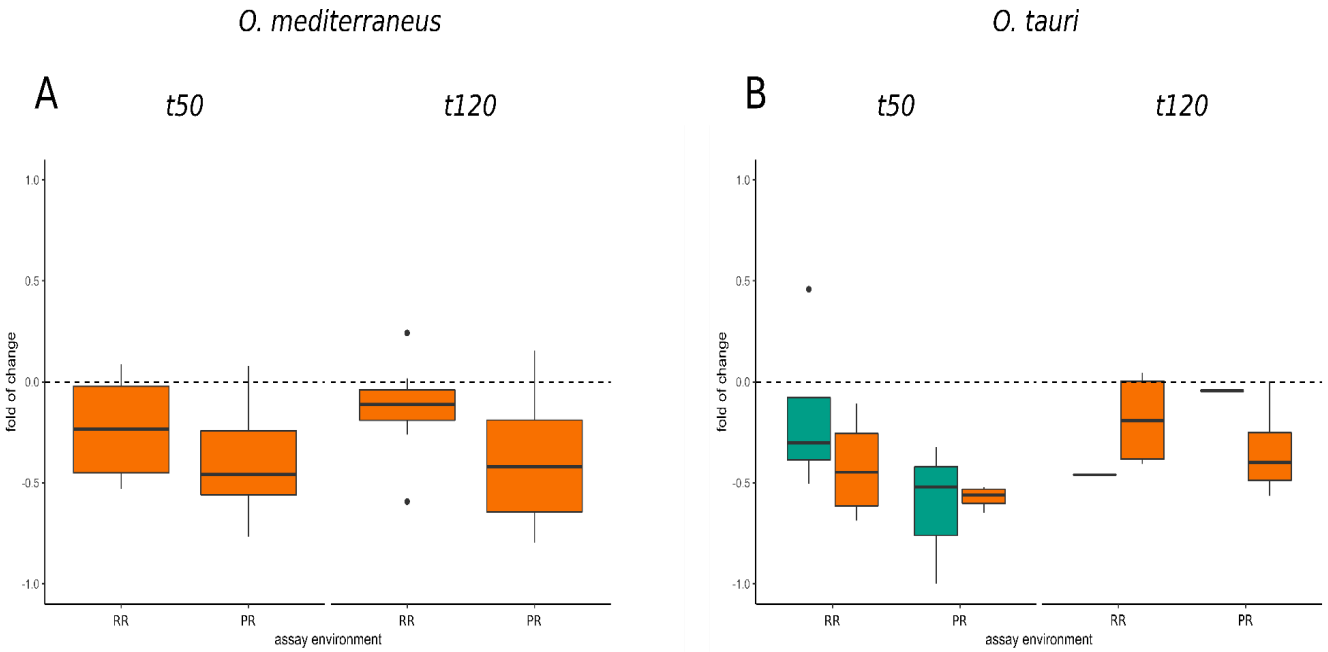


Figure 2.3.5. Short-term plastic responses of RR evolved samples assayed in the RP and SP treatment. **A** panel shows responses for *O. mediterraneus* after 50 and 120 generations of selection, **B** for *O. tauri* after 50 and 120 generations. The dashed line at 0 indicates no changes in the short-term responses. Negative values indicate a decrease in short-term plastic response and positive values an increase. Colours are displayed as for the other plots (orange for the Kiel Area and blue for the Bornholm Basin). Boxplots are displayed as is standard.

Figure 2.3.6. Short-term plastic responses of RP evolved samples assayed in the RR and PR treatment. **A** panel shows responses for *O. mediterraneus* after 50 and 120 generations of selection, **B** for *O. tauri* after 50 and 120



generations. The dashed line at 0 indicates no changes in the short-term responses. Negative values indicate a decrease in short-term plastic response and positive values an increase. Colours are displayed as for the other plots (orange for the Kiel Area and blue for the Bornholm Basin). Boxplots are displayed as is standard.

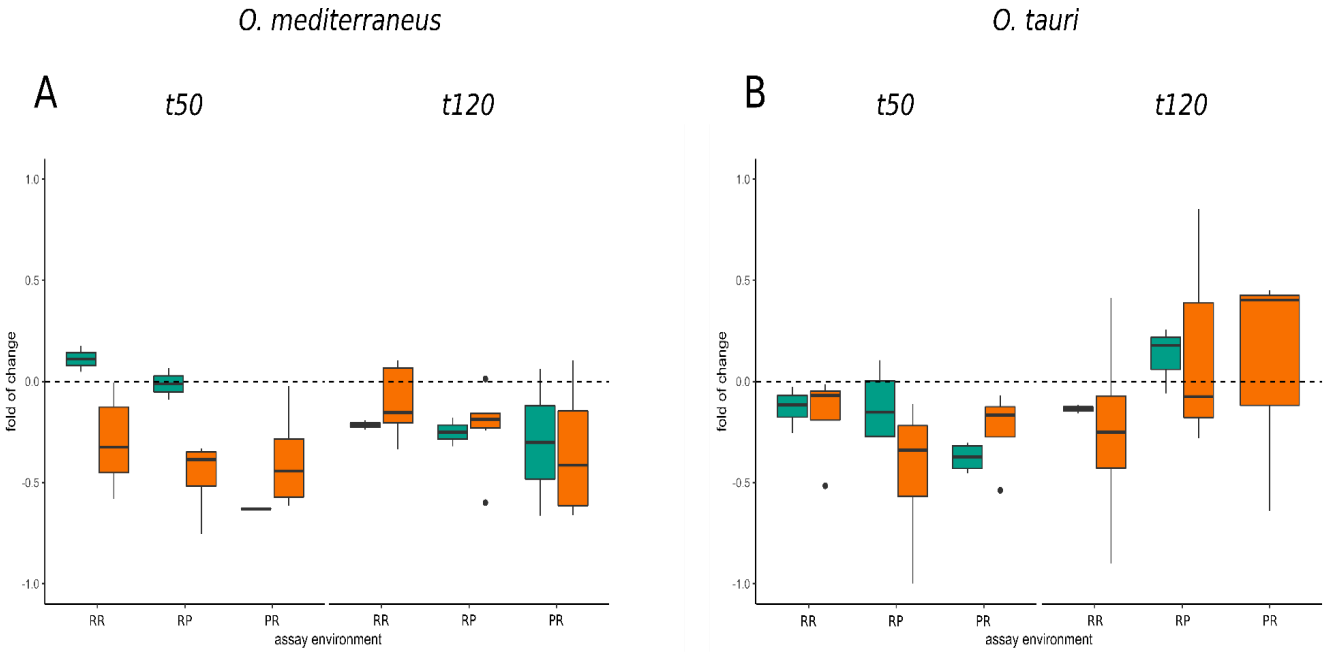


Figure 2.3.7. Short-term plastic responses of PP evolved samples assayed in the RR, RP and PR treatment. **A** panel shows responses for *O. mediterraneus* after 50 and 120 generations of selection, **B** for *O. tauri* after 50 and 120 generations. The dashed line at 0 indicates no changes in the short-term responses. Negative values indicate a decrease in short-term plastic response and positive values an increase. Colours are displayed as for the other plots (orange for the Kiel Area and blue for the Bornholm Basin). Boxplots are displayed as is standard.

I compared short-term plastic responses measured at t50 and t120 of the evolved and assayed treatments. Plasticity at t50 did not display correlations with plasticity at a later point in time, indicating that evolution of plasticity at a previous point in time, is not predicting the evolution of plasticity in a fluctuating environment at a later point in time (Fig. S2.3.4). The samples evolved in the control condition also showed a similar trend.

Discussion

Evolution but not adaptation: growth rate trajectories and metabolism

I did not detect a substantial or significant increase in fitness (here, described as growth rates) during the experiment in any of the treatments. Under control conditions (CC treatment), growth rates also did not change throughout the duration of the selection experiment showing that in fairly 'fresh' natural isolates, adaptation to laboratory conditions may either be happening on a longer time scale or happen very quickly, i.e. before the selection experiment was started. In prior experiments (see Study I) conducted for immediate physiological (within the same generation) responses, I showed that community samples coming from the more unpredictable Kiel Area were able to widen their metabolic thermal tolerances on seasonal time-scales. Short-term responses (spanning from 1 to 20 generations; Study II) proved that samples from the Kiel Area were more plastic in terms of fitness, too. Plasticity is argued to facilitate evolution (Levis and Pfennig 2016) when helping maintain high fitness (and thus higher population size) and increasing the pool of organisms and genes selection acts on. While our previous studies suggested that there should be selection for higher (i.e. Kiel Area) or lower (i.e. Bornholm Basin) leves of plasticity, we did not detect any differences in terms of fitness´ plasticity or growth rate values between samples. Slow growth rates are not commonly related to adaptive mechanisms, but a growing body of literature indicates slow growth as an advantageous mechanism. Growing slowly may help reduce damages in daughter cells and prevent nutrient limitations (Sinead Collins and Schaum 2021; Lindberg and Collins 2020). Preliminary results from measurements of mitochondrial potential, an indirect measurement of cellular stress (C. E. Schaum and Collins 2014), also pointed toward a generally low level of stress. I observed that, particularly in the Random/Random and Random/Predictable treatments, values of mitochondrial membrane potential returned to initial values (Fig. S2.3.5). Stable phenotypes (i.e. growth rates and metabolism) over time in fluctuating conditions, may be due to either plasticity being costly to maintain (especially in unpredictable environments Reed et al. 2010) or could be caused by phenotypic buffering (M. Pigliucci and Kaplan 2010), when a change in the environment causes no changes in a trait.

Phenotypic buffering should select for organisms able to maintain cellular physiological processes and metabolism at levels beneficial enough to cope with changing conditions rather than to outcompete faster growing organisms. In this case, I would expect metabolism to be maintained at rates similar to what organisms showed at the beginning of the experiment. I suggest that the pattern of slow growth I found is mostly related to the non-detrimental thermal conditions the organisms faced and phenotypic buffering phenomena. In my experiment, the only stressful conditions were - potentially - the fluctuations themselves. The chosen range of temperatures spans from suboptimal conditions (where growth rates were approx. 0.5 ± 0.1 divisions per day for all samples), to slightly supra-optimal temperatures (where growth rates were about 25% less than peak rates). I argue that if temperature conditions are not stressful, the environment selects for low growth due to costs of maintaining high growth rates and absence of risks to the survival of the population imposed by the environment. Nevertheless, slow growth could also reflect higher fitness (e.g., if fast growing cells accumulate more damages). In order to fully disentangle the effect of slow growth on fitness, more tests across a fine-grained scale of environmental quality, such as having all tested temperatures as control stable conditions, are needed. Moreover, net primary production (NP) was not declining over time and rates at t120 were restored to ancestral values. In the treatments where fluctuations were unpredictable (RR and RP treatments), NP showed similar values through all tested temperatures after showing a higher level of variation at t50. Reversion of plastic responses is a common strategy (C-Elisa Schaum, Rost, and Collins 2016; Lohbeck, Riebesell, and Reusch 2012) detected in long-term experiments and can be related to the damages imposed by fast increase in trait values (Sinéad Collins 2016). Metabolic rates are a phenotypically plastic trait as well, and one that is directly linked to fitness when carbon is allocated primarily into growth. The decrease of rates at t120 in RR and RP treatments indicated a bet-hedging response, where values in unpredictable conditions are kept similar to each other.

Regression slopes from beginning to end of the growth rate trajectories showed a small, increase in fitness for *O. mediterraneus* coming from the Bornholm Basin for all fluctuating treatments. This increase is not statically significant though, when taking into account that growth varied from transfer to transfer. Even if not significant, this pattern can specifically address the eventual influence of microevolutionary processes. Micro- and macroevolution have varied definitions in literature (Hautmann 2020), but here I consider macroevolution as evolution above the species level and guided by sorting within a population caused by external drivers (in our case, fluctuations) as opposed to microevolution, which instead

requires sorting of intraspecific variation. The differences in the regression slopes of the two species from the two areas, showed an interaction of phenomena on microevolutionary timescales (i.e., few generations), which are masking longer macroevolutionary processes. Interestingly, this result revealed the possibility for organisms of the same genus to display several strategies to overcome environmental changes in the same area, overtaking local adaptation patterns, which would be ultimately determined here by geographical areas.

Evolution of plasticity: reciprocal transplants and habitat tracking

I tested how and if plasticity evolved in response to a change in the fluctuation pattern by calculating short-term plastic responses for growth rates at two timepoints (t50 and t120). To do so, I carried out a reciprocal transplant assay at each of these time points. I found that plastic responses in growth rates in the completely unpredictable treatment (RR) decreased at t50 when fluctuations type changes. Responses remained stable between 50 and 120 generations of selection, with the only two exceptions being *O. tauri* strains from the Bornholm Basin, where responses to the RP treatment increased through time, and *O. mediterraneus* strains from the Bornholm Basin, where plasticity was lost instead . Nevertheless, in both of the latter strains, the increase of plasticity in *O. tauri* and the decrease in *O. mediterraneus* did not influence the long-term growth rates in the selection environment. I suggest that, even if there is a cost associated with plasticity (and maintaining the same plasticity levels in an unpredictable environment), it was not in this case limiting the magnitude of plastic responses. In the RP treatment, where only the frequency of the fluctuations was kept unpredictable, plasticity was completely lost for *O. mediterraneus* strains coming from the Bornholm Basin. I argue that having an unpredictable frequency in the fluctuations pattern might hinder the evolution of plasticity. Under this condition, there might be costs and limits to plasticity for two main reasons: a cost for maintaining plastic responses can be directly associated to information acquisition (Van Tienderen 1991) and a limit can be posed by lag-time between environmental cues and occurrence of plasticity (DeWitt 1998). In fact, when the time available to sense the changing conditions is unpredictable, the mismatch between sensing and responding to the environmental cues may be enhanced and on longer lag-time plasticity can be a disadvantage (Abley et al, 2016). Here, a clear influence of past evolution and ecological constraints is noticeable, since plasticity is indeed lost only for strains that already evidenced a lower degree of phenotypic plasticity and are coming from a more thermally predictable area (Santelia et al, 2022, preprint).

Organisms subjected to completely predictable fluctuations, instead evolved a higher degree of phenotypic plasticity. In addition to noticeable increases in the short-term plastic responses, I also found evidence for habitat-tracking in *O. tauri* strains, probably guided by plastic responses. When the environment changes in a predictable way, with predictable amplitude and frequency, organisms may have time to sense the environmental cues and respond accordingly, "tracking" and even anticipating changes. I used a segmented regression analysis approach (see methods) to identify changes in fitness related to experienced temperature. I analysed each replicate (see Fig. S2.3.6 in supplementary for analysis and plots for each replicates) individually because all samples evolved independently and were subjected to temperature changes regarding their generation times. Growth matched remarkably well with selection regime temperature in *O. tauri,* in the completely predictable selection environment. Mean growth rates decreased at 26°C (a slightly supra-optimal temperature) and increased when facing lower temperatures (15-22-24°C). It is not clear from the results if we can consider this habitat tracking responses to be caused by active plasticity, with a precise anticipation of the environmental cues, or mostly by passive plasticity, determined by changes in the phenotype imposed by the environment (Kurashige and Callahan, 2007). Increase in plasticity when the environment is predictable, is in accordance with previous experimental and modelling studies, which suggest that plasticity should be more favourable than other processes, such as bet-hedging, in more predictable environments (Tufto 2015; Botero et al. 2015).

Conclusions

In conclusion, I hypothesise that a high degree of phenotypic plasticity evolves under fluctuations that are predictable in both frequency and amplitude, while unpredictable frequency and amplitude of the fluctuations do not select for high plasticity. Regardless, maintaining a stable level of plasticity was not associated with a high cost either. Moreover, fluctuating conditions were detrimental mostly when the frequency is unpredictable on timescales of 4-11 generations and there is not a fixed time to sense and adjust to changes, in contrast with the genetic assimilation theory, which is described as the expression of a phenotype determined by an environmental cue, even when the stimulus is not evoked (e.g., (Braendle and Flatt 2006).

Interestingly, plasticity evolved differently in growth rates and metabolic traits. In the latter, plasticity strongly decreases in the RR and PR treatments at t120 in response to different

fluctuations patterns. Growth rate is an emergent trait that could be strongly influenced by synergies with other traits and trade-offs with metabolic responses can be present. These results indicated that physiological traits other than growth rates, may behave differently from growth rate and from each other. In order to better understand the correlation between multiple phenotypic traits, I analysed trait-scape trajectories (see Study IV). This allows us to explain complex interactions within traits using an intuitive approach and can thus crucially contribute to our understanding of evolutionary trajectories and phenotypic movements in response to changing environmental conditions.

My results offer a novel perspective on phytoplankton responses in fluctuating environments. I proved that the predictability of frequency and amplitude of the fluctuations, greatly influence the evolution of phenotypic plasticity. This proves particularly important for predicting population persistence under and different strategies used to cope with climate change. Monitoring data are often readily available for many marine regions, so that natural regimes of fluctuations (e.g., more frequency or amplitude driven) can easily be related to my experimental set-up and findings. Specifically, a further increase in unpredictability of temperatures at sea might prove particularly detrimental for organisms evolved in environments where frequency of fluctuations is already unpredictable.

2.4 Study IV: Trait-scape and trait correlations over 120 generations

Differences in thermal predictability lead to microevolution of trait values and trait correlations

Maria Elisabetta Santelia, Jana Hinners, C-Elisa Schaum

Abstract

The trait-scape approach has been recently used to explore phenotypic plasticity and how much it contributes to evolutionary dynamics. Here, we isolated strains of *Ostreococcus* spp. from areas of the Baltic Sea with different thermal predictability and investigated how phenotypes vary in response to past and present predictability of thermal fluctuations and the direction and strength of trait correlations through time. We used data collected during a long-term selection experiment (ca. 120 generations) after 20, 50 and 120 generations of selection. We isolated strains of *Ostreococcus* spp. from areas of the Baltic Sea with different thermal predictability. *Ostreococcus* strains were allowed to evolve in 4 selection regimes with different levels of predictability of frequency and amplitude of thermal fluctuations. We found that long-term evolution (here, evolutionary history as imposed by characteristics of the sampling location) constrained the trait-scape in respect to present (selection treatment) characteristics of fluctuations . Trait correlations were influenced by selection treatments, with lower correlation strength found in the more predictable environment. Our findings demonstrate that evolutionary history can limit plastic responses and can therefore differentially shape evolutionary trajectories.

Introduction

Phytoplankton are ubiquitous and they play a pivotal ecological role (Falkowski 1994). However, our understanding of phytoplankton physiology derives primarily from responses in predictable or constant environments.

Fluctuating environmental conditions have the potential to change the direction and pace of evolutionary trajectories. For example, experiments show that in the long-term thermal fluctuations could lead to more generalistic individuals (e.g., Ketola et al. 2013). Also, depending on the speed and predictability of fluctuations, plastic responses (i.e. the ability of a phenotype to change according to the environment) can vary through time and the topic regarding evolution of plasticity arose in conspicuous debates as reviewed by Bitter et al. (2021).

Studies (e.g.,Botero et al. 2015; Lande 2009) demonstrated that predictability of the environmental factors plays a role in the plastic responses arising in the long-term. Specifically, stronger plastic responses evolve in predictable environments, whereas a lesser degree of plastic responses is expected in highly unpredictable environments.

However, evolution is not a tale with one main character (growth rate); instead, organisms can be characterised by a plethora of functional traits with different reaction norm shapes (Litchman and Klausmeier 2008) which together can influence fitness in ways that may not be easy to disentangle. Even when there is only one main selective driver, trait correlations can be positive or negative. Well-studied trade-offs usually trade one trait against the other, for example as in trade-offs between cell size and nutrient affinity (Litchman 2022; Lindemann et al. 2016). Nevertheless, the existence of a three-way trade-off was demonstrated, showing the multidimensional nature of trait correlations (Edwards et al. 2011). Trade-offs and trait correlations have the potential to constrain evolution of phenotypes (Blows and Hoffmann 2005). A key insight from multivariate trait correlations can be to explain the ecological success of species, linking fitness to phenotypic traits.

Multivariate trait-based approaches allow us to capture the complexity of phenotypes including multiple traits and trait correlations (Walworth et al. 2021; Hinners et al. 2022). In order to visualise the movements of phenotypes, i.e. the way that phenotypes change through time in response to changed or changing environments, on as few axes (or dimensions) as possible, we used a principal component analysis (PCA) to create a "trait-scape" (Walworth et al. 2021; Argyle et al. 2021). Even if recently some pivotal studies (Hinners et al. 2022; Argyle et al. 2021) started to apply multivariate approaches to explore covariance between traits, there is a paucity of observations on phenotypic movements in complex environments. Here, I investigated the movements of phenotypes in the trait-scape of different strains of two picophytoplankton species, *Ostreococcus tauri* and *Ostreococcus mediterraneus,* evolving in four environments characterised by different levels of predictability in the timing and amplitude of temperature fluctuations. I investigated how multitrait phenotypes evolve through time and if the trait scape approach is applicable to explain multitrait adaptation in *Ostreococcus* spp. I hypothesised that phenotypic movements can be affected by previously experienced thermal variability (i.e., evolutionary histories (Follows et al. 2007) here the species original sampling location) and present selection regimes (here, the selection treatments), with populations from the more unpredictable Kiel Area and evolved under fluctuating conditions, showing more phenotypic movements than population previously adapted to predictable environments. In order to test this assumption, we analysed subsets of strains coming from two thermally distinct areas in the Southwestern Baltic Sea (Kiel Area, warmer and less predictable and Bornholm Basin, cooler and more predictable; (Santelia et al. 2022)) and evolved for 120 generations in differently predictable fluctuating environments. Specifically, in this study I wanted to assess: i) how phenotypes vary in response to past and present predictability of environmental fluctuations ii) If evolving traits will retain the original correlations between them or will the strength of those correlations change as well iii) and if plasticity creates divergent phenotypes or fluctuations impose a stronger constraint on the possible movements.

