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Plastic and evolutionary responses of *Chlamydomonas reinhardtii* to multiple environmental drivers

THE UNIVERSITY of EDINBURGH

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Supervisors

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Nick Colegrave
Signed declaration

I declare that I have composed this thesis myself, that the work is my own, and that the thesis has not been submitted for any other degree or professional qualification. Below, I outline the contribution of my supervisors and colleagues to the paper based in chapter 2, and technical assistance in collecting data used in this thesis.

For the Nature Climate Change paper based on chapter 2, the experiment was designed by Sinead and I, and discussed with Nick Colegrave. I performed the experiments, analysed data and both Sinead Collins and I wrote the manuscript (see appendix papers). In addition, Sinead Collins produced the model used to measure the effect of sampling from finite number of environments and provided Figure 7.9 (appendix 1), discussed in chapter 2. Sinead Collins also provided Figure 7.10 (see appendix 1).

For equation 2-4, Jarrod Hadfield provided the mathematical formula to calculate the proportion of the variation explained my fixed and random effects in a linear mixed-effects model.

FACS sorting was performed by Martin Waterfall at the Flow Cytometry Core Facility, Institute of Immunology & Infection Research. Proteomic data presented in Appendix 4 and discussed in chapter 4, was collected and analysed by Theirry Le Bihan. I performed further analysis following discussion with Theirry.

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Lay summary

Under future global change scenarios predicted by the IPCC (Intergovermental Panel on Climate Change), many aspects of the environment will change simultaneously. Within aquatic environments, environmental changes will include warmer temperatures, elevated $\text{CO}_2$ and lower pH. However, these changes will not occur in isolation, and changes in light intensity, nutrients, UV radiation and man-made alterations, will likely have further impacts on wild populations. Environmental changes that produce a change to traits associated with fitness (here I used growth rates) are referred to as “drivers”. There are urgent calls for investigations to increase the number of environmental drivers (NED) that they investigate, so that better predictions on the outcome of multiple environmental drivers (MEDs) can be made for lab organisms and extended to wild populations. Of particular importance are the small photosynthetic plants that live in aquatic environments, these are called microalgae. Fifty percent of the oxygen we breath is produced by microalgae. They are the primary producers upon which aquatic ecosystems depend, forming the base of food chains and are therefore important for larger organisms such as fish, sharks, whales and humans. They consume more between 30 – 50% of the global carbon dioxide in the atmosphere, and so play an important role in future atmospheric $\text{CO}_2$, and $\text{CO}_2$ driven changes, such as ocean acidification. In short, no other plant does so much. Microalgae have huge potential to persist under MEDs, as they grow in large populations and reproduce quickly, and this allows microalgae to adapt to MEDs relatively quickly.

My Ph.D work tests the effect of adding more environmental drivers on the growth rate of the microalga *Chlamydomonas reinhardtii*. This will reveal how evolution in the face of a single environmental driver differs from evolution to many (up to eight) simultaneous environmental drivers. For this experiment, I used environmental drivers that are relevant to understanding how populations of microalgae may evolve to MEDs in natural environments (including elevated temperatures, increased $\text{CO}_2$, reduced light intensity, reduced phosphate, UV radiation, reduced pH, general
nutrient depletion and the addition of a herbicide). I found that populations of *C. reinhardtii* do evolve in response to increasing NED, and environmental quality is a good predictor of how much populations will evolve in their environment. Using ninety-six different environments (with 0 to 8 environmental drivers) I disentangled the effect of the NED and the identity of environmental drivers and found that the single environmental drivers, including the environmental driver with the largest effect on growth rates, are important for making predictions on the short- and long-term effects of MEDs. Furthermore, short- and long-term growth rates in environments with MEDs are remarkably similar, allowing me to make predictions on the long-term effect of MEDs, using short-term experiments.
Abstract

In my thesis I present data collected from a long-term selection experiment using the freshwater model organism *Chlamydomonas reinhardtii*. The selection experiment was designed to disentangle the effects of the number of multiple environmental drivers (MEDs) and the identity of those environmental drivers including high CO$_2$, high temperature, general nutrient depletion, reduced light intensity, reduced phosphate availability, the addition of a herbicide, UV radiation and reduced pH. Using up to eight environmental drivers, I show how simple organisms such as *C. reinhardtii* evolve in response to MEDs.