Methods

Experimental set-up

We performed a long-term experiment, cultivating *Ostreococcus tauri* and O. *mediterraneus* strains coming from two different areas of the South-Western Baltic Sea. Samples were collected during two oceanographic cruises in the Kiel Area and the Bornholm Basin. Cells

were isolated upon returning to the lab. Single cells were propagated on a semi-solid medium (2% agar plates) in order to obtain five different clonal strains.
Samples were exposed to five treatments assessing the effect of predictability of amplitude and frequency of temperature fluctuations on evolutionary and acclimative responses (see Study III for detailed information about the experimental design).
Samples were kept in incubators (INFORS HT, Switzerland) for the whole duration of the experiment (approx. 10 months) at the same light irradiance of 100 µmol photons m^{-2} s^{-1} on a 12:12 light:dark cycle with constant shaking (60 rpm). Samples were kept in semi-continuous batch cultures and 3000 cells mL^{-1} were transferred every 14 days into fresh f/2 media.

Growth rates

Growth rates were assessed every three days using a flow cytometer (ACCURI B6, Bioscience). 100 µL of cultures were transferred into a multiwell plate and cells counting were determined. Thresholds were applied on both FSC (accounting for size) and FL3 (chlorophyll a) signals and pre-determined comparing with already established *Ostreococcus* strains from culture collection. Growth rates were calculated as running mean over three days with the following formula:

$$\mu = \frac{ln\,(N_{tf}) - ln\,(N_{ti})}{\Delta t}$$

Where N_{tf} is the population density at the end time point, N_{ti} is the population density at the initial time point and Δt is the time in days between the two time points.

Collection of flow cytometric traits

We collected the traits at specific time points during the evolution experiment. Specifically, after 20, 50 and 120 generations of selection, to ensure the samples spent the same number of generations in the experimental conditions.
Size (FSC), internal complexity of the cell (hereby referred to as cellular internal complexity. Larger internal complexity reflects in more structures in the cytoplasm), chlorophyll a (FL3, excitation wavelength 488 nm, emission wavelength >670 nm) and phycoerythrin (FL2, excitation wavelength 488 nm, emission wavelength 585/40 nm) content, were collected with a flow cytometer (ACCURI B6, Biosciences). For this purpose, an aliquot of the sample was collected during the light phase (at least two hours after the light switched on) and analysed applying the same threshold used for the growth rates measurements. Values were normalised by size to account for differences.

Mitochondrial health: Rhodamine 123 fluorescence.

We stained cells with rhodamine 123 to determine mitochondrial transmembrane potential as a proxy for cellular health (Baracca et al. 2003). Rhodamine fluorescence was detected on the FL1 (excitation wavelength 488 nm, emission wavelength 533/30 nm) channel as green fluorescence on the flow cytometer. We transferred 190 µL of samples into a 96 multiwell plate and added 1µL of a 0.2 µg mL^{-1} of rhodamine solution. Stained cells were left to incubate wrapped in aluminium foil for at least 30 minutes and measurements were conducted in the dark to avoid degradation of the dye. Furthermore, we run the same stained sample twice, at the beginning and at the end of the measurements. FL1 signal of unstained cells was subtracted to the stained cells´ signal to correct for anomalous fluorescence levels and background noise and subsequently normalised by FSC to account for differences in cell size.

Photophysiological measurements

Photosynthesis vs. irradiance (PI) curves relative to PSII electron transfer rates were measured using a LabSTAF fluorometer (FRRf, Chelsea Technologies). We acclimated samples in the dark chamber of the instrument for at least 10 minutes prior to the data collection. We set the instrument to an excitation wavelength of 450 nm and used a single turnover mode, with a saturation phase of 100 flashlets on a 20 µs pitch and a relaxation phase of 40 flashlets of 1 µs on a 50 µs pitch. rP (maximum relative photosynthesis), alpha (photosynthetic rate during the light-saturated curve) and E_k (irradiance at which the onset of saturation occurs) were calculated with the RunStaf software, using the Webb model for the alpha phase (Webb et al. 1974).

Data analysis

Data analyses were performed in the R environment (v. 4.0.2). Principal component analysis (PCA) was carried out using the "vegan" package (v. 2.5.7). Trait data for the PCA were collected from 6 strains (4 biological replicates per strain), from 5 treatments at 3 different time points. The data were standardised with mean = 0 and sd = 1 to account for different measurement units. Loading and scores were extracted using the "factoextra" package prior to plotting. Correlation matrices were done using the "corrplot" package (v. 0.89).

Results

Multitrait evolution in response to temperature fluctuations

To investigate multitrait evolution of *Ostreococcus* spp. in response to environments differing in the predictability of their fluctuations, I characterised all experimental lineages by a total of 9 traits. Measured traits comprise fitness responses (i.e., growth rates) and physiological characteristics (i.e., size, internal cellular internal complexity, phycoerythrin and chlorophyll a content, mitochondrial potential, as well as characteristics of the photosynthesis-irradiance curve: E_k, alpha, and rP). Single trait analyses revealed clear changes in trait values for several traits over time (Supplementary Fig. S2.4.1). For example, cell size and cellular internal complexity decreased over time across strains and selection regimes, whereas the growth rate showed fluctuations. Photosynthesis-related traits (E_k, rPm and alpha) showed little variation.

I combined measurements taken at different time points, after 20, 50 and 120 generations of selection, into one trait-scape (principal component analysis). We expected a larger degree of plastic responses at t20, whereas starting from t50, responses should be largely attributable to evolutionary responses.The first two principal components captured a total of 57.6% of the variation. Consistent with previous studies (Argyle et al. 2021; Walworth et al. 2021), this multivariate approach was effective in encapsulating the majority of variation in the examined traits. The first two dimensions (PC1 and PC2) accounted for similar percentages of the variation (PC1 30.6% and PC2 27%), but different groups of traits were highly correlated with the two dimensions. Specifically, most of the cytometric traits (size, chlorophyll a, phycoerythrin) correlated with PC1, while photophysiology traits (alpha, E_k, rPm) accounted for variations captured by PC2. Instead, growth rates were highly correlated with PC3 (11.9%; Table 2.4.1), indicating that variations in this trait could not be captured accurately in the two-dimensional trait-scape of PC1 and PC2

Table 2.4.1. Trait contributions to PCA dimensions

Traits	Traits Description	PC1	PC2	PC3
Size	Cell diameter	26.41	1.86	0.02
Chlorophyll a	Chlorophyll content	24.85	3.22	0.005
Cellular internal complexity	Granularity	12.93	0.16	1.69
Phycoerythrin	Putative breakdown products of chlorophyll	22.67	3.18	3.07
Rhodamine	Mitochondrial potential, proxy for cellular health	3.53	1.27	25.05
Alpha	Photosynthetic rate during the light-saturated curve	0.92	27.06	0.89
E_k	Maximum irradiance	4.56	28.76	0.003
rPm	Relative maximum photosynthetic rates	4.02	34.21	0.11
Growth rate	Proxy for fitness	0.1	0.31	69.16

Movement within the trait scape in relation to geographical origin

I considered the movements within the trait-scape at different time points, to assess the evolution of multitrait phenotypes in response to predictably and unpredictably fluctuating temperature conditions. In order to simplify the results and thoughtfully analyse the impact of predictability on phenotypic movements, we reported only the two more extreme selection environments, RR (random amplitude and frequency) and PP (predictable amplitude and frequency) treatments and the control environment (CC). Results for the other two treatments are reported in the supplementary information (Supplementary Fig. S2.4.2).

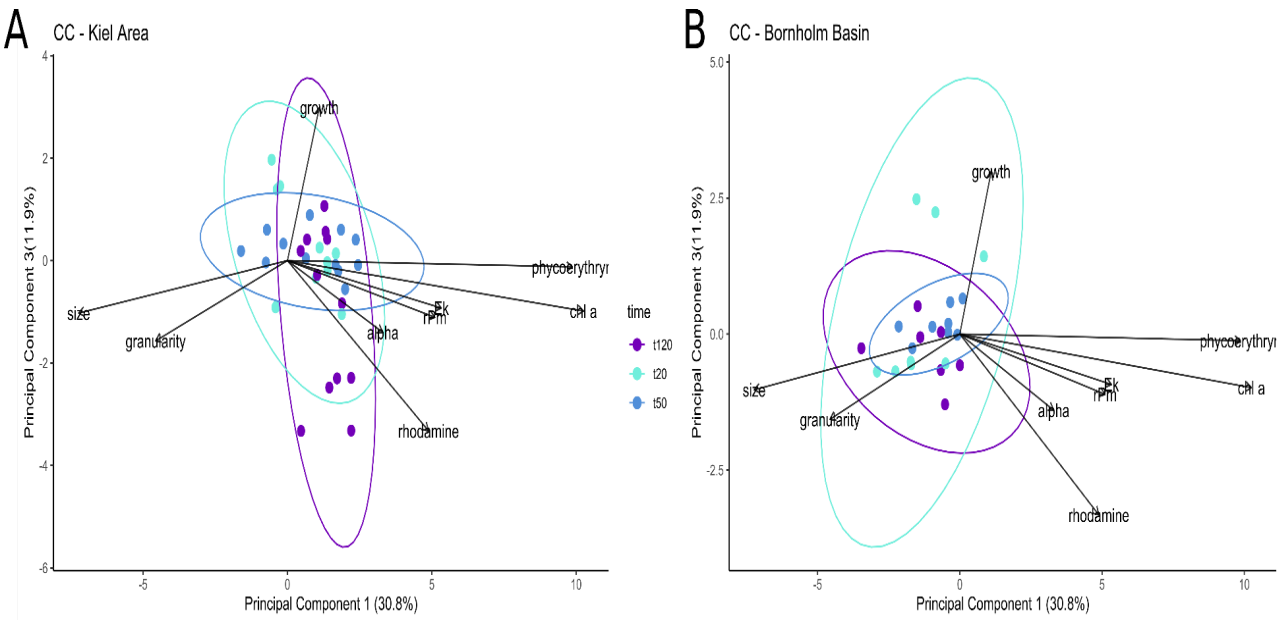
The trait-space within PC1 and PC2 was uniformly occupied, with samples not deviating considerably from the t20 trait-scape across fluctuating treatments (supplementary Fig. S2.4.3 - S2.4.4 - S2.4.5). Thus, based on the trait-scape analysis using PC1 and PC2, strains did not substantially alter phenotypic trait value combinations in response to selection under different thermal fluctuation regimes.

Growth rate is a fitness proxy and explained almost 70% of the variance in PC3. Thus, I also analysed the movements in the trait-scape along PC1 and PC3, which, taken together, explain more than 40% of variation.

In the control treatment (CC), the strains from the less thermally predictable Kiel Area, phenotypically diversified at t120 compared to the other time points. Strains at t120 displayed two different strategies, with populations either developing higher or lower growth rates (Fig. 2.4.1 A). In the samples from the Bornholm Basin, instead, the strains from the control treatment developed reduced phenotypic diversity at t50 and t120 (Fig. 2.4.1 B). Here, the t120 population was characterised by a higher rhodamine signal, which represents a proxy for cellular stress in *Ostreococcus* spp. In the completely unpredictable treatment (RR) in samples from the Kiel Area, strains first developed lower phenotypic diversity at t50, to then expand it again at t120 (Fig. 2.4.2 A). For the Bornholm Basin samples in the RR treatment, we recognized an expansion of phenotypic diversity at t120 along the PC3, corresponding to a diversification of growth strategies (Fig. 2.4.2 B).

In the completely predictable (PP) fluctuating environment, in the Kiel Area the trait-scape showed the same variations we found in the control treatment (Fig. 2.4.3 A). In the Bornholm Basin strains, we did not detect any clear movements in trait-scape, except a contraction of diversity at t50 (Fig. 2.4.3 B).

In summary, while little variation is observed in the PC1-PC2 trait-scape, the analysis of the PC1-PC3 traitscape, which explains more than 40% of variation, reveals different movements



in the trait-scape. Depending on treatment and point in time, we observed diversification and narrowing of growth strategies.

Figure 2.4.1. Trait-scape created from 9 traits. Traits were assessed in samples of *Ostreococcus spp.* in the CC (control) treatment. Trait-scapes are displayed for samples from the thermally unpredictable Kiel Area (A) and from more predictable Bornholm Basin (B). Colours identify the time points at which the traits were assessed (t20, t50 and t120, corresponding respectively to 20, 50 and 120 generations of selection). Ellipses represent the 95% confidence intervals.

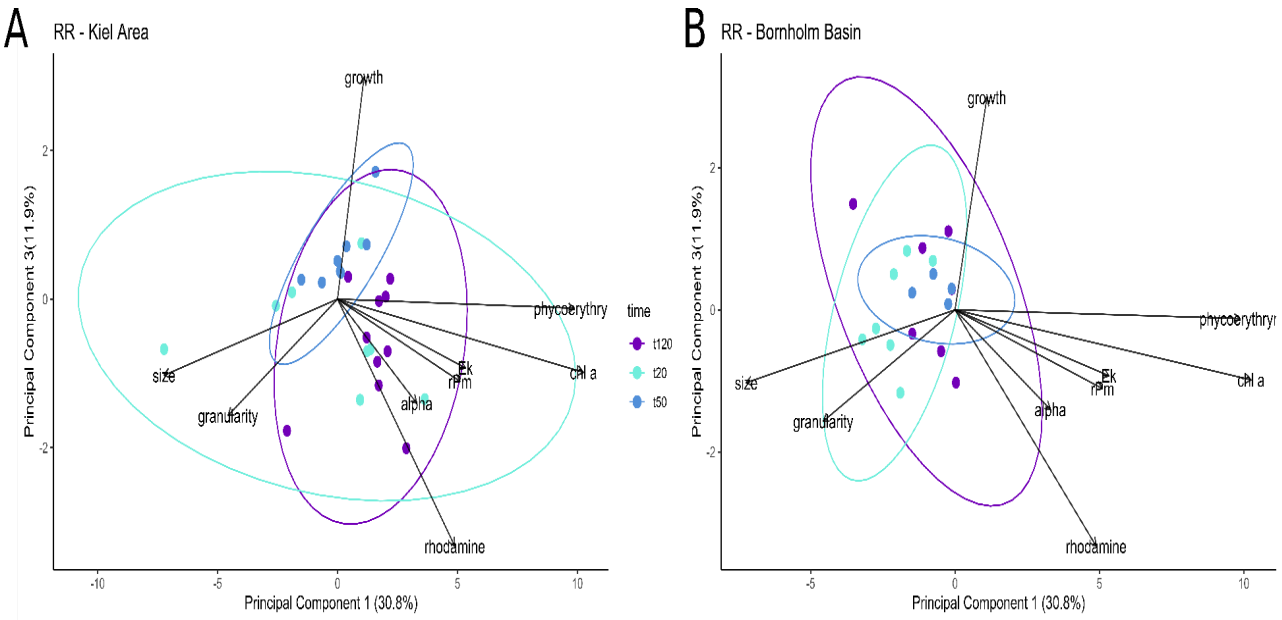


Figure 2.4.2. Trait-scape created from 9 traits. Traits were assessed in samples of *Ostreococcus spp.* in the RR (unpredictable timing, unpredictable amplitude) treatment. Trait-scapes are displayed for samples from the thermally unpredictable Kiel Area (A) and from more predictable Bornholm Basin (B). Colours identify the time points at which the traits were assessed (t20, t50 and t120, corresponding respectively to 20, 50 and 120 generations of selection). Ellipses represent the 95% confidence intervals.

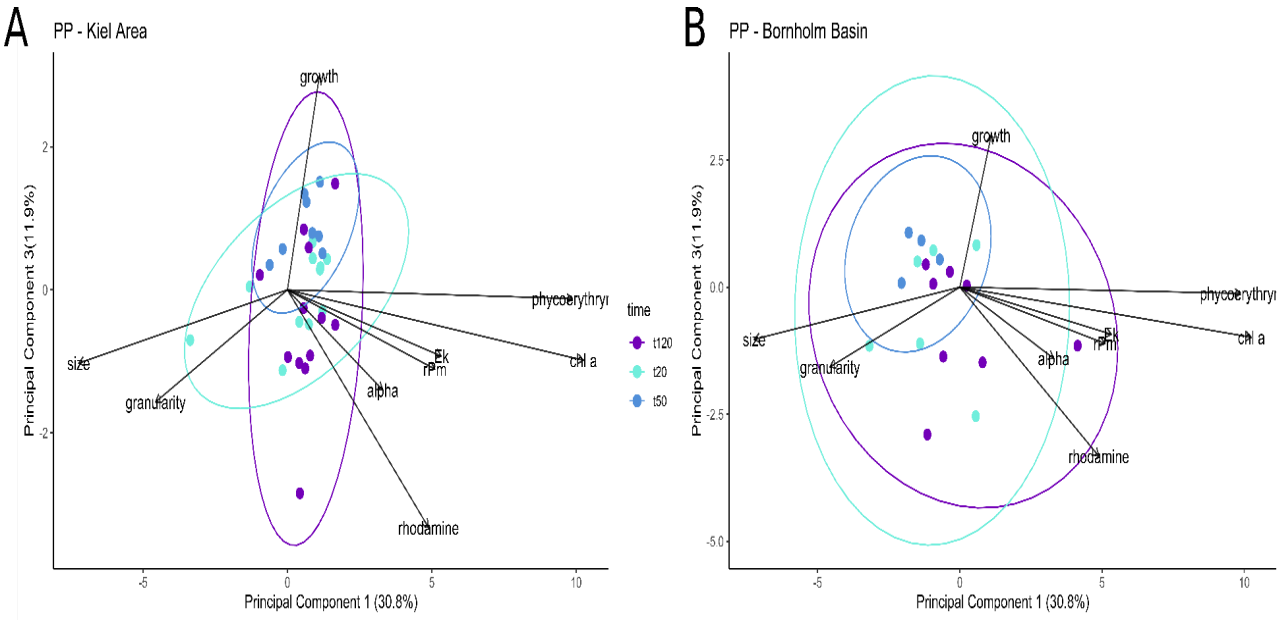


Figure 2.4.3. Trait-scape created from 9 traits. Traits were assessed in samples of *Ostreococcus spp.* in the PP (predictable timing and predictable amplitude) treatment. Trait-scapes are displayed for samples from the thermally unpredictable Kiel Area (A) and from the more predictable Bornholm Basin (B). Colours identify the time points at which the traits were assessed (t20, t50 and t120, corresponding respectively to 20, 50 and 120 generations of selection). Ellipses represent the 95% confidence intervals.

Trait correlations

To investigate in more depth the evolution of trait correlations over the course of the experiment, we analysed correlation matrices for the different time points. Only few trait correlations appeared to be consistent across strains, treatments, geographical areas and time. In particular, only E_k, rP, chlorophyll a and phycoerythrin content were positively correlated and preserved over time. Growth rates were never positively correlated with size at the end of

the selection experiment (t120) apart from two cases (CC and RR treatment in the Bornholm Basin Fig. 2.4.5 C and 2.4.7 C) and only in a few cases (RR treatment in the Bornholm Basin, Fig. 2.4.7 and CC treatment, Fig. 2.4.4 and 2.4.5) were correlated with rP. In general, the correlations of growth rate with other traits were (when present) mostly lost or turned negative toward t120 in all treatments. It is interesting to notice that at t120, fitness trajectories tended to be mostly homogenous in all levels of predictability and generally slow (see Study III).

In the RR treatment, correlations between size and chlorophyll a were negative or undetected. Also, correlations with rhodamine tended to be reversed at t120, indicating low levels of cellular stress (Fig. 2.4.6 and 2.4.7).

In the PP treatment, instead, we observed a drastic decrease in trait correlation strength with time in the Kiel Area (Fig. 2.4.8), while in the Bornholm Basin we did not detect drastic changes but the strength of correlations fluctuated with time (Fig. 2.4.9). In this case rhodamine´s correlations with other traits became more negative at t50 to then being lost over time.

Overall, even though phenotypic multitrait changes appeared strongly constrained in the trait-scape, trait correlations showed substantial changes across traits and selection regimes.

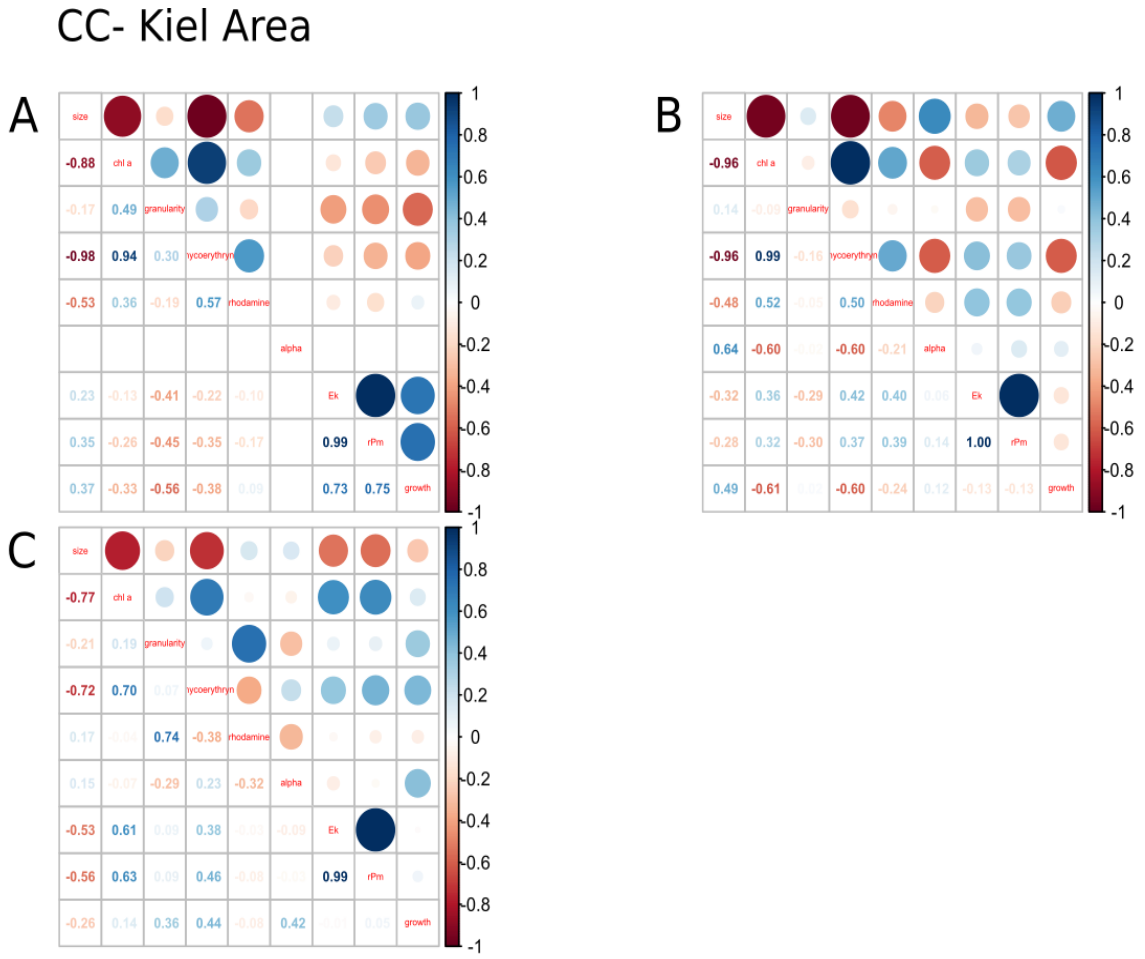


Figure 2.4.4. Trait correlations between the 9 examined traits in samples from the Kiel Area under CC (control) treatment. Plots correspond to correlations at t20 (**A**), t50 **(B**) and t120 (**C**). The blue colour indicates positive correlation, while the red negative correlation among traits. The size of the circles denotes the strength of the correlation and when the cell is blank means no significant correlation was found. Values are Pearson´s linear correlations.

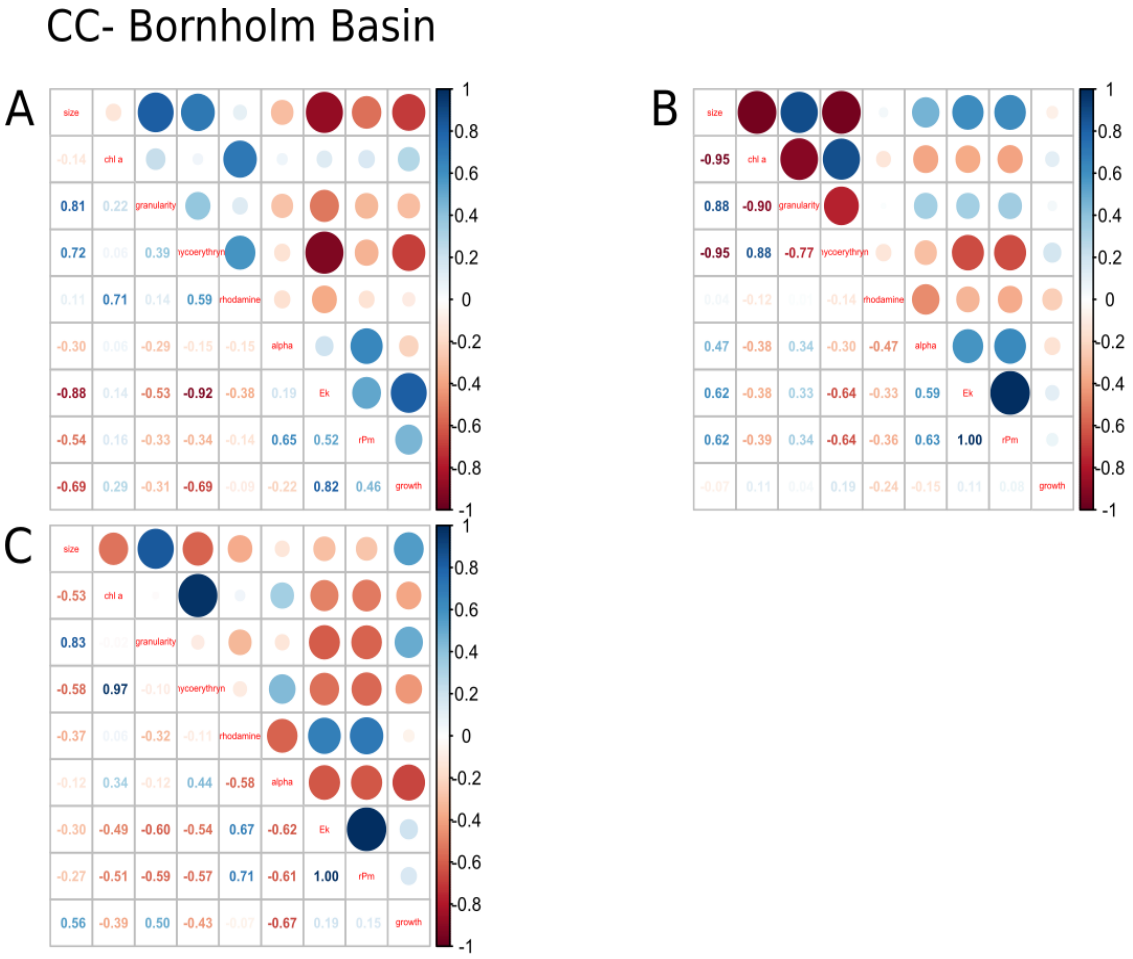


Figure 2.4.5. Trait correlations between the 9 examined traits in samples from the Bornholm Basin under CC (control) treatment. Plots correspond to correlations at t20 (**A**), t50 **(B)** and t120 (**C**). The blue colour indicates positive correlation, while the red negative correlation among traits. The size of the circles denotes the strength of the correlation and when the cell is blank means no significant correlation was found. Values are Pearson´s linear correlations.

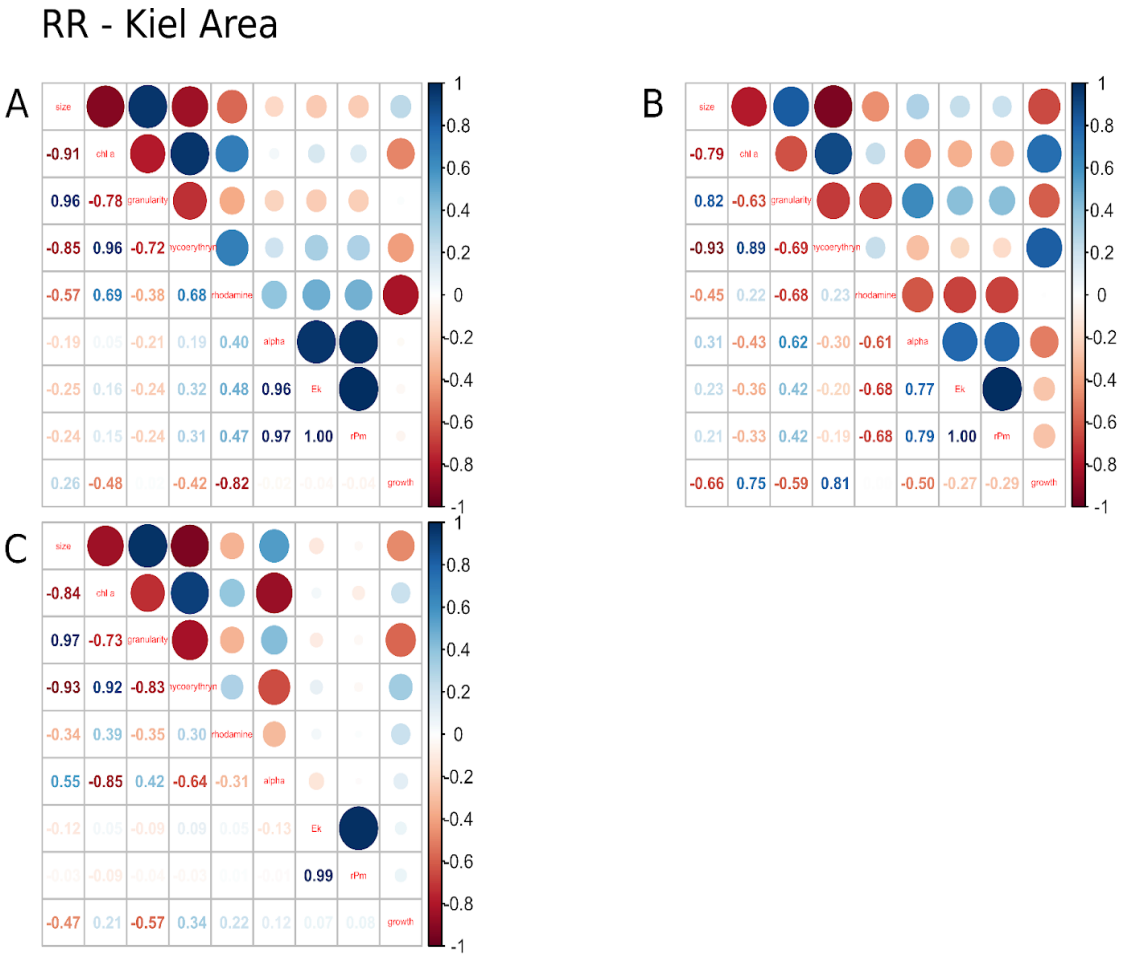


Figure 2.4.6. Trait correlations between the 9 examined traits in samples from the Kiel Area under RR (unpredictable timing, unpredictable amplitude) treatment. Plots correspond to correlations at t20 (**A**), t50 **(B)** and t120 (**C**). The blue colour indicates positive correlation, while the red negative correlation among traits. The size of the circles denotes the strength of the correlation and when the cell is blank means no significant correlation was found. Values are Pearson´s linear correlations.

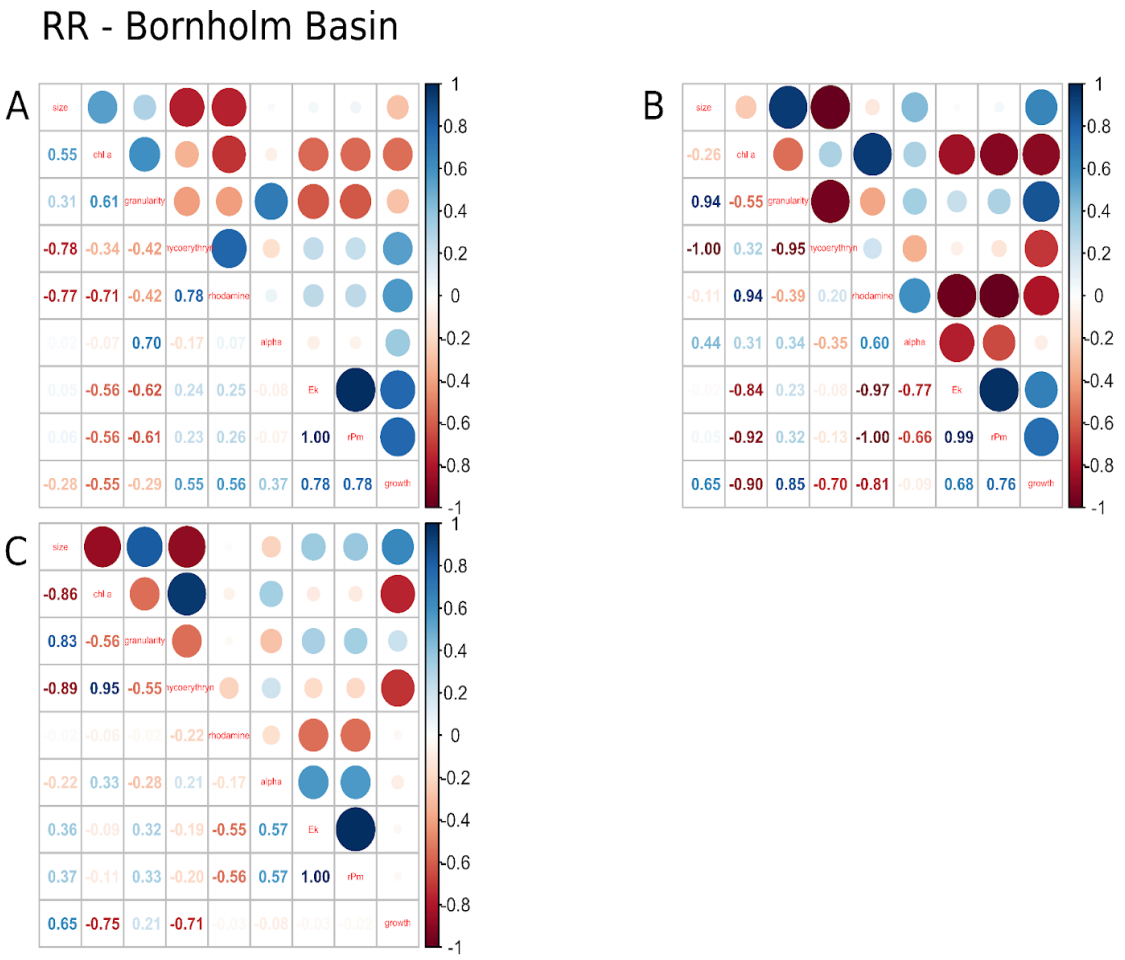


Figure 2.4.7. Trait correlations between the 9 examined traits in samples from the Bornholm Basin under RR (unpredictable timing, unpredictable amplitude) treatment. Plots correspond to correlations at t20 (**A**), t50 **(B)** and t120 (**C**). The blue colour indicates positive correlation, while the red negative correlation among traits. The size of the circles denotes the strength of the correlation and when the cell is blank means no significant correlation was found. Values are Pearson´s linear correlations.

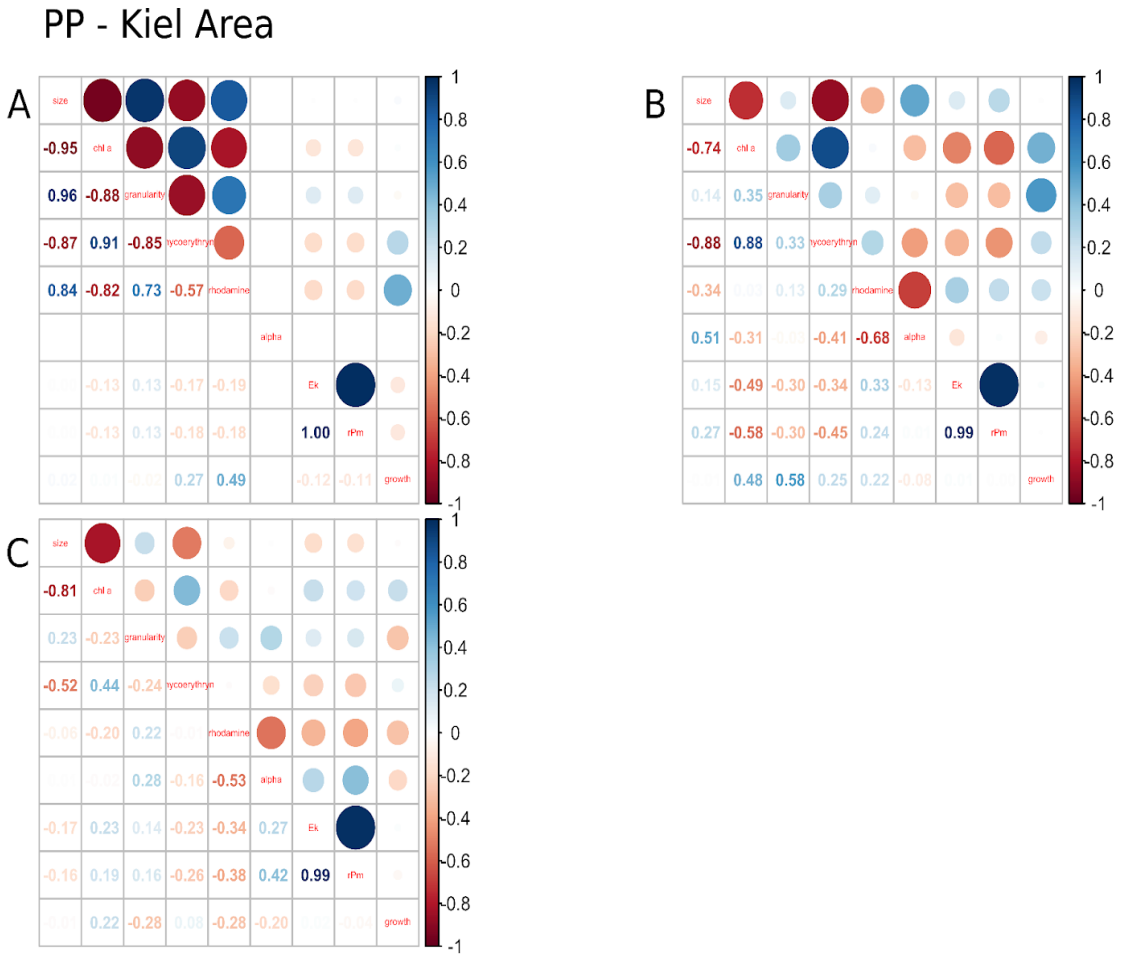


Figure 2.4.8. Trait correlations between the 9 examined traits in samples from the Kiel Area under PP (predictable timing, predictable amplitude) treatment. Plots correspond to correlations at t20 (**A**), t50 **(B)** and t120 (**C**). The blue colour indicates positive correlation, while the red negative correlation among traits. The size of the circles denotes the strength of the correlation and when the cell is blank means no significant correlation was found. Values are Pearson´s linear correlations.

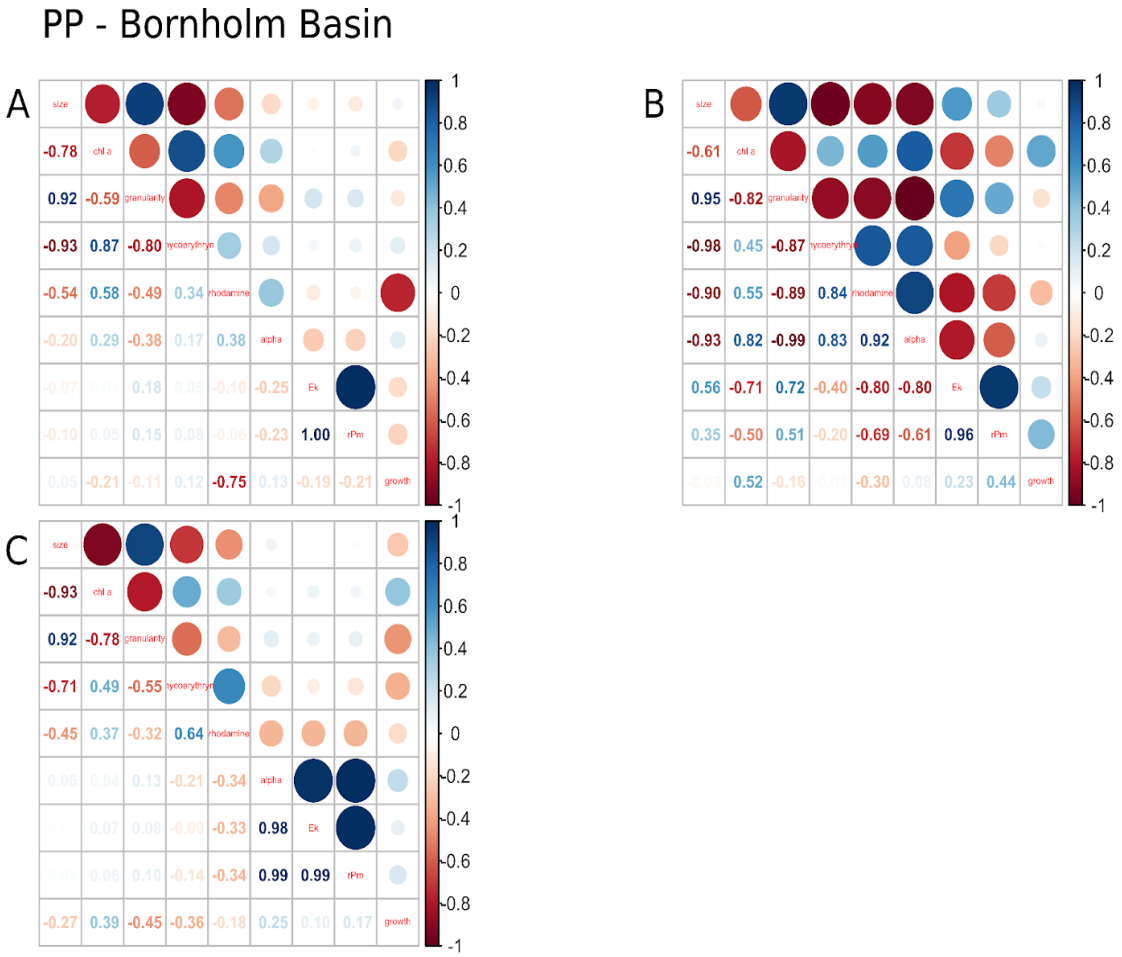


Figure 2.4.9. Trait correlations between the 9 examined traits in samples from the Bornholm Basin under PP (predictable timing, predictable amplitude) treatment. Plots correspond to correlations at t20 (**A**), t50 **(B)** and t120 (**C**). The blue colour indicates positive correlation, while the red negative correlation among traits. The size of the circles denotes the strength of the correlation and when the cell is blank means no significant correlation was found. Values are Pearson´s linear correlations.

Discussion

A growing body of literature demonstrates how phenotypic trait correlations transcends pairwise correlations and can change on evolutionary timescales (Walworth et al. 2021; Edwards et al. 2011). Here, we used selection regimes with different levels of thermal predictability to study evolutionary trajectories in the green alga *Ostreococcus* spp. Our study reveals that remarkable changes in single traits and trait correlations arise in response to differently predictable selection pressures. However, these changes in traits and trait correlations appeared on hidden dimensions of variation that are not necessarily captured on the first two principal components of the trait-scape. This highlighted the stronger effect of long-term evolution (evolutionary past) compared to evolution during the selection experiment.

In fact, against our expectations, our analysis of the trait-scape of populations evolved in fluctuating conditions (RR and PP treatments), did not show clear movements across the experiment (i.e., after 20, 50 and 120 generations of selection). This may indicate either that the investigated traits and trait correlations were very constrained or not influenced by selection regimes, such that no movement in the trait-scape took place. A second possible explanation is that even though trait correlations and traits evolved, their evolution was not captured by the first two principal components.

Using single trait analyses, I detected strong changes in the single traits, e.g., a clear decrease in cell size and cell complexity across treatments and strains (Fig. S2.4.1). Moreover, growth rate showed fluctuations over the course of the experiment, but only little correlation with other traits. In particular, positive correlations with other traits tend to consistently decrease after 120 generations in all fluctuating selection environments. The un-correlation of growth rates is also depicted in the trait being separated from the others on PC3. This could signify that if a directional selection is missing, as in the case of a fluctuating environment, organisms tend to express a higher variance in growth, which becomes independent from any other physiological changes. Little change was observed in photosynthetic traits, and this shows a phenotypic mismatch between fitness and metabolism.

I further investigated changes in trait correlations over the course of the experiment. Trait correlations proved to be evolutionary flexible, although not following a clear pattern. In fact, I detected multiple combinations of traits that ultimately produced similar growth rates.

I did not investigate the molecular basis of trait changes, but the shown pattern can be explained by compensatory mutations (Moore et al. 2000). In fact, I detected in all treatments an increase in trait correlations after 50 generations of selection, which is then reversed toward the end of the experiment. This suggests that, although growth rates appear to be stable, trait correlations were under strong selection. Trait correlations became weaker in particular in the more predictable treatment (PP). The absence of clear effects of this strategy on growth rates makes it more difficult to draw causal links, but it seems clear that differences in strength in correlations are not responsible for the similarities in fitness patterns. A reduction in the strength of the trait correlations can be a result from a low genetic diversity (Roff 2000) imposing a genetic constraint on evolution.

In conclusion, our study demonstrates that microevolution can manifest itself in changes in trait values and trait correlations that are not necessarily visible in the trait-scape. Whereas previous work suggested that the evolution of traits and trait correlations follows a reduced set of allowable combinations (Hinners et al. 2022), our results suggest that the evolution of traits and trait correlations may not follow clear patterns but may express itself along cryptic hidden dimensions of variation that are not necessarily captured on the first two principal components. This might be explained by differences in the two experimental approaches. Here, we used fluctuating selection regimes and non-detrimental thermal selection pressures, while in Hinners et al. (2022), regimes were stable and non-detrimental. Stressful or more extreme experimental conditions could have broken established correlations between traits and led to more hidden movements along further dimensions of the phenotypic space. But phytoplankton will likely experience change, and even changes in fluctuations, as gradual. This may lead to more generation time to adjust to environmental changes. Moreover, we here investigated a genus of green alga, while previous literature (Hinners et al. 2022; Argyle et al. 2021) explored the trait-scape in diatoms. Whereas the diatom trait-scape was able to capture more than 70% of trait variation on principal component 1 and 2, our Ostreococcus trait-scape was only able to capture about 50% of variation, leaving half of the variation across traits hidden. This might point toward substantial differences across phytoplankton functional groups, or due to cellular complexity, that can justify the observed differences. This consideration opens up to the possibility that multitrait phenotypes evolution strongly varies across functional groups.

Phytoplankton host a large taxonomic and functional diversity and plasticity and adaptation will shape their responses to climate change. The trait-scape approach can be useful in order to explain phenotypic differences and make valuable predictions for ecosystem-based

modelling, especially when models allow for changes in ecologically relevant traits on evolutionary time scales. My study highlights the need of considering biogeography and functional group-specific physiology in selection experiments.While the world is changing, adaptive potential will largely depend on local conditions experienced by organisms and this can strongly influence how ecological “winners and losers” in future scenarios are selected.

3 Summary and conclusions

Contents

In this thesis, I have thoroughly analysed the differences in adaptive potential of phytoplankton when organisms are subjected to environments that differ in thermal predictability (in their past adaptive history and during the experiments themselves) and therefore are characterised by different selective pressures. I analysed phenotypic plasticity and evolutionary trajectories on communities and single species isolated from two naturally variable areas on subsequent time-scales with increasing theoretical complexity. I mostly focused on plastic thermal responses and responses to fluctuating temperatures. After 3 main experiments, and 4 years later, I was able to answer the questions I posed at the very beginning and below I will elaborate on them one by one:

1. **How does a history of variability influence thermal tolerance?**
2. **From few to many: Which are the challenges upscaling single species responses?**
3. **Time goes by: can we relate immediate responses to short and long-term responses?**
4. **What are the effects of different levels of predictability of thermal fluctuations on evolution and maintenance of phenotypic plasticity and on evolutionary responses?**

1. How does a history of variability influence thermal tolerance?

To answer this question, I investigated immediate and short-term responses of natural phytoplankton communities coming from two distinct regions in the Baltic Sea: the Kiel Area, overall warmer and less thermally predictable, and the Bornholm Basin, cooler and more predictable. In Studies I and II, I first analysed immediate or acute (i.e. plasticity within one generation) metabolic thermal tolerances, and then growth rate responses in the short-term to a range of temperatures (spanning 20 generations). In order to analyse evolution using either time-series or space-for-time approaches, I compared the obtained results from each of the study across seasons and geographical area of origin.

Phenotypic plasticity can be a locally adapted trait and shaped by environmental conditions (Somero 2010). Understanding the influence of local adaptation on plastic responses and thermal tolerances is important in order to merge evolutionary biology (here, the direction of plastic and evolutionary responses) and ecology (here, evolutionary history) and thus better predict niche width and their modifications in light of the future climate crisis.

We expected to find evidence of local adaptation, especially regarding the degree of plasticity expressed by the populations. Particularly, theories for terrestrial organisms (Vázquez et al. 2017; Bozinovic, Calosi, and Spicer 2011) predict that plasticity should evolve and be favoured in less predictable environments. Interestingly, this trend is not always supported by studies on marine organisms (Leung et al. 2020).

In Study I, I analysed thermal tolerance curves, which represent a measure of within-generation plasticity of metabolic traits. In order to see how flexible within-generation plasticity is, I compared thermal optima across seasons. I found that the direction of the change in metabolism with warming on seasonal timescales was similar in the two areas. But, in the Kiel Area, community composition did not change at the functional group level throughout the seasons. In the Bornholm area, instead, functional group composition drastically changed on a seasonal time scale. This proves that in the Kiel Area a history of variability left ample scope for phenotypic plasticity of individuals - or at the very least for rapid genotype sorting within the same species.

Moreover, in Study II, I analysed plasticity in growth rates responses after 20 generations of acclimation to temperatures spanning from 15 to 30°C in the pico-phytoplankton communities from both regions. In this case, the Kiel Area community isolated during summer (and during a heatwave), was able to widen thermal tolerances considerably. Interestingly, growth rates remained on average the same for all tested temperatures (from 15 to 28°C), which resulted in a horizontal thermal reaction norm at all survivable temperatures. The strategy in this case, was a fast re-shuffling of the populations, denoted by a drastic reduction of alpha diversity

during the last 10 generations of the experiment, whereas in the Bornholm Basin communities, species sorting on seasonal time scales (from winter to summer), prevented plastic responses to happen at fast ecological timescale.

While answering this question, an observation emerged. Lande (2009) suggested that in the absence of a cost of plasticity, the expected responses would depend on environmental variability. Specifically, plasticity is predicted to be high in more variable and predictable environments and lower with low variance and predictability. I do argue that costs (and constraints) on phenotypic plasticity in relation with environmental predictability, could be overlooked when dealing with complex and natural assemblages. I suggest that the cost imposed by the constant adjustment of phenotypic characteristics, decreases when the community *in toto* is capable of responding fastly to environmental changes (i.e. re-shuffling of functional groups and/or genotypic sorting).

A history of variability increases the intrinsic plastic potential of communities coming from less predictable areas, therefore influencing adaptive potential.

2. From few to many: Which are the challenges upscaling single species responses?

One of the major challenges in evolutionary biology is to understand adaptive responses in complex environments. This includes evolution and acclimation in the presence of biotic interactions (e.g. competition, mutualism or coexistence). This question is extremely important in light of predictions on how evolutionary responses will affect ecosystem properties.

Biotic interactions can affect evolutionary dynamics and plastic responses in several ways. The first,more intuitive scenario is that a change in population size can increase rates of evolution, especially in the presence of competition for limited resources and increasing diversity (Johansson 2008). Other theories focus on the influence of ecological sorting. In this case, if the environment changes, species pre-adapted to grow better in the new conditions will outcompete the others, preventing adaptation of existing populations to occur (Fowler and MacMahon 1982). But the topic is not short of controversies, with other theories instead predicting biotic interactions to facilitate co-evolution, imposing a stronger selective pressure than the changes in the environments themselves. This is the main *dictat* of the Red Queen Hypothesis formulated by Liow et al. (2011).

Most selection experiments and plasticity assays are carried out on a single species (but in many cases including their cohabitating bacteria). While this is an efficient way to understand underlying genetic mechanisms, it also imposes that relationships between organisms exerts a non significant effect compared to the environmental drivers.

In my thesis, I instead measured plasticity and evolution focusing on natural phytoplankton communities´ responses. In Study I, whole community respiration proved to not be more sensitive to seasonal temperature changes than photosynthesis. This is in sheer contrast with laboratory experiments on single species (e.g., Barton et al. 2018). Only when facing extreme conditions, such as the heatwave event which occurred during the Summer cruise in 2018, the influence of environmental temperature on respiration was significant. Moreover, in Study II, I highlighted the importance of the phenotypic composition of the starting communities and the intrinsic adaptive potential gained from the environment, on short-term fitness responses. Also, when looking at whole community responses, metabolism did not clearly explain fitness. Results were instead in the opposite direction when considering a single species, *Ostreococcus tauri*, in isolation. In this case, I detected an increase in fitness thermal tolerance in the Bornholm Basin´s strains. The increase was mainly led by an upregulation of

gross photosynthesis, in particular for what concerns the E_a (i.e. steepness of the reaction norm before the optimum) and thermal optimum, denoting a rapid adaptation to warming.

Community plasticity, especially driven by species or genetic sorting, do not predict single species´ plastic responses

3. *Time goes by: can we relate immediate responses with short and long-term responses?*

In this thesis, I examined the plastic and adaptive potential of phytoplankton on three different time scales: immediate (or acute, within the same generations), short-term (20 generations) and long-term (120 generations).

Contemporary evolution to environmental variability has the potential to re-shape population dynamics and it is in my opinion important to examine physiological responses to environmental drivers on several time scales (Table 3.1), in order to fully grasp the factors affecting contemporary evolution and the strength and magnitude of the interplay between evolution and ecology.

Table 3.1 Overview of the different timescales and temporal levels considered in this thesis

	Immediate responses	Short-term	Long-term
length of the experiment	1 generation	20 generations	120 generations
evolutionary history influence	yes	yes	yes
level of the timescale	physiological/seasonal	ecological/seasonal	microevolutionary
seasonal influence	within seasons/across seasons	within /across	not considered

Evolutionary responses in microorganisms can happen within relatively few generations, and these also translate to relatively short 'real' time scales , such as a single bloom period. These time frames are on par with ecological time scales, and this imposes a number of sub-questions:

> ***Are responses measured on ecologically relevant time scales (i.e. short-term) related to responses within one generation (i.e ., their within-generation plasticity potential)? How much of an influence has the timing of occurrence of plasticity on community structure?***

In Study I, I found that when measuring plasticity of metabolic traits within one generation across several seasons (Study 1), Kiel Area communities were evidently able to adjust to seasonal changes (i.e., thermal optima for gross photosynthesis followed the seasonal increase in temperature), without sorting on the functional group level. In direct contrast, when we

used these communities from the Kiel Area to assay growth rates across temperatures ranging from 15 ºC to 30 ºC for 20 generations (Study II), we found evidence for a fast turn-over on the functional group level for samples from the summer season and across seasons for the Bornholm Basin communities.

Physiological responses to temperature can be fast. Acute responses can be either caused by "passive" plasticity, which is merely stress-induced and includes non-adaptive acute physiological responses (Ghalambor et al. 2007) or "active" plasticity which asks for physiological changes induced by a recognition of the environmental cue. Passive plasticity is thought to be difficult to measure due to the logistical time needed to perform measurements, which usually involve a level of acclimatisation (Schulte et al. 2011).

The differences between the two types of plasticity, raise some questions. Usually, plasticity is measured as the slope of a linear reaction norm, meaning that if the slope is horizontal the trait under consideration lacks plasticity. Following this interpretation, we should conclude that summer communities from the Kiel Area in Study II, totally lack plasticity. However, passive plasticity acts on acute timescales, and the only way to achieve a reaction norm with zero slope at acclimation temperatures, is for active plasticity to maintain physiological responses at previously experienced temperatures.

We argue here that the acute and short-term responses overlap in terms of responses if, alongside changes in traits and reaction norms slopes, we consider also underlying changes in the communities. This suggests two main conclusions: i) at least when considering communities, responses within one generation are clearly an effective way of predicting the magnitude of phenotypic plasticity, which translates into less effort for acclimation procedures. ii) evolutionary past dictates the pace of organisms´ (at least fast growing ones) phenotypic changes, making global predictions more challenging and directing phenotypic plasticity´s directions.

Have past evolutionary responses shaped the evolutionary responses to environmental predictability in the selection environment?

I then show that in the long-term (Study III), plastic short-term growth responses are lost for strains that already had a lower degree of plasticity, in fact, Bornholm Basin strains evolved in a fluctuating environment unpredictable in timing, and are unable to cope with diversely predictable treatments).

While we do not have the data here to directly compare the magnitude of plastic responses of communities with the ones for populations originated from single clones, I ran a pilot study to investigate short-term responses in *Ostreococcus* spp. from the two areas (see Supplementary Information Study II). In that experiment, local adaptation to different degrees of variability in the past environment, proved to have an effect on phenotypic plasticity which was comparable to what happened in Study III.

Phenotypic plasticity in the short-term, seems not to be then related with evolutionary dynamics in the long-term. Nevertheless, I cannot here disentagle here if it is due to a unpairing between plasticity and evolution, or to the increased complexity of the environmental cues (i.e., predictability of the fluctuations).

Immediate responses are good predictors of the flexibility of plastic changes related to the past environmental conditions.
However in more natural (fluctuating) conditions, short-term changes rarely reflect long-term directional changes.

4. Isolating the effects of predictable and unpredictable fluctuations

A plethora of scientific studies has been focused on the effects of increasing temperatures (e.g., Jin and Agustí 2018; Baker et al. 2018; Barton et al. 2020). Nevertheless, the frequency of extreme events and variability of the environment are also expected to increase (IPCC 2007). Several studies have suggested that thermal extremes may have a strong effect on spatial distributions of phytoplankton (e.g., Mulholland et al. 2009). Nevertheless, little is known about when and how the predictability of fluctuations as well as the frequency or the amplitude of the fluctuation play a role. I extensively tested this question and proved that local adaptation (here determined by the area of provenience of the strains) plays a large role in determining the direction of plastic responses. However, in Study I and II, I tested responses in constant assay environments. In order to better understand the contribution of predictability per se, and to test in concept whether changes in frequency or amplitude shape evolutionary trajectories, I used different levels of predictability of thermal fluctuations.
In Study III and IV, I analysed the evolutionary trajectories, the evolution of plasticity and the trait-scape of two species belonging to the *Ostreococcus* genus. Cells were subjected for more than one hundred of generations to fluctuating thermal conditions, unpredictable in both frequency and amplitude. According to our hypothesis, the Kiel Area thermal regime should more closely resemble the completely unpredictable treatment (RR, unpredictable both in the timing and the amplitude), while the Bornholm Basin natural regime should be more similar to the treatment where only timing is kept unpredictable (RP). I found evidence supporting these assumptions, showing that during a reciprocal transplant experiment, Bornholm Basin RP-evolved strains, completely lost fitness plastic responses. In the RR treatment, instead, plastic responses for the Kiel Area strains remained on average similar throughout the experiment. While the geographical area and previous local adaptation played a role when it came to plastic responses, growth rate trajectories did not follow the same pattern. Here,

instead, the shared strategy was to grow slow. Previous studies already proved slow growth to be advantageous, especially in a fluctuating environment and it was correlated with lower cellular stress (C. E. Schaum and Collins 2014). In order to identify constraints on observed phenotypes, we also used principal component analysis to analyse the "trait-scape" of phenotypes through time. My analysis revealed that microevolution can manifest itself in changes in trait values and trait correlations that are not necessarily visible in the trait-scape, which was in turn unresponsive or constrained by previous local adaptation.

Fluctuating environments (both predictable and unpredictable) force organisms to exhibit more strategies. While traits dynamics are constrained by previous local adaptation, fitness and metabolic plastic responses are related with previously experienced environments

Future perspective

This thesis is an important stepping stone to improve our understanding of phytoplankton evolutionary potential, however further questions arise and more improvements are needed:

Community-wide analysis: further perspective on natural assemblages and interactions

Single-species responses cannot always be used to upscale responses at the whole communities level, and my thesis shows that this is particularly the case when considering the environments in which they previously evolved and not merely a global average, i.e. average sea surface temperature or increases therein would not be a good predictor. I explained before how species interactions can strongly interfere, negatively and positively, with evolutionary dynamics.

These results evoke a variety of new questions (and limitations we had to face). I think it will be important in the future to continue assessing trait responses of whole communities, but improving identification of species in the community. We used cytometric fingerprints as a proxy for diversity, and, even if it is a powerful and easy tool to estimate functional group diversity, can overlook genotype sorting and miss detailed and important information regarding a clear identification of the species. I want to stress the importance of applying more detailed methods, such as metabarcoding.

Moreover, it would be interesting to assess the main determinants of evolutionary rates and estimate the evolutionary potential of single species in the community. Monocultures of single species in isolation could be used to assess single species evolutionary potentials and compare the obtained results with evolution in coexistence. Obviously one major problem regarding this approach would be the gargantuan isolation effort and the difficult to track single species dynamics in mixed cultures.

Local adaptation in different areas

In this thesis, we obtained some clear indications regarding the effect of evolutionary histories (i.e. the environment in which organisms lived and adapted) on evolutionary potential, both at single species and whole community level. Our findings deepened our knowledge of interactions between local adaptation and evolutionary dynamics. Nevertheless, our study was limited to the small, yet intriguing, area of the South-Western Baltic Sea. The area offered some peculiarities (i.e. consistency in confounding effects such as nutrients and light availability but differences in the thermal regimes) that made it perfectly fit as a model study area. I do argue that to clearly generalise our results, a more global approach is needed. For example, the Antarctic environment, which is already strongly impacted by climate change. Or the Mediterranean Basin, considering the strong nutrients concentration East-West

gradient. These areas are also well monitored either through stations or research cruises, which will ensure a full evaluation of previous and present environmental conditions.

Direction of coevolution to different drivers

In my thesis, my main focus was represented by temperature as a driver. Although temperature is considered to be one of the main factors influencing phytoplankton physiology and spacial range (e.g., Thomas et al. 2012), nature is complex and changes in not only temperatures, but also pH, nutrients availability and salinity will occur together. A large body of recent literature has been focusing on multi-driver evolution (e.g., Brennan and Collins 2015; Boyd et al. 2018), but a smaller effort has been put on understanding co-evolution to different drivers when organisms were already selected to face changing environmental conditions. For example, I conducted (alongside my co-authors, Jana Hinners and Laura Kaiser), a side-project using long-term evolved samples. We tested how strains evolved at different levels of predictability of thermal fluctuations responded when suddenly subjected to supra-optimal conditions in temperature (mimicking heat waves), light limitation and nutrient limitation. We highlighted co-evolution in temperature and light traits, but no dependency was weirdly found between temperature and nutrients. Thermal fluctuations also seemed to have increased the differences between strains, even if we used a reduced set of samples compared to the long-term in Study III. More astoundingly, we found that, at the level of trait correlations patterns, the unpredictable treatment (RR) enhanced correlations patterns in all strains tested in a similar fashion. The selection pressure had therefore a strong impact on strains and affected a wide variety of traits and relations within them. We proved that previous environmental changes have the potential to alter stress responses to new conditions. Even if this manuscript is, at the current state, still in preparation and it is thus not part of this thesis, the preliminary results open up the possibilities of co-evolution of traits with different

environmental drivers. More effort is needed in understanding both within-species genotypic sorting and effects of superimposing new drivers at different time steps, which can be ecologically relevant in the context of migrations pattern, dispersion or subsequent modifications of the environment.

4 Supplementary Information

Contents

4.1 Supplementary Information Study I

Table S2.1.1. Statistical results from a One-way Anova using the "Anova" function in the *car* package on R. The test was conducted on means for the main abiotic factors (salinity, temperature and nutrient concentration) describing the Kiel Basin and the Mecklenburg Bight. No significant differences were present, and we therefore group the two regions as 'Kiel Area' throughout the paper. (df: degree of freedom; SS: sum of squares; F: F-value; p: p-value).

Variable	*df*	SS	*F*	*p*	
Salinity (PSU)	1	22.038	3.521	0.085	
Temperature (° C)	1	0.04	6e-04	0.98	
Nitrogen	1	312.2	0.251	0.627	
Silicates	1	1.421	0.11	0.747	
Phosphate	1	47.84	0.483	0.503	

Table S2.1.2. Ranges of temperatures used for metabolism analysis. Increments are uneven and higher temperature may vary due to tolerance differences of the samples.

	Temperature (°C)																					
Cruise id	**3**	**5**	**8**	**10**	**12**	**14**	**15**	**18**	**20**	**22**	**24**	**25**	**26**	**28**	**29**	**30**	**31**	**32**	**34**	**35**	**38**	**40**
AL505 (March 2018)	✔	✔			✔		✔	✔	✔			✔										
AL513 (July 2018)				✔		✔		✔		✔			✔			✔			✔		✔	✔
AL520 (March 2019)	✔	✔	✔	✔	✔		✔	✔	✔	✔		✔										
AL521 (April 2019)		✔	✔	✔	✔		✔	✔	✔	✔												
AL522 (May 2019)		✔	✔	✔	✔		✔	✔	✔	✔	✔		✔									
AL524 (July 2019)			✔	✔	✔		✔	✔	✔			✔			✔		✔	✔		✔		
AL530 (October 2019)		✔	✔	✔	✔		✔	✔	✔	✔	✔		✔	✔								

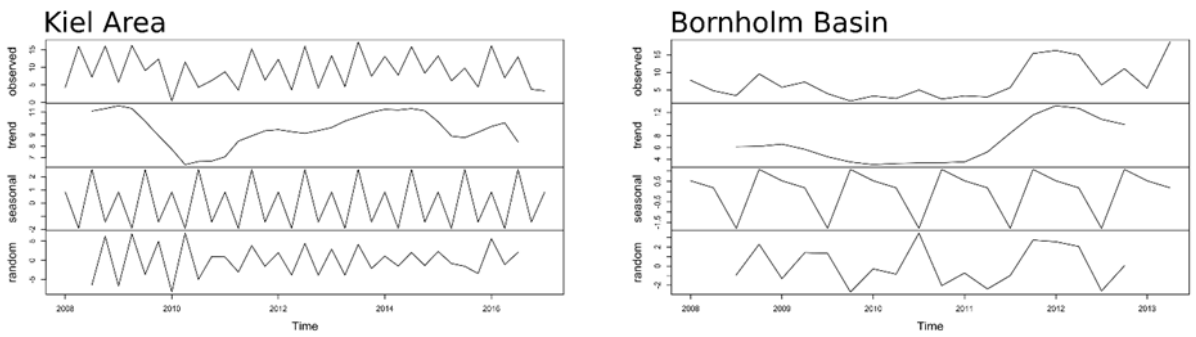


Figure S2.1.1. Decomposition analyses of surface temperatures (-8 m) of the last 5 years for the Kiel Area and the Bornholm Basin. Absence of a reproducible and clear pattern of seasonality in the Kiel Area, indicates a less predictable trend in the area.

Table S2.1.3. Random components outcomes produced from decomposition analysis performed on sea surface temperatures time series for Bornholm Basin and the Kiel Area, using the function decompose of the anomalize package (0.2.0). We used an additive (seasonal + trend + random) approach, assuming a quarterly seasonality (frequency = 4. The quarterlies are reported in the table as Qtr1, Qtr2, Qtr 3 and Qtr4). Statistical results from a One-way ANOVA comparing the two geographical areas are reported at the beginning (df: degree of freedom; SS: sum of squares; F: F-value; p: p-value). Mean values for random effect were higher in the Kiel Area, meaning that the time series is less constant and consequently more variable (KA: 1202.95 ± 682.67; BB: 151.94 ± 87.47). No seasonal component was found for the Kiel Area (also using a multiplicative approach assuming a monthly seasonality).

Variable	*df*	SS	*F*	*p*
Geographical Area	1	3.001e+08	718.9	>2e-16

Bornholm Basin

Time points	**Qtr1**	**Qtr2**	**Qtr3**	**Qtr4**
1	NA	NA	0.122195559	0.239398849
2	0.110243914	-0.464088322	-0.154304441	-0.316601151
3	0.707993914	0.055411678	-0.260179441	0.055711349
4	0.352743914	-0.387150822	0.023695559	-0.455913651
5	-1.074443586	1.117911678	2.453695559	-0.941913651
6	1.572181414	-1.914775822	-0.814804441	0.118898849
7	0.171368914	-0.377838322	0.014320559	0.310711349
8	0.104118914	-0.117088322	0.101258059	-0.628351151

9 -0.999631086 1.091974178 0.735508059 -0.041726151

10 0.086743914 -0.714400822 0.297820559 -0.145788651

11 -0.502506086 -0.827588322 1.854320559 0.633086349

12 -0.887943586 -0.967025822 1.688508059 -0.330101151

13 -0.547693586 -0.623400822 1.216445559 -0.360976151

14 -0.049881086 4.050161678 -3.393616941 -0.806601151

15 0.262868914 -0.470963322 0.140883059 0.274273849

16 -0.268068586 -0.948900822 0.766633059 0.371273849

17 -0.001193586 -0.104025822 -0.128929441 0.367273849

18 0.052868914 -0.460213322 0.053508059 -0.120601151

19 -0.160631086 -0.914838322 1.822445559 -0.089851151

20 -0.342943586 -0.446963322 -0.224616941 2.462898849

21 0.952675164 -2.115238322 -0.895104441 0.024586349

22 0.282212664 -0.296225822 -0.003866941 0.265398849

23 -0.062381086 -0.987088322 0.690008059 0.608648849

24 -0.094881086 -0.329838322 -0.051304441 0.133773849

25 0.391493914 -0.289775822 0.208633059 0.326336349

26 -0.579506086 0.427286678 -0.334429441 -0.791476151

27 0.590993914 -0.146963322 0.065445559 0.191336349

28 0.044118914 -0.287025822 -0.068929441 -0.339476151

29 0.581368914 -0.543713322 1.052695559 -0.069851151

30 -0.604881086 0.636224178 1.335570559 -1.088913651

31 -0.357756086 -0.441900822 0.044758059 0.135836349

32 -0.148506086 -0.112838322 0.064445559 0.327148849

33 -0.116443586 -0.709213322 0.416820559 0.473148849

34 -0.310381086 -0.176588322 0.356695559 0.309836349

35 -0.598443586 0.143786678 0.512070559 -0.493726151

36 -0.274631086 -0.839838322 1.243570559 0.217398849

37 -0.161756086 -0.483838322 -0.447491941 -0.646288651

38 2.714681414 -0.878900822 -0.463179441 -0.089788651

39 0.782556414 -1.649025822 -4.133429441 4.272836349

40 0.690868914 0.046536678 -0.003679441 0.096648849

41 -1.839506086 1.859036678 2.977570559 -2.390851151

42 -2.989506086 1.615286678 0.696320559 0.377898849

43 2.079243914 -0.822213322 -0.791179441 0.164461349

44 2.250743914 -2.525463322 -0.659804441 -0.013163651

45 0.212681414 -0.408338322 0.015195559 -0.108351151

46 -0.962318586 2.329536678 -0.927179441 0.082961349

47 -0.283568586 0.558786678 -0.664929441 -0.890913651

48 -3.284318586 5.379411678 0.797070559 -1.172101151

49 -0.389506086 -0.634713322 0.027570559 0.676148849

50 2.856618914 -2.427025822 -0.767429441 0.167836349

51 -0.033881086 0.023099178 -0.197179441 0.019711349

52 -0.048256086 -0.097213322 -0.059929441 -0.059601151

53 -0.131193586 0.658224178 -0.813429441 0.275398849

54 1.223118914 -0.848650822 -0.005554441 -1.229726151

55 -3.858068586 3.690161678 1.161820559 0.415398849

56 -0.014506086 -0.634713322 0.158820559 0.640398849

57 0.022993914 -1.034713322 -0.053679441 1.052898849

58 -0.252006086 0.202786678 -0.541179441 0.040398849

59 0.329243914 -0.428463322 0.071320559 0.465398849

60 0.047993914 -0.197213322 2.283820559 -1.997101151

Kiel Area

Time

points	Qtr1	Qtr2	Qtr3	Qtr4
1	NA	NA	0.1501879117	-0.0414275030
2	-0.1850069240	-0.0138159846	-0.0983120883	0.0445724970
3	0.0692430760	-0.0346284846	-0.1024995883	0.0576974970
4	0.0686180760	-0.0298784846	-0.0514995883	-0.0048025030
5	-0.0518194240	0.0516215154	0.1470629117	-0.0595525030
6	-0.0146944240	0.0221215154	-0.0716870883	0.0135724970
7	-0.0296319240	0.1144965154	-0.0788120883	-0.0411775030
8	0.0273680760	0.0908715154	0.0528129117	-0.0043025030
9	-0.0236319240	0.0149965154	-0.0714370883	-0.1023025030
10	-0.0007569240	0.0528715154	0.0075629117	-0.0033025030
11	0.0016180760	0.0333715154	0.0158129117	-0.0014275030
12	0.0433680760	0.0303715154	0.0039379117	-0.1061775030
13	-0.0153819240	0.0504965154	-0.0601870883	-0.1453025030
14	0.0199930760	0.0281215154	0.0334379117	-0.0116775030
15	0.0176180760	0.1341215154	-0.0663745883	-0.0459900030
16	0.0499930760	0.0196215154	0.0032504117	0.0301349970
17	-0.0341319240	-0.0130034846	-0.0115620883	-0.0605525030
18	-0.0178819240	0.0238715154	0.0470629117	0.0209474970
19	0.0043680760	0.0186215154	-0.0199370883	-0.0260525030
20	0.0212430760	0.0044965154	0.0216879117	0.0301974970
21	0.0022430760	0.0486215154	0.0004379117	-0.0446775030
22	-0.0653819240	-0.0140034846	-0.0253120883	-0.0005525030
23	-0.0516319240	-0.0021284846	0.0456879117	0.0778849970
24	-0.1276319240	-0.0991284846	0.3184379117	-0.1776150030
25	-0.0646319240	0.1593715154	0.0635629117	-0.1306775030
26	-0.0940069240	0.0672465154	-0.0588120883	0.1370099970
27	-0.2221319240	-0.0476284846	0.0931254117	0.1151349970
28	0.0109930760	-0.1062534846	-0.1401245883	0.1551974970
29	0.0139930760	-0.0377534846	-0.0246245883	0.0885724970

30 0.0649305760 0.0836840154 -0.0731870883 -0.1101775030

31 -0.0060694240 -0.0728159846 0.0291254117 0.2403224970

32 -0.0641319240 -0.3026909846 -0.2269370883 0.3764474970

33 -0.0027569240 0.0843715154 0.2526254117 -0.4258025030

34 0.3583680760 0.6531215154 -1.0286245883 0.0111349970

35 -0.0467569240 -0.2799409846 -0.1325620883 -0.0846775030

36 0.2113680760 0.8166215154 0.1023129117 -0.0884900030

37 0.2693055760 -0.1147534846 -0.7823120883 -0.1128025030

38 0.3785555760 0.0572465154 -0.3061870883 0.2485099970

39 -0.0381319240 -0.1323159846 -0.0834370883 0.3056349970

40 -0.0375069240 0.0130590154 -0.1800620883 -0.1345525030

41 -0.2428819240 0.7088715154 0.2513129117 -0.1168025030

42 -0.1827569240 0.0041215154 -0.0965620883 -0.1425525030

43 0.1004930760 0.1007465154 -0.0121870883 -0.0575525030

44 0.0057430760 0.1002465154 0.1120004117 -0.0971775030

45 0.2220555760 -0.5903784846 0.1380004117 0.3321974970

46 0.9124305760 0.0132465154 -0.2468120883 -0.4562400030

47 -0.7778819240 -0.5606284846 0.2115004117 0.6186974970

48 -0.5977569240 0.3321215154 0.3550004117 0.8751974970

49 0.4993680760 -1.4892534846 -1.0240620883 0.3529474970

50 0.3849930760 0.2703090154 0.4673129117 0.8678849970

51 -0.6358819240 -0.2889409846 0.3766254117 -0.7394900030

52 0.2584930760 0.0163715154 -0.2472495883 -0.5719900030

53 -0.0421944240 -0.7845659846 0.9648129117 0.2585724970

54 0.0863055760 0.0184340154 -0.0138120883 0.3085724970

55 0.0091180760 0.0313715154 -0.0339370883 -0.2651775030

56 0.1598680760 0.1334965154 0.0074379117 0.0691349970

57 -0.1311319240 0.0387465154 0.2155004117 -0.0087400030

58 -0.3813819240 -0.0492534846 -0.2754370883 0.0946349970

59 0.3343680760 -0.0148784846 -0.0678120883 0.0819474970

60 -0.0251319240 -0.0315034846 -0.0011870883 0.0270099970

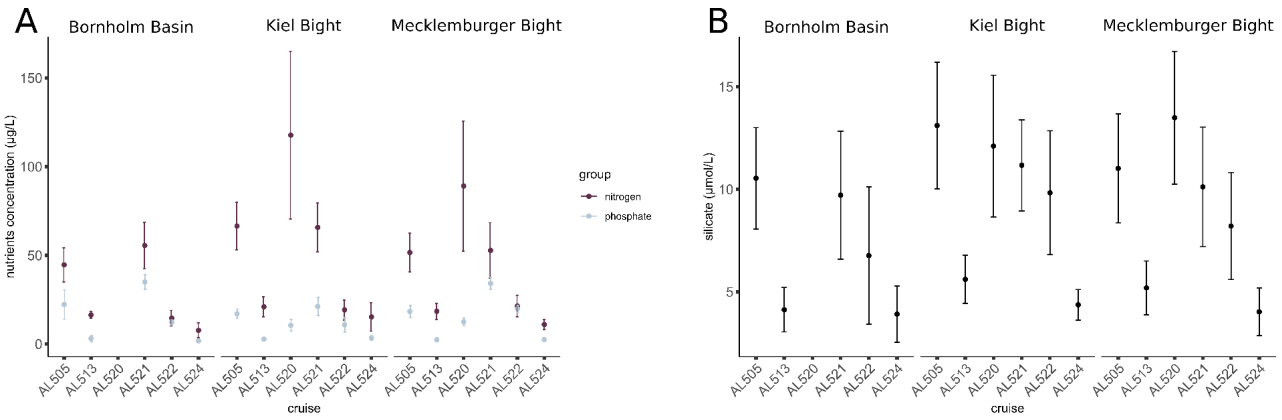


Fig S2.1.2. Nutrient concentrations at sampling locations during the cruises. (A) Total nitrogen (nitrate and nitrite), in purple and phosphate, in grey. Concentrations are expressed in µg L^{-1} while silicates (B) are expressed in µmol L^{-1}. Kiel Area is hereby presented as Mecklenburg Bight and Kiel Basin separately, to highlight the overall non-significant differences between the two areas (hence, throughout considered unitary as Kiel Area). Error bars indicate ±SD around the mean.

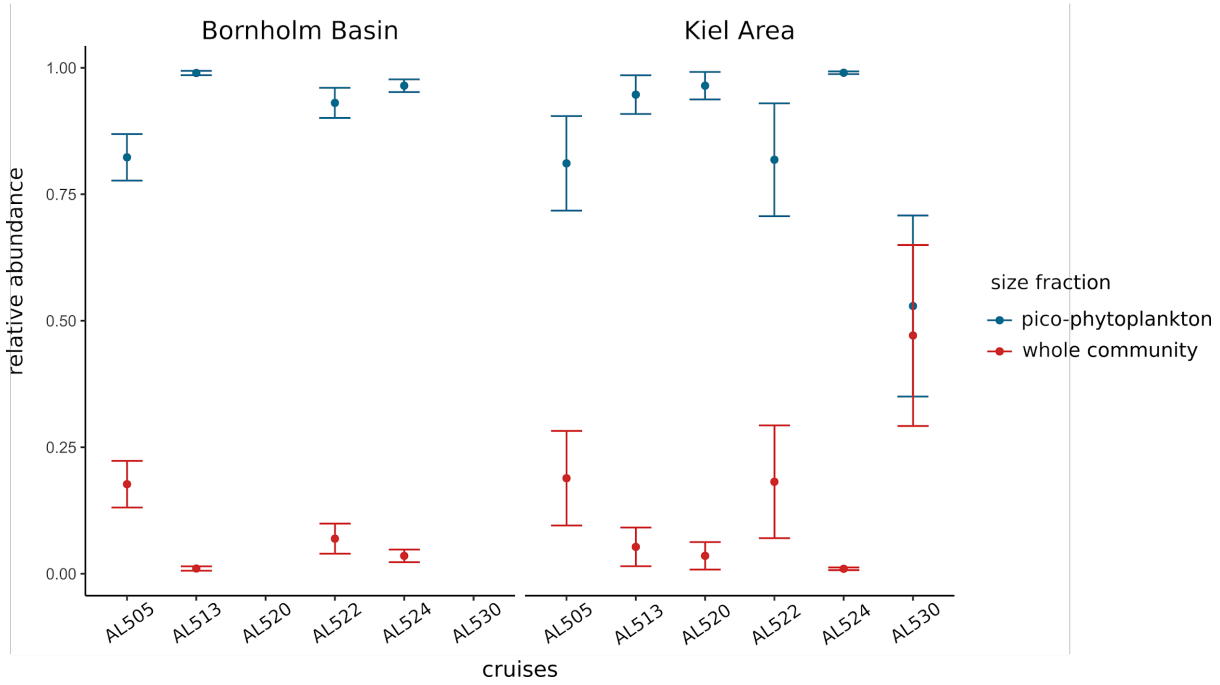


Fig S2.1.3. Relative abundance of pico-phytoplankton (in blue) and bigger (> 3 µm in diameter) organisms (in red) composing the whole community throughout the cruises. Contribution of the bigger cells was calculated from cytometer enumeration of events bigger than reference size beads. Error bars indicate ±SD around the mean.

Table S2.1.4. Outcome of model selections. Mean sampling temperature (meanT), size fraction (fraction), geographical areas (geo) and interaction between size fraction and geographical areas (fraction:geo) are here considered as fixed effects. The first line corresponds to the most parsimonious model. A plus states the variable is even slightly significant. Reduction was carried out using the function *dredge* in the *MuMIn* package (1.43.6) in the R environment. Models are ranked according to the AICc scores. Where delta AICc <2, models are averaged until delta AICc exceeds 2. (A,C) were fitted on the thermal optima for gross photosynthesis, excluding and including the heat wave event respectively. (B,D) were fitted on thermal optima for respiration, excluding and including the heatwave event respectively. (df: degree of freedom; logLik: log-likelihood; delta: delta AIC). The averaged models are highlighted in bold.

A Thermal optima gross photosynthesis excluding heatwave

Intercept	fraction	geo	meanT	fraction:geo	df	logLik	AICc	delta	weight
8.43	**+**	**NA**	**0.61**	**NA**	**5.00**	**-125.24**	**262.30**	**0.00**	**0.56**
10.21	+	+	0.55	NA	6.00	-124.88	264.39	2.09	0.20
12.93	NA	NA	0.41	NA	4.00	-129.02	267.21	4.92	0.05
10.47	+	+	0.55	+	7.00	-124.84	267.29	4.99	0.05
14.42	+	NA	NA	NA	4.00	-129.12	267.41	5.12	0.04
16.81	+	+	NA	NA	5.00	-127.83	267.47	5.17	0.04
16.49	NA	NA	NA	NA	3.00	-131.00	268.68	6.38	0.02
13.82	NA	+	0.38	NA	5.00	-128.94	269.70	7.41	0.01
18.17	NA	+	NA	NA	4.00	-130.43	270.03	7.74	0.01
16.83	+	+	NA	+	6.00	-127.82	270.27	7.98	0.01

B Thermal optima respiration excluding heatwave

Intercept	fraction	geo	meanT	fraction:geo	df	logLik	AICc	delta	weight
15.72	**NA**	**NA**	**NA**	**NA**	**3**	**-119.27**	**245.26**	**0.00**	**0.42**
14.42	**NA**	**NA**	**0.17**	**NA**	**4**	**-118.90**	**247.05**	**1.79**	**0.17**
15.31	NA	+	NA	NA	4	-119.21	247.68	2.42	0.13
15.94	+	NA	NA	NA	4	-119.24	247.73	2.47	0.12
13.92	NA	+	0.17	NA	5	-118.83	249.60	4.34	0.05
14.61	+	NA	0.16	NA	5	-118.88	249.70	4.44	0.05
15.53	+	+	NA	NA	5	-119.18	250.30	5.04	0.03
14.12	+	+	0.17	NA	6	-118.81	252.41	7.16	0.01
15.17	+	+	NA	+	6	-119.13	253.06	7.80	0.01
13.86	+	+	0.16	+	7	-118.77	255.41	10.15	0.00

C Thermal optima gross photosynthesis including heatwave

Intercept	fraction	geo	meanT	fraction:geo	df	logLik	AICc	delta	weight
9.06	**+**	**NA**	**0.58**	**NA**	**5**	**-166.43**	**344.22**	**0.00**	**0.40**
11.26	**+**	**+**	**0.56**	**+**	**7**	**-163.82**	**344.31**	**0.10**	**0.38**
9.53	+	+	0.56	NA	6	-166.38	346.72	2.50	0.11
12.37	NA	NA	0.49	NA	4	-169.29	347.46	3.25	0.08
11.91	NA	+	0.50	NA	5	-169.26	349.88	5.67	0.02
19.50	+	+	NA	+	6	-170.24	354.43	10.22	0.00
17.97	NA	NA	NA	NA	3	-174.05	354.61	10.40	0.00
16.54	+	NA	NA	NA	4	-173.02	354.93	10.72	0.00
17.99	+	+	NA	NA	5	-172.21	355.78	11.56	0.00
19.12	NA	+	NA	NA	4	-173.70	356.30	12.08	0.00

D Thermal optima respiration including heatwave

Intercept	fraction	geo	meanT	fraction:geo	df	logLik	AICc	delta	weight
10.77	**NA**	**NA**	**0.76**	**NA**	**4**	**-164.01**	**336.92**	**0.00**	**0.55**
9.86	NA	+	0.77	NA	5	-163.79	338.98	2.06	0.20
10.41	+	NA	0.76	NA	5	-163.92	339.24	2.32	0.17
9.56	+	+	0.77	NA	6	-163.72	341.45	4.53	0.06
9.77	+	+	0.77	+	7	-163.70	344.13	7.20	0.02
19.02	NA	NA	NA	NA	3	-173.86	354.25	17.33	0.00
18.49	+	NA	NA	NA	4	-173.73	356.37	19.44	0.00
18.97	NA	+	NA	NA	4	-173.86	356.62	19.70	0.00
18.49	+	+	NA	NA	5	-173.73	358.85	21.93	0.00
18.40	+	+	NA	+	6	-173.73	361.45	24.53	0.00

4.2 Supplementary Information Study II

Isolation of *Ostreococcus* spp.

Upon returning to the laboratory, we performed serial dilutions to isolate clonal isolates of single strains of *Ostreococcus* spp. Pico-phytoplankton assemblages were counted on a flow cytometer (BD Accuri C6 Plus). Cells were gated according to cytometric fingerprints (based on FSC and FL3 signals) first established using known cultures of the species of interest. The samples were then diluted with f/2 media of respective salinity to obtain a desired number of cells (0.5 cells/well) in 96-multiwell plates. The plates were kept in shaking incubators (Multitron, Infors HT) at a temperature close to the sampling one with a 12:12h dark:light cycle at 100 $\mu mol^{-1}\ m^{-2}\ s^{-1}$. This procedure was repeated at least twice to ensure clonality. When cellular densities increased, suitable strains were selected first according to cytometric fingerprints and then sequenced to ensure the presence of *Ostreococcus*. We isolated 4 strains, 3 from the Kiel Area and 1 from the Bornholm Basin.

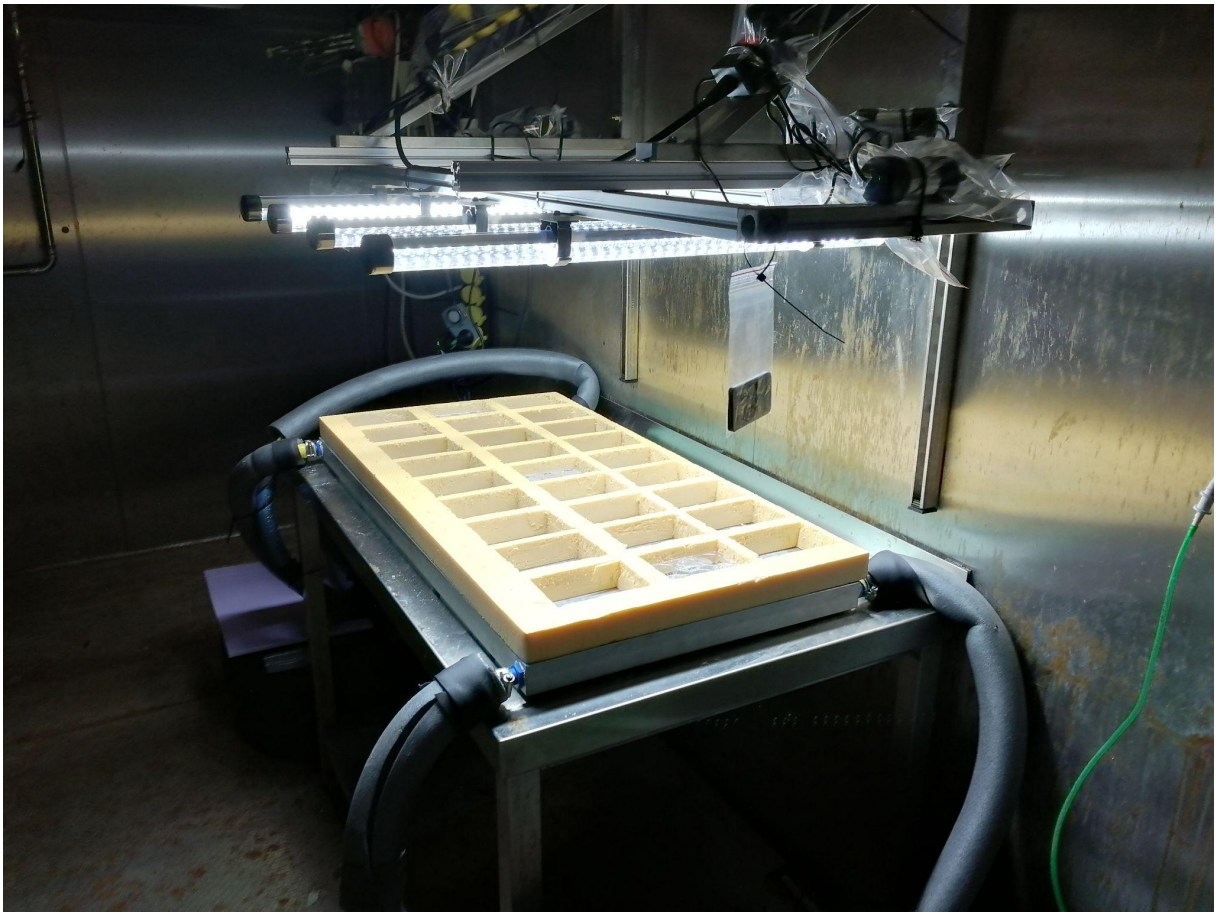


Figure S2.2.1. Picture of the temperature gradient table used for the experimental set-up.

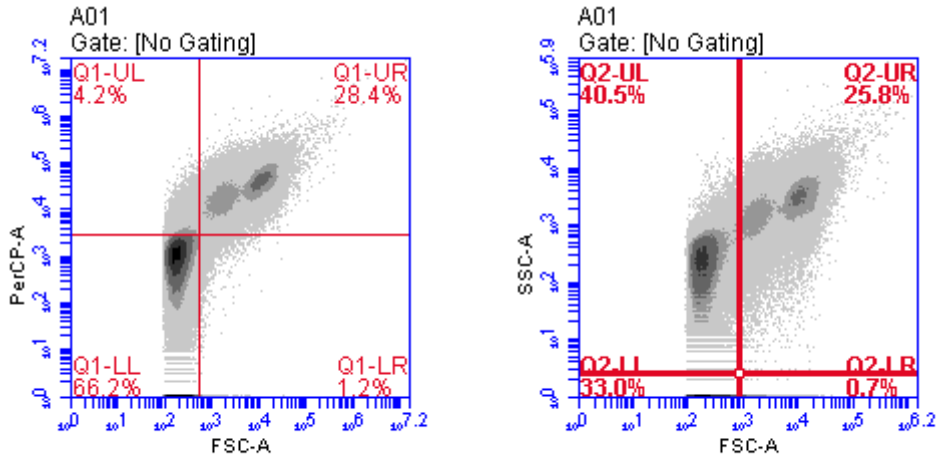


Figure S2.2.2: An example of FL3 (PerCP) against FSC and SSC against FSC plots. Gating strategy is depicted here with the Q2-UL gatings corresponding to the bacteria and debris gate as previously assessed (see Santelia et al, preprint). For further analysis throughout the paper, we considered the Q2-UR gate.

Table S2.2.1. Outcomes of GAMs for growth rates. Temperature is here considered as smooth terms and geographical area and sampling stations (nested; geostation) are considered as fixed effects. Cruise 1 and 2 refer respectively to winter and summer cruise (df: degree of freedom; F: f-value).
formula: gr.mean~cruise*geostation+s(assayT, by=geo.cruise, bs= cr, k=3)

Geographical area: cruise	edf	Ref.df	F	p-value	
Kiel Area:cruise 1	1.93	2	8.17	0.000308	***
Kiel Area:cruise 2	1	1	0.06	0.812642	
Bornholm Basin:cruise 1	1.95	2	16.03	1.81E-07	***
Bornholm Basin:cruise 2	1.97	2	20.97	2.46E-09	***

Rank: 16/24		
R-sq.(adj) = 0.325	Deviance explained = 35.90%	
GCV=0.029296	Scale est. = 0.027697	n = 272

Table S2.2.2: Outcome of GAMs on alpha diversity indexes.

Assay temperature and geographical area (Kiel Area and Bornholm Basin) are considered as fixed effects. Table A refers to winter cruise (cruise 1) and Table B to summer cruise (cruise 2)

A

Geographical area	edf	Ref.df	F	p-value
Bornholm Basin	1	1	1.277	0.261
Kiel Area	1	1	0.575	0.449

R-sq.(adj) = 0.00094	Deviance explained = 2.30%
GCV = 4.90E+05	Scale est. = 4.76E+05 n = 137

B

Geographical area	edf	Ref.df	F	p-value	
Bornholm Basin	1	1	105.05	<2.00E-16	***
Kiel Area	1.707	1.914	4.694	0.00772	**

R-sq.(adj) = 0.608	Deviance explained = 62.30%
GCV = 76846	Scale est. = 72998 n = 94

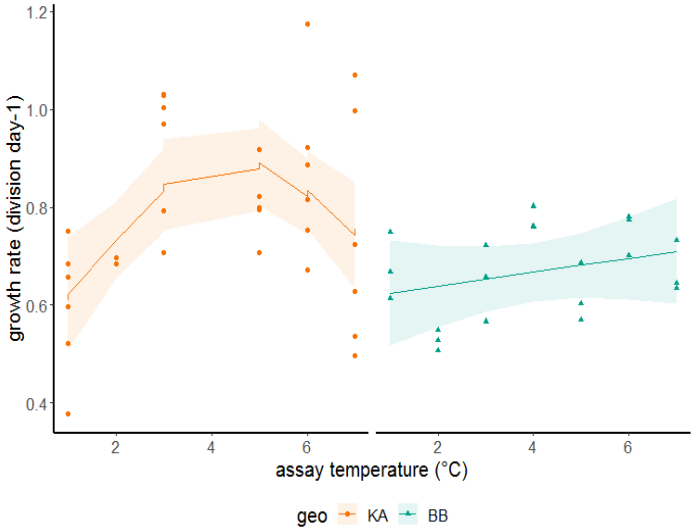


Figure S2.2.3 : growth rates over assayed temperatures for *Ostreococcus spp.* isolates. The two geographical area of interests are depicted in orange (Kiel Area) and blue (Bornholm Basin). Individual points are single replicates of different samples pooled by geographical area. We fitted a GAM (generalized additive model) model including geographical area and assayed temperatures as fixed effects. The shaded areas depict the 95% confidence interval

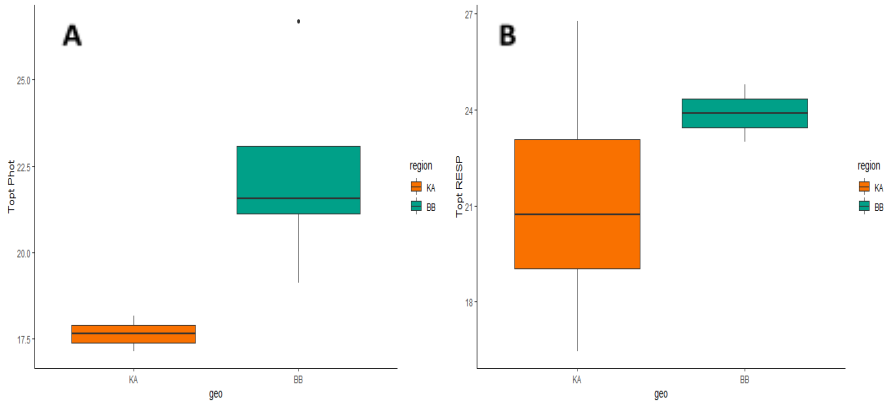


Fig. S2.2.5: Thermal optima (Topt) for gross photosynthesis (A) and respiration (B) for each geographical area (Kiel Area and Bornholm Basin, respectively depicted as KA and BB) for Ostreococcus spp. isolates. The two areas are colored in orange (Kiel Area) and blue (Bornholm Basin). Boxplots are displayed as standard: bold lines represent the media, whiskers the highest and lowest values in the lower and upper quartile and boxes the ends of the lower and upper quartile.

4.3 Supplementary Information Study III

Table S2.3.1. Detailed information regarding the different treatment conditions used during the experiment.

Treatment id	Amplitude	Frequency
CC	22°C	—----------
RR	Unpredictable (range of temperatures: 15 - 18 - 20 - 21 - 22 - 24 -26°C)	Unpredictable (range of generations: 4 - 5 - 6 - 7 - 8 - 9 - 10 - 11 generations)
RP	Predictable (temperature cycle: 15 - 22 - 24 - 26°C)	Unpredictable (range of generations: 4 - 5 - 6 - 7 - 8 - 9 - 10 - 11 generations)
PR	Predictable (temperature cycle: 15 - 22 -	Unpredictable (range of generations: 4 - 5 - 6 - 7 - 8 - 9 - 10 - 11 generations)
PP	Predictable (temperature cycle: 15 - 22 - 24 - 26°C)	Predictable (10 generations)

CC	22°C	22°C	22°C	22°C	22°C	22°C	22°C	22°C	22°C	22°C	22°C	22°C	22°C	22°C	22°C	22°C	22°C	22°C	22°C	22°C	22°C	22°C	22° C	22° C	22° C	22° C	22° C	22° C
RR	10 gen 15°C	5 gen 22°C	7 gen 24°C	8 gen 22°C	8 gen 24°C	11 gen 15°C	7 gen 21°C	5 gen 18°C	9 gen 26°C	10 gen 20°C	4 gen 20°C	5 gen 18°C	5 gen 22°C	7 gen 15°C	11 gen 24°C	10 gen 20°C	10 gen 21°C	11 gen 15°C	8 gen 21° C	4 gen 24°C	7 gen 26°C	4 gen 22°C	6 gen 21° C	5 gen 24° C	6 gen 22° C	9 gen 21° C	11 gen 20° C	5 gen 26° C
RP	11 gen 15°C	7 gen 22°C	8 gen 24°C	8 gen 26°C	4 gen 15°C	10 gen 22°C	7 gen 24°C	8 gen 26°C	6 gen 15°C	8 gen 22°C	8 gen 24°C	7 gen 26°C	11 gen 15°C	10 gen 22°C	8 gen 24°C	7 gen 26°C	4 gen 15°C	11 gen 22°C	11 gen 24°C	8 gen 26°C	6 gen 15°C	7 gen 22°C	10 gen 24° C	9 gen 26° C	8 gen 15° C	6 gen 22° C	10 gen 24° C	5 gen 26° C
PR	10 gen 15°C	10 gen 20°C	10 gen 18°C	10 gen 20°C	10 gen 18°C	10 gen 24°C	10 gen 20°C	10 gen 22°C	10 gen 18°C	10 gen 26°C	10 gen 21°C	10 gen 22°C	10 gen 26°C	10 gen 21°C	10 gen 24°C	10 gen 15°c	10 gen 15°C	10 gen 21°C	10 gen 22°C	10 gen 24°C	10 gen 22°C	10 gen 26°C	10 gen 24°c	10 gen 21° C	10 gen 22° C	10 gen 24° C	10 gen 20° C	10 gen 18° C
PP	10 gen 15°C	10 gen 22°C	10 gen 24°C	10 gen 26°C	10 gen 15°C	10 gen 22°C	10 gen 24°C	10 gen 26°C	10 gen 15°C	10 gen 22°C	10 gen 24°C	10 gen 26°C	10 gen 15°C	10 gen 22°C	10 gen 24°C	10 gen 26°C	10 gen 15°C	10 gen 22°C	10 gen 24°C	10 gen 26°C	10 gen 15°C	10 gen 22°C	10 gen 24° C	10 gen 26° C	10 gen 15° C	10 gen 22° C	10 gen 24° C	10 gen 26° C

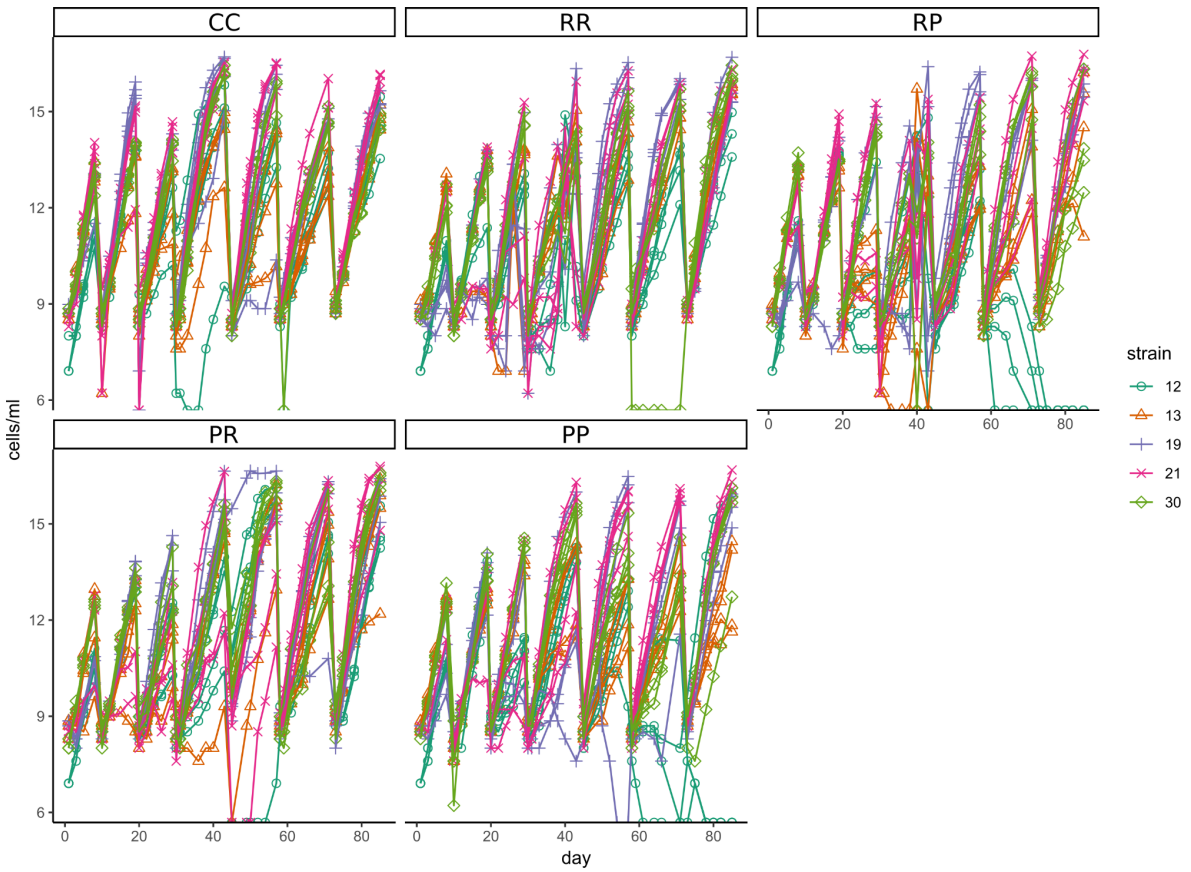


Figure S2.3.1 Growth curves for all strains and replicates in the different treatments (see methods for details about the acronyms used).

Table S2.3.2: **Outcome of GAMs on regression slopes beginning-end of growth rates trajectories.**

Species (*O. tauri* and *O. mediterraneus*) and geographical area (Kiel Area and Bornholm Basin) are considered as fixed effects.

Formula: slopes ~ geo * species + s(treat, by = geo.species, bs = 'cr'), random = ~ 1|strain/rep

	(Intercept)	geo	s(week)	species	treat	geo:species	geo:treat	species:treat	geo:species:treat	df	logLik	AICc	delta	weight
256	**0.45**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**23 1**	**232 5.5**	**-41 80**	**0.0**	**1.0**
128	0.43	+	+	+	+	+	+	+		22 7	230 3.82	-41 44	35. 2	0.0
96	0.43	+	+	+	+	+		+		22 3	229 3.19	-41 31	49. 1	0.0
64	0.45	+	+	+	+	+	+			22 2	228 3.52	-41 13	67. 1	0.0
112	0.45	+	+	+	+		+	+		22 5	228 5.24	-41 10	70. 0	0.0

Table S2.3.3: **Outcome of GAMs on growth rates trajectories.**

Species (*O. tauri* and *O. mediterraneus*), geographical area (depicted as "geo" , representing Kiel Area and Bornholm Basin) and selection treatments ("treat") are considered as fixed effects.

Formula: growth rates ~ treat * geo * species + s(week, by = treat.geo.species, bs = 'cr'), random = ~1|treat.geo.species/rep

Intercept	geo	s(week)	species	treat	geo:species	geo:treat	species:treat	geo:species:treat	df	logLik	AICc	delta	weight
0.45	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**231**	**2325.5**	**-4179.6**	**0.0**	**1.0**
0.43	+	+	+	+	+	+	+		227	2303.8	-4144.5	35.2	0.0
0.43	+	+	+	+	+		+		223	2293.2	-4130.5	49.1	0.0
0.45	+	+	+	+	+	+			222	2283.5	-4112.6	67.1	0.0
0.45	+	+	+	+		+	+		225	2285.2	-4109.6	70.0	0.0
0.45	+	+	+	+			+		221	2275.0	-4097.5	82.2	0.0
0.47	+	+	+	+		+			221	2264.9	-4077.5	102.2	0.0
0.46	+	+	+	+	+				214	2248.2	-4058.4	121.2	0.0
0.47	+	+	+	+					213	2230.6	-4025.2	154.4	0.0
0.48	<NA>	+	+	+			+		221	2211.8	-3972.9	206.7	0.0

Table S2.3.4. **Outcome of GAMs on rates of net photosynthesis (NP).**
Geographical area (depicted as "geo" , representing Kiel Area and Bornholm Basin) and selection treatments ("treat") are considered as fixed effects. Table **A** depicts the summary for net photosynthesis rates after 20 generations of selection, **B** after 50 generations and **C** after 120 generations.

Formula: logNP ~ treat * geo + s(week, by = treat.geo.species, bs = 'cr'), random = ~1|treat.geo.species/rep

A

Smooth term	edf	Ref.df	F	p-value	
s(assayT):treat.geoCC:BB	0.16844	0.16844	0.273	0.83042	
s(assayT):treat.geoCC:KA	0.08847	0.08847	0	0.99728	
s(assayT):treat.geoRR:BB	1.91014	1.99192	5.209	0.00751	**
s(assayT):treat.geoRR:KA	1.59242	1.83388	0.818	0.47277	
s(assayT):treat.geoRS:BB	1	1	0.246	0.62054	
s(assayT):treat.geoRS:KA	1.6065	1.84516	0.977	0.27149	
s(assayT):treat.geoSR:BB	1	1	0.016	0.90086	
s(assayT):treat.geoSR:KA	1	1	0.041	0.84067	
s(assayT):treat.geoSS:BB	1.57868	1.82249	0.819	0.48451	
s(assayT):treat.geoSS:KA	1.37936	1.6148	0.483	0.67217	

R-sq.(adj) = 0.102 Deviance explained = 19%

GCV = 4.0021 Scale est. = 3.5888 n = 204

B

Smooth term	edf	Ref.df	F	p-value	
s(assayT):treat.geoCC:BB	0.2356	0.2356	0	0.9917	
s(assayT):treat.geoCC:KA	1.6015	1.8412	0.933	0.4719	
s(assayT):treat.geoRR:BB	1.3945	1.6333	0.767	0.5663	
s(assayT):treat.geoRR:KA	1.8325	1.972	2.468	0.0894	.
s(assayT):treat.geoRS:BB	1.8603	1.9805	4.56	0.0176	*
s(assayT):treat.geoRS:KA	1.1254	1.2351	1.424	0.2786	
s(assayT):treat.geoSR:BB	1.8093	1.9636	3.094	0.0698	.
s(assayT):treat.geoSR:KA	1	1	0.08	0.778	
s(assayT):treat.geoSS:BB	1	1	0.283	0.595	
s(assayT):treat.geoSS:KA	1	1	0.368	0.5448	

R-sq.(adj) = 0.0758 Deviance explained = 15.8%

GCV = 8.49 Scale est. = 7.71 n = 245

C

Smooth term	edf	Ref.df	F	p-value
s(assayT):treat.geoCC:BB	1	1	1.2	0.2744
s(assayT):treat.geoCC:KA	1	1	2.967	0.0862 .
s(assayT):treat.geoRR:BB	1	1	0.876	0.3502
s(assayT):treat.geoRR:KA	1	1	0.017	0.8972
s(assayT):treat.geoRS:BB	1	1	0.08	0.7772
s(assayT):treat.geoRS:KA	1	1	1.19	0.2764
s(assayT):treat.geoSR:BB	1	1	0.255	0.6143
s(assayT):treat.geoSR:KA	1	1	0.102	0.75
s(assayT):treat.geoSS:BB	1	1	2.588	0.109
s(assayT):treat.geoSS:KA	1	1	2.078	0.1507

R-sq.(adj) = 0.0749 Deviance explained = 14.10%

GCV = 5.2922 Scale est. = 4.8943 n = 266

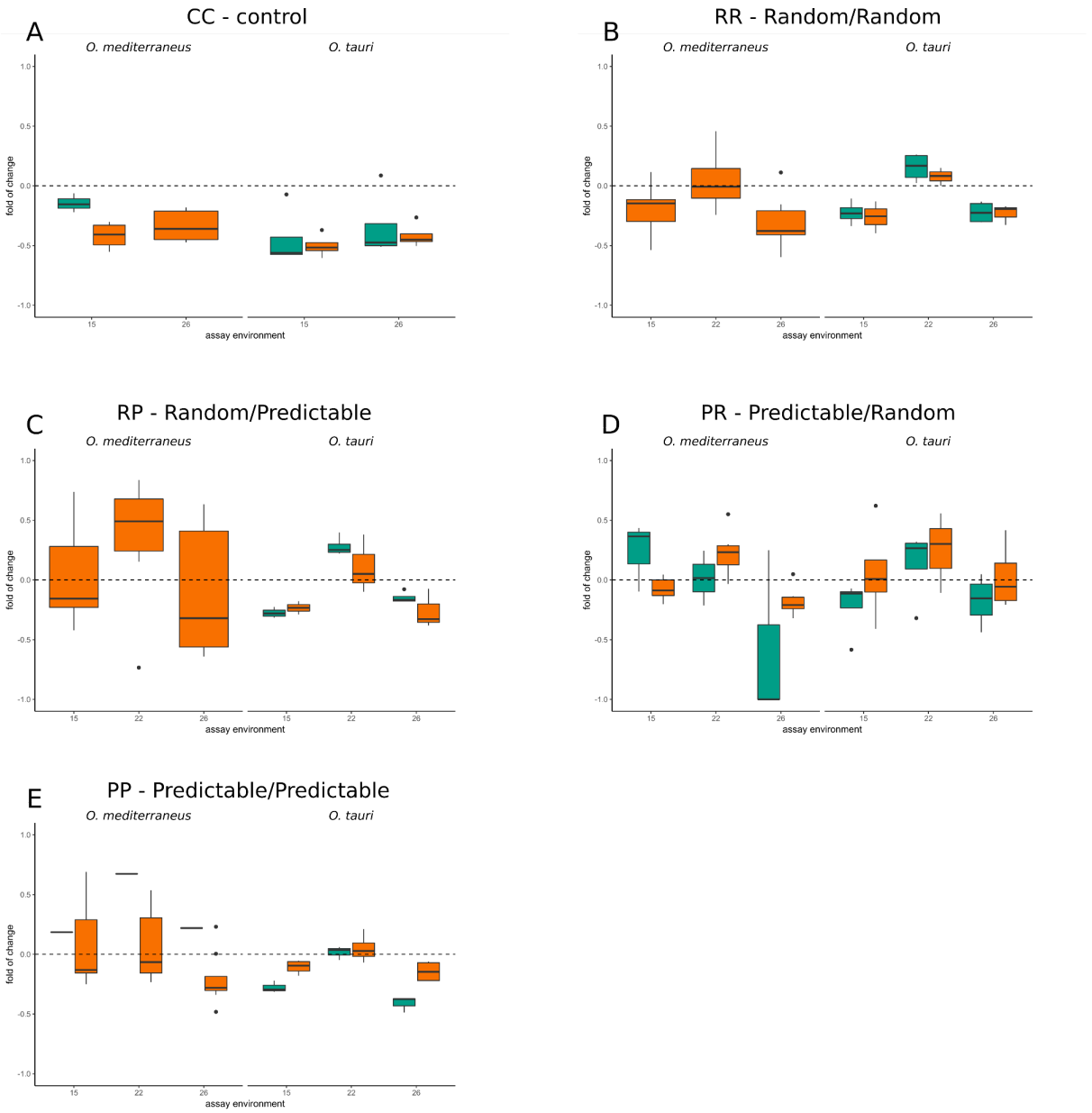


Figure S2.3.2. Short-term plastic responses after 20 generations of selection for *O. mediterraneus* and *O. tauri* strains. **A** panel shows responses for CC (control) treatment, **B** for RR (Unpredictable timing/Unpredictable amplitude) treatment, **C** for RP (Unpredictable timing/Predictable amplitude) treatment, **D** for PR (Predictable timing/Unpredictable amplitude) treatment and **E** for PP (Predictable timing/Predictable amplitude) treatment. The dashed line at 0 indicates no changes in the short-term responses. Negative values indicate a decrease in short-term plastic response and positive values an increase. Colours are displayed as for the other plots (orange for the Kiel Area and blue for the Bornholm Basin). Boxplots are displayed as is standard.

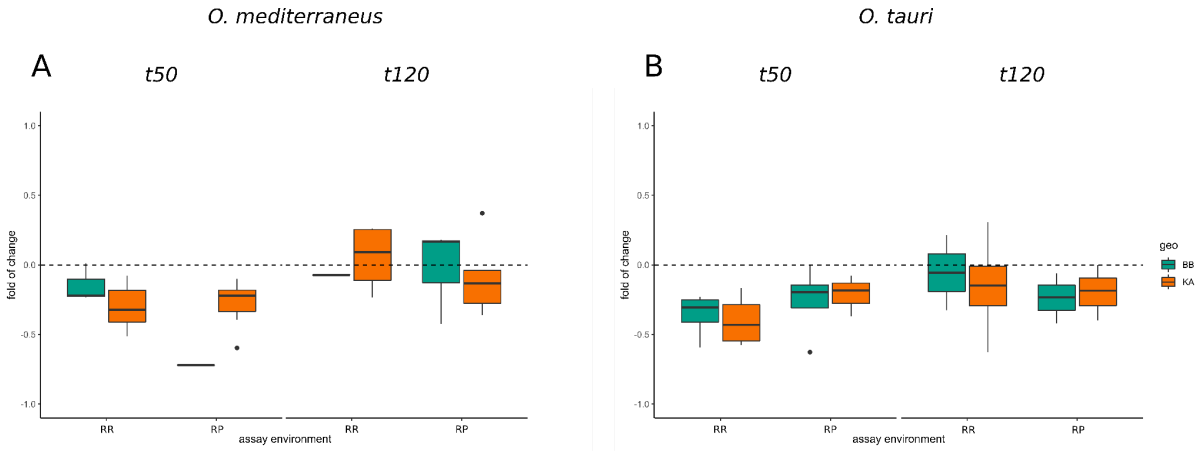


Figure S2.3.3. Short-term plastic responses of PR evolved samples assayed in the RR and RP treatment. **A** panel shows responses for *O. mediterraneus* after 50 and 120 generations of selection, **B** for *O. tauri* after 50 and 120 generations. The dashed line at 0 indicates no changes in the short-term responses. Negative values indicate a decrease in short-term plastic response and positive values an increase. Colours are displayed as for the other plots (orange for the Kiel Area and blue for the Bornholm Basin). Boxplots are displayed as is standard.

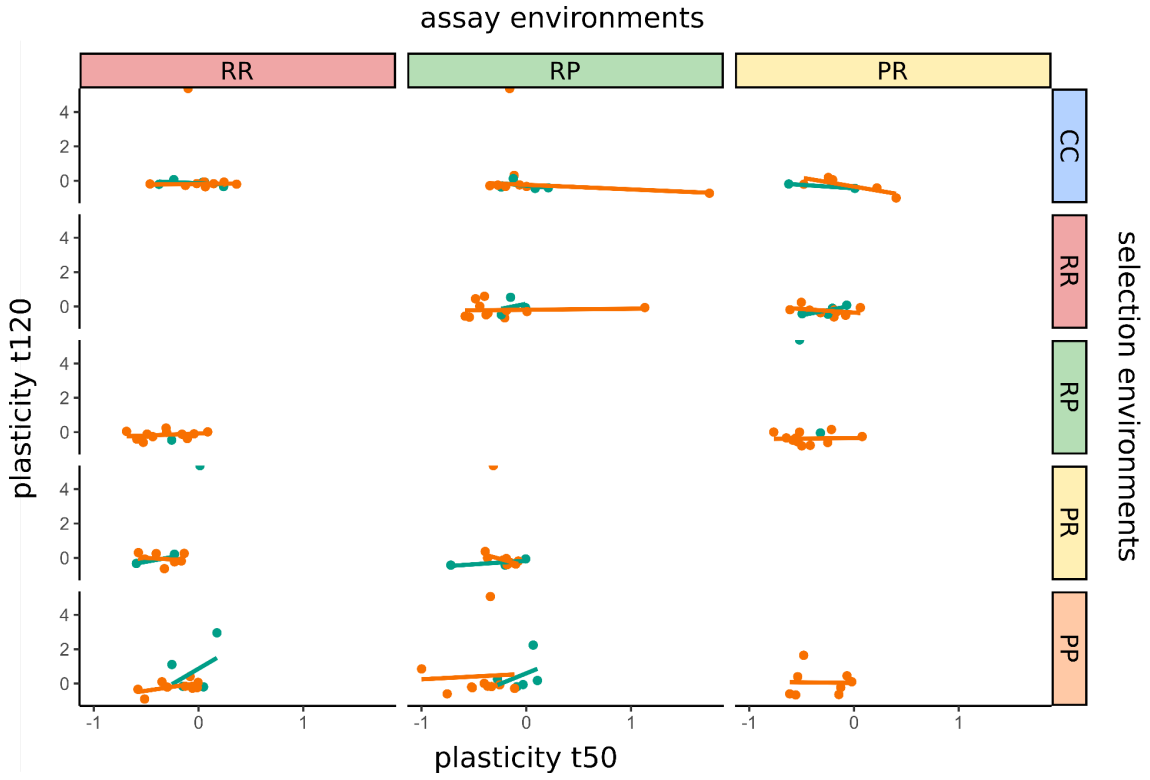


Figure S2.3.4. Correlation plots between plasticity (calculated as short-term responses as reported in the text) at t50 and t120 (after, respectively, 50 and 120 generations of selection). Colours represent the two geographical areas (orange for Kiel Area and blue Bornholm Basin) and shape represent the two species (circles for *O. mediterraneus* and triangles for *O. tauri*).

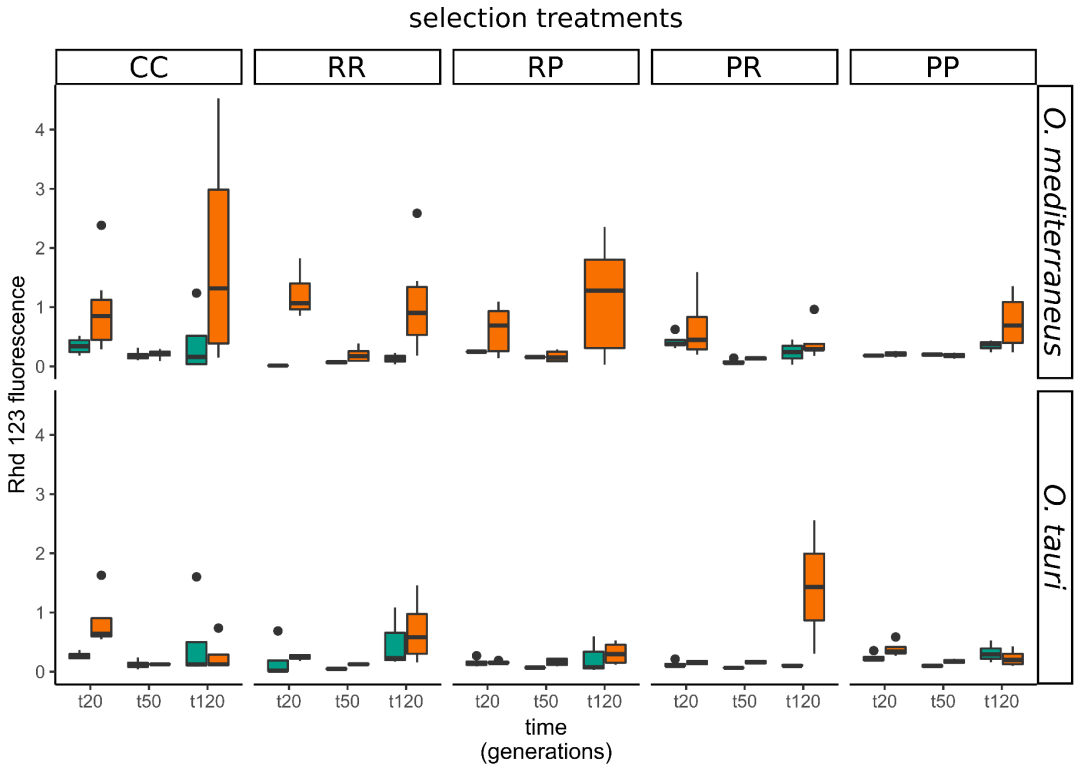
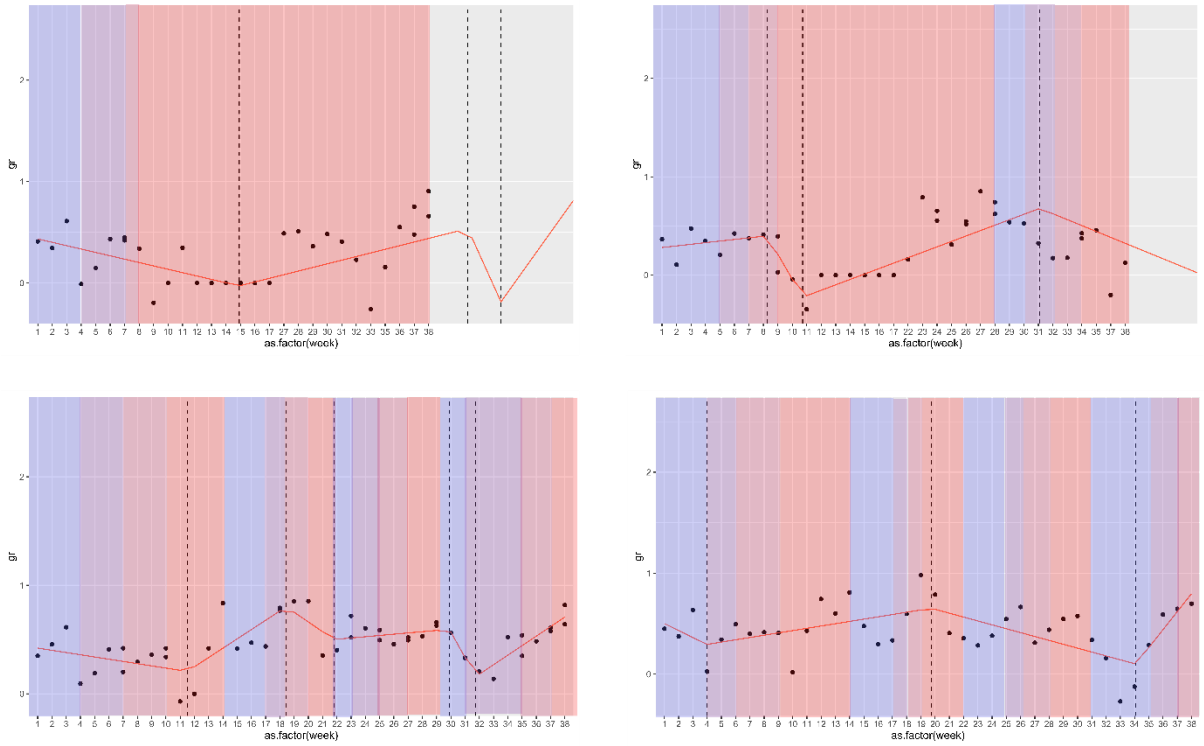


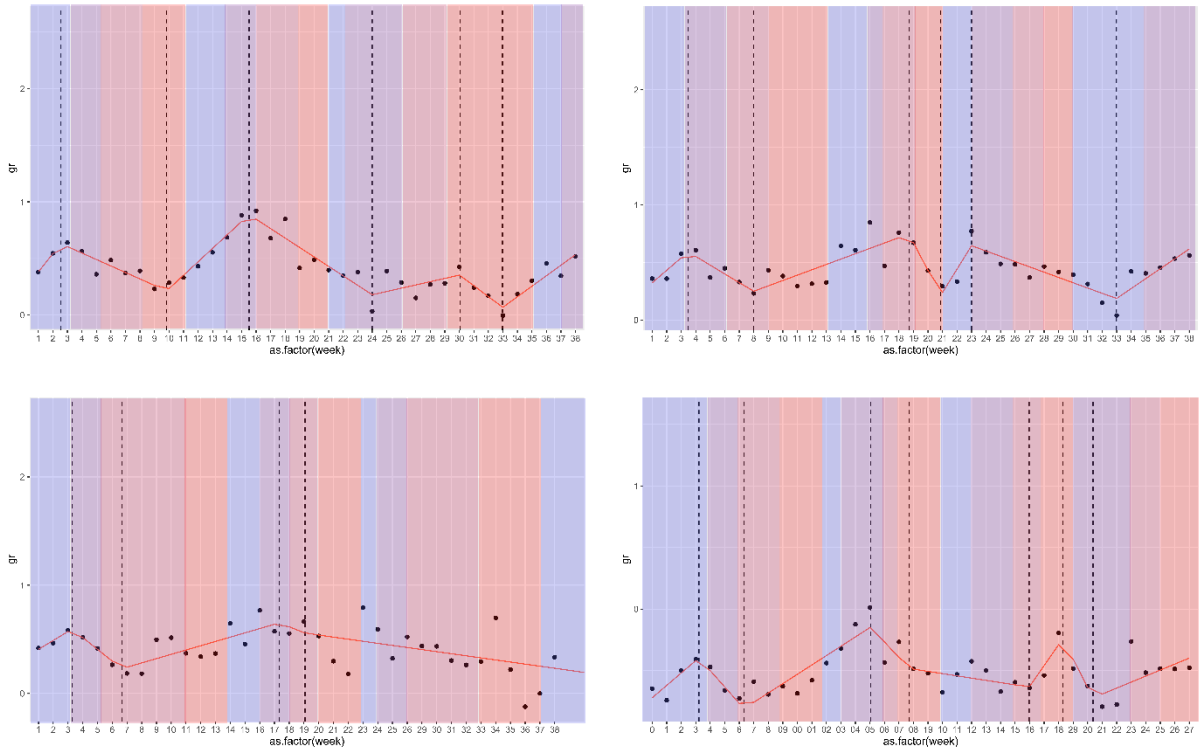
Figure S2.3.5. Rhodamine 123 fluorescence for all the selection treatments (CC, RR, RP, PR and PP) and the two species (*O. mediterraneus* and *O. tauri*) measured after 20, 50 and 120 generations of selection. Colours represents the geographical area of origin, with Kiel Area in orange and Bornholm Basin in blue. Boxplots are depicted as standard.

Figure S2.3.6. Fitted lines of the segmented regression analysis of time series for the growth rates. Points represent the weekly average growth rates. Colours indicate the temperature experienced at the time (from 15 to 26°C), indicated as a gradient from blue (colder) to red (warmer). The analysis was fitted singularly on each biological replicate of each strain. **A** depicts strain 12, **B** depicts strain 13, **C** depicts strain 30, **D** depicts strain 21, **E** depicts strain 19.

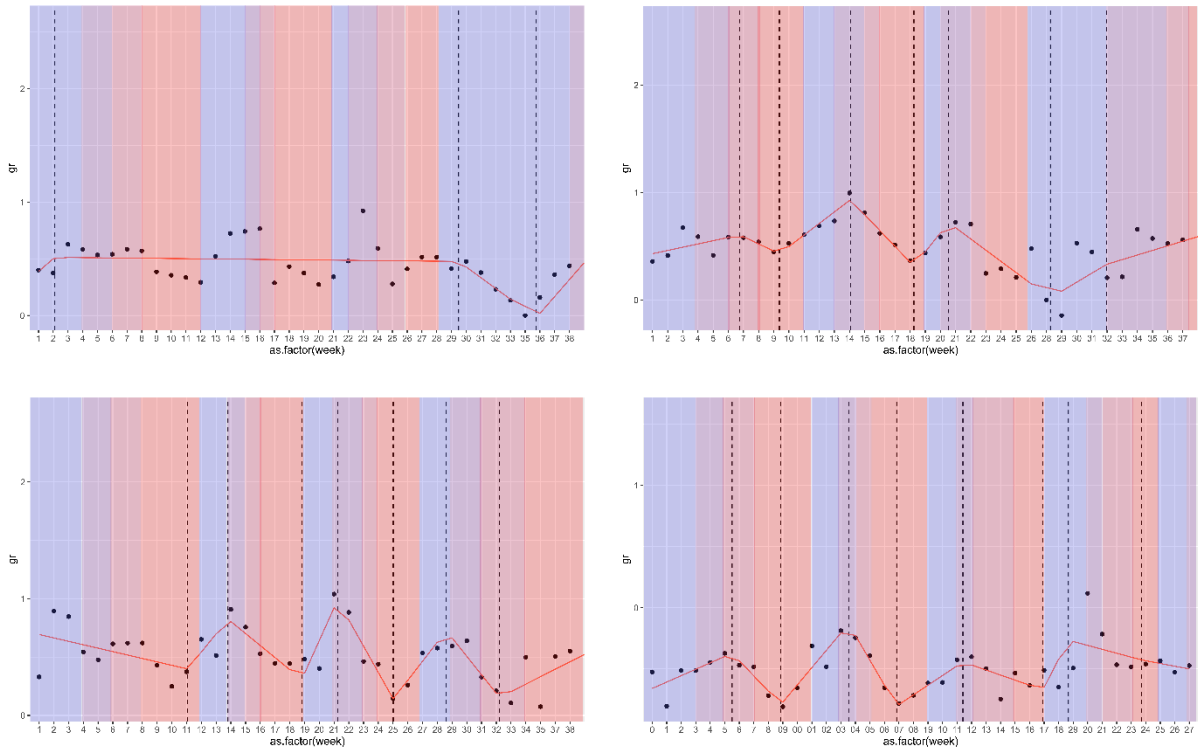
A Strain 12 (*O. mediterraneus* from the Bornholm Basin). The four panels represent each of the single biological replicates.



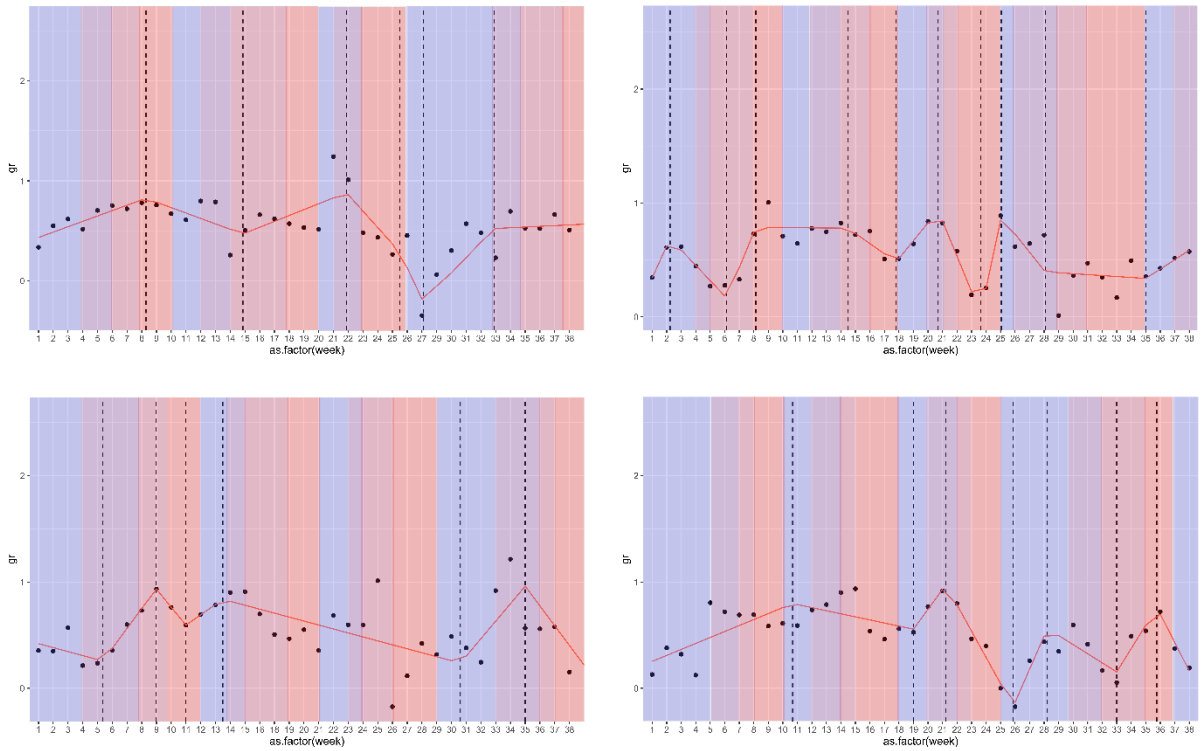
B Strain 13 (*O. mediterraneus* from the Kiel Area). The four panels represent each of the single biological replicates.



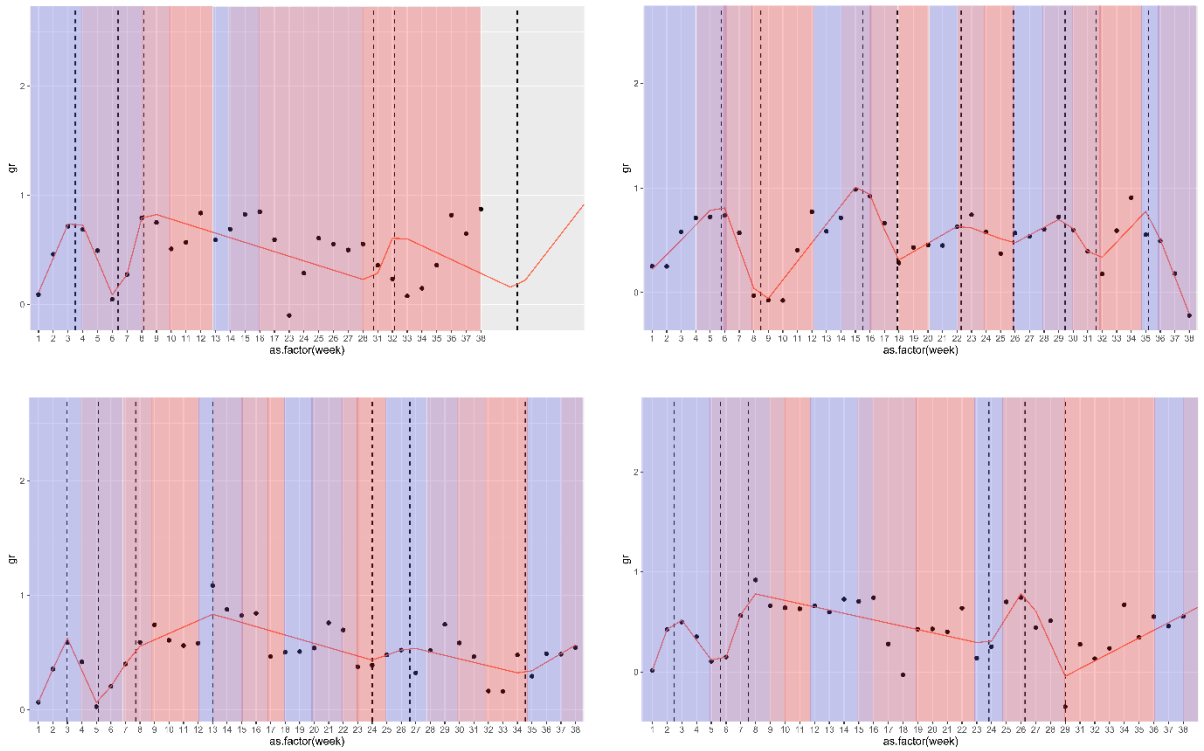
C Strain 30 (*O. mediterraneus* from the Kiel Area). The four panels represent each of the single biological replicates.



D Strain 21 (*O. tauri* from the Kiel Area). The four panels represent each of the single biological replicates.



E Strain 19 (*O. tauri* from the Bornholm Basin). The four panels represent each of the single biological replicates.



4.4 Supplementary Information Study IV

Figure S2.4.1 Analysis of single traits changes over time (after 20, 50 and 120 generations of selection) for each geographical area (Kiel Area, in orange, and Bornholm Basin, in blue). The 9 traits are the same used to build the trait-scape: size, or cellular diameter (**A**), chlorophyll a content (**B**), mitochondrial potential, or rhodamine 123 content (**C**), granularity, or internal cellular complexity (**D**), phycoerythrin content (**E**), growth rates (**F**), Ek (**G**), alpha (**H**) and rPm (**I**). Boxplots are displayed as standard.

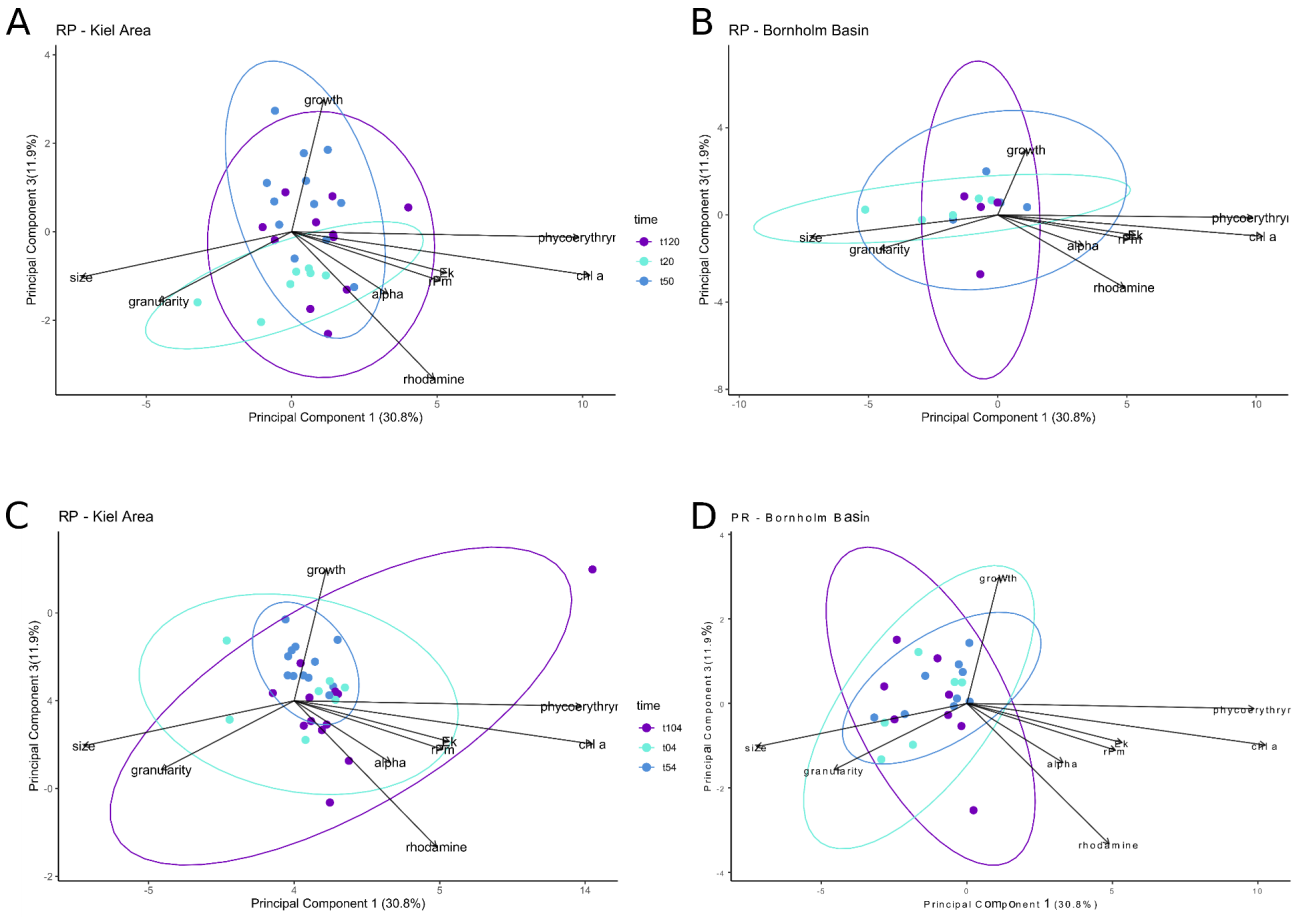


Figure S2.4.2.Trait-scape created from 9 traits. Traits were assessed in samples of *Ostreococcus spp.* in the RP (unpredictable timing, predictable amplitude; **A-B**) and in the PR (predictable timing, unpredictable amplitude; **C-D**) treatment. Trait-scapes are displayed for samples from the thermally unpredictable Kiel Area (**A and C**) and from the more predictable Bornholm Basin (**B and D**). Colours identify the time points at which the traits were assessed (t20, t50 and t120, corresponding respectively to 20, 50 and 120 generations of selection). Ellipses represent the 95% confidence intervals.

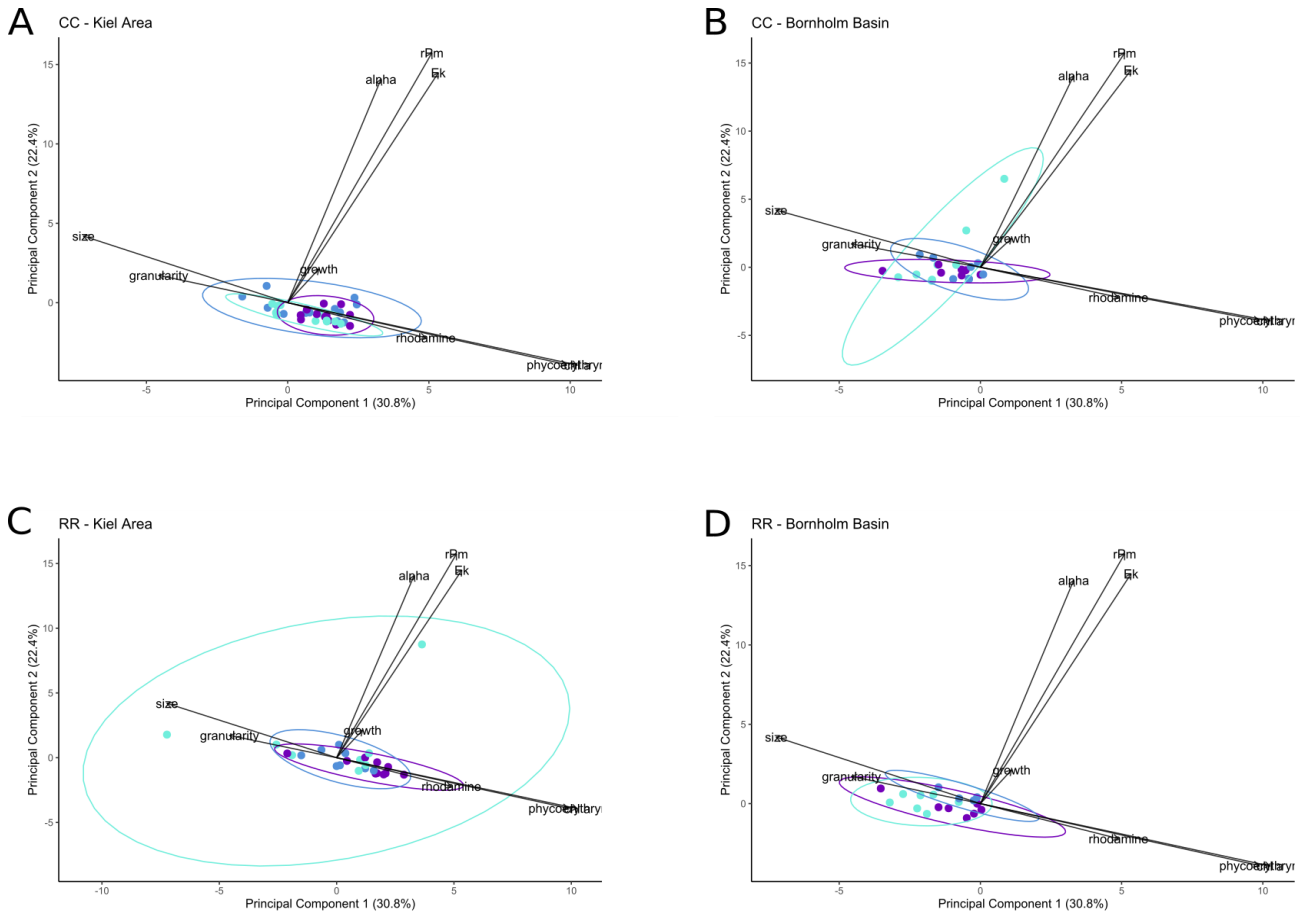


Figure S2.4.3.Trait-scape created from 9 traits displaying phenotypic movements along PC1 and PC2. Traits were assessed in samples of *Ostreococcus spp.* in the CC (control; **A and B**), RR (unpredictable timing, unpredictable amplitude; **C and D**) treatment. Trait-scapes are displayed for samples from the thermally unpredictable Kiel Area (**left side**) and from the more predictable Bornholm Basin (**right side**). Colours identify the time points at which the traits were assessed (t20, t50 and t120, corresponding respectively to 20, 50 and 120 generations of selection). Ellipses represent the 95% confidence intervals.

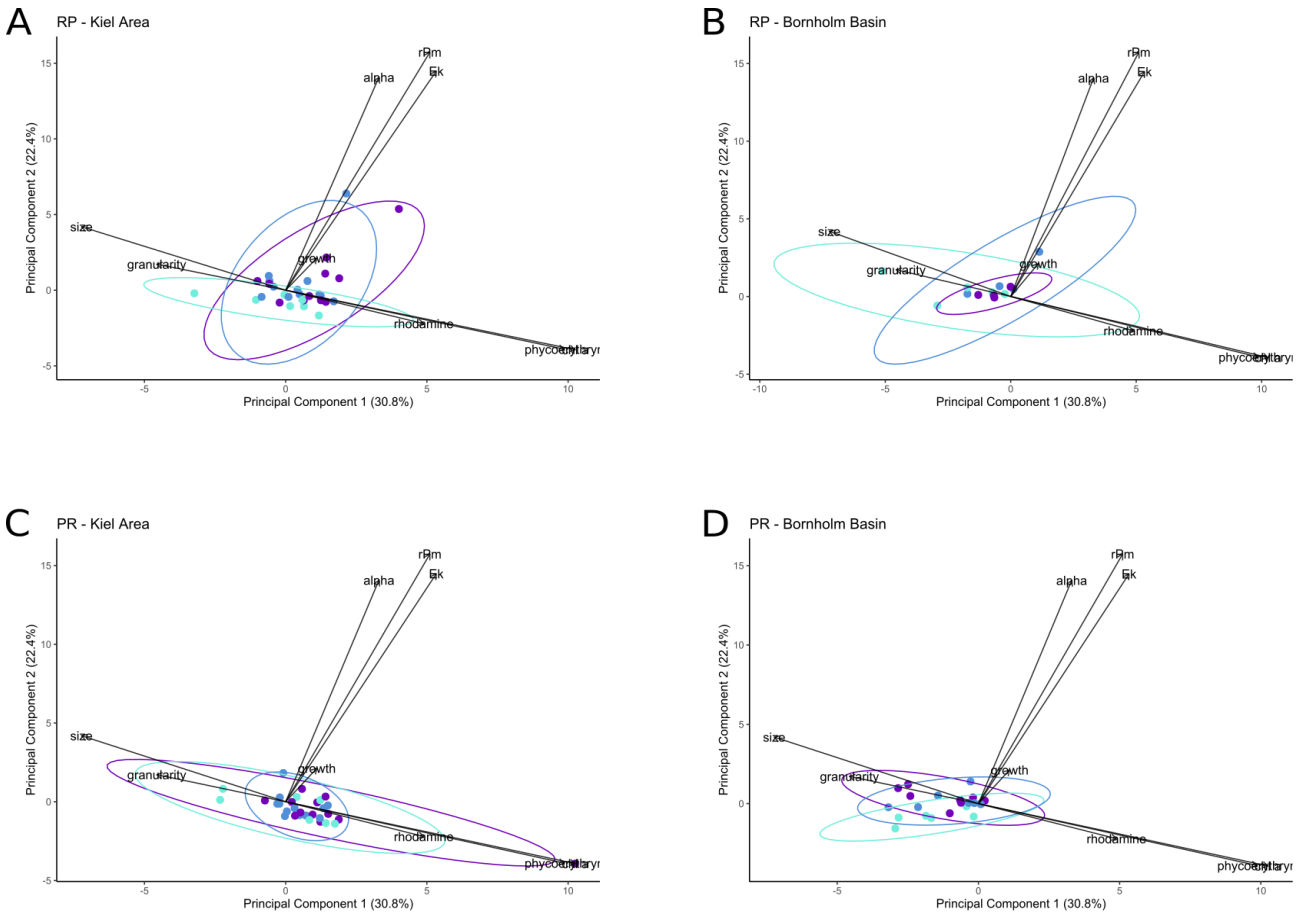


Figure S2.4.4.Trait-scape created from 9 traits displaying phenotypic movements along PC1 and PC2. Traits were assessed in samples of *Ostreococcus spp.* in the RP (unpredictable timing, predictable amplitude; **A and B**), PR (predictable timing, unpredictable amplitude; **C and D**) treatment. Trait-scapes are displayed for samples from the thermally unpredictable Kiel Area (**left side**) and from the more predictable Bornholm Basin (**right side**). Colours identify the time points at which the traits were assessed (t20, t50 and t120, corresponding respectively to 20, 50 and 120 generations of selection). Ellipses represent the 95% confidence intervals.

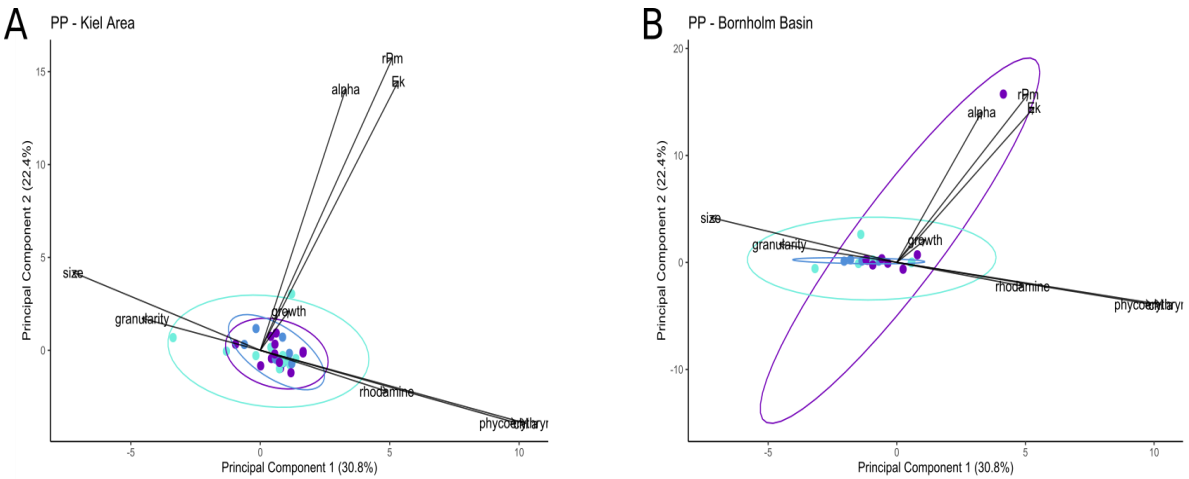


Figure S2.4.5.Trait-scape created from 9 traits displaying phenotypic movements along PC1 and PC2. Traits were assessed in samples of *Ostreococcus spp.* in the PP (predictable timing, predictable amplitude; **A and B**) treatment. Trait-scapes are displayed for samples from the thermally unpredictable Kiel Area (**left side**) and from the more predictable Bornholm Basin (**right side**). Colours identify the time points at which the traits were assessed (t20, t50 and t120, corresponding respectively to 20, 50 and 120 generations of selection). Ellipses represent the 95% confidence intervals.

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Eidesstattliche Versicherung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertationsschrift selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe. 2 | I hereby declare upon oath that I have written the present dissertation independently and have not used further resources and aids than those stated in the dissertation.

Ort, den | City, date

Hamburg, 13/10/2022

Unterschrift | Signature

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