The first step in this investigation is to examine the short-term response of MEDs. Data collected at the beginning of the selection experiment will provide insight into the early stages of microevolution by investigating key differences in the short-term (plastic) responses to few vs. many MEDs. Here, I focus on how the data collected from the responses to single environmental drivers can help us predict the responses to MEDs by using ecological models (additive, comparative, multiplicative). I show that the short-term plastic responses to single environmental drivers can predict the effect of MEDs using the comparative model because the response is largely driven by the single dominant driver present. I also demonstrate the importance of the number of environmental drivers (NED) for making predictions from the single environmental drivers and show that predictions become more reliable as the NED increases. The results gathered from short-term responses provide evidence that single environmental driver studies are useful for predicting the effect of MEDs. After evolution, I found that the strength of selection varies with NED in a predictable way, which connects the NED to the evolutionary response (size of the direct response) through the strength of selection. Here, I used statistical models to quantify the effect of NED on the evolutionary response to MEDs and then interpreted this by considering the possible genetic constraints on adaptation to MEDs.
A subset of populations evolved in environments with five environmental drivers and all populations evolved in the single environmental driver environments are used to examine how adapting to single vs. many environmental drivers affect local adaptation. I examine how populations selected in environments with one environmental driver, five environmental drivers and the evolved control, differ in their response to new environments with the same NED, environments with different NED, and a novel environment. I found that there is a relationship between local adaptation and the strength of selection in the local environment and patterns of local adaptation are affected by the NED of new environments. Lastly, I present the phenotypic consequences of evolution under MEDs. I found that before evolution, measures of chlorophyll content and cell size decline with increasing NED. However, after evolution the relationship between chlorophyll content and cell size with NED is weaker because populations converge on the same phenotypes as they evolve. I also present a case-study of how mass spectrometry methods can be used to better understand underlying molecular mechanisms of two phenotypes (chlorophyll positive and chlorophyll negative cells).

This selection experiment is a good example of how laboratory investigations and model organisms can be used to design experiments with enough replication to have high statistical power in order to make more accurate predictions on the short- long-term effects of MEDs. Whilst there have been some studies on the effects of MEDs, these studies rarely have more than three environmental drivers (sometimes 5 environmental drivers) and there are only a handful of long-term MED studies. This study can be used to develop *a priori* hypotheses for investigating how environmental change will shape natural microbial communities, and is especially useful for organisms where long-term studies with multiple environmental drivers are unfeasible.
Specific Terminology

**Control plastic response:** plastic response of the evolved control populations in new environments.

**Correlated response:** The correlated response to selection was calculated by measuring the difference in population growth rate between the MED-evolved populations and evolved control populations when assayed in the control environment.

**Direct response:** The direct response to selection was calculated by measuring the difference in population growth between the MED-evolved populations and evolved control populations when assayed in the selection environments.

**Environmental Drivers:** The individual environmental drivers that make up each regime (e.g. temperature). It was required that they impact positively or negatively on growth rate.

**Evolved control populations:** Populations that have evolved in the control environment.

**MED-evolved populations:** Populations that have evolved in environments with multiple environmental drivers (NED = 1 – 8).

**Evolved NED = 1 populations:** Populations selected in environments with one environmental driver.
**Evolved NED = 5 populations:** Populations selected in environments with five environmental drivers.

**MED-evolved plastic response:** Plastic response of MED-evolved populations to new environments.

**MED:** Multiple Environmental Drivers.

**NED = 1 Assay Environments:** Assay environments with a single environmental driver.

**NED = 5 Assay Environments:** Assay environments with a five environmental drivers.

**NED:** The number of environmental drivers in a regime (e.g. temperature is NED =1, temperature/CO₂/pH is NED = 3).

**Negative Pleiotropy:** Mutations that effect multiple traits and at least one new trait value is in the opposite direction of selection. Trait values with consistently negative effects in new environments are also referred to as negative pleiotropy or selectional pleiotropy (see Paaby & Rockman (2013)).

**Overlap:** A measure of how much overlap (or shared environmental drivers) exist between regimes within the same NED level. A value of 0 indicates that no environmental drivers are shared between different regimes within the same NED, and a value of one indicates that all environmental drivers are shared between the regimes within the same NED (note that all regimes are unique and therefore all measures of overlap are less than one).
**Pleiotropy:** A single mutation that has an effect on multiple traits under selection.

**Positive Pleiotropy:** Mutations that effect multiple traits and new trait values are all in the same direction of selection. Trait values with consistently positive effects in new environments are also referred to as positive pleiotropy (see Paaby & Rockman (2013)).

**Regime:** the identity of the combination of environmental drivers (e.g. temperature/CO$_2$/pH).

**Selection environments:** General term referring to all regimes where population evolved (NED 0-8).

**Similarity:** Similarity is calculated between selection and assay environments (or home and away environments). A value of zero indicates that no environmental drivers are shared between the selection and assay environment. A value of one indicates that all environmental drivers are shared between the selection and assay environment.
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Chapter 1 - General Introduction

The focus of my thesis is uncovering the short- and long-term responses of a photosynthetic microalga to multiple environmental drivers (MEDs). Global change will involve multiple environmental changes in all ecosystems. For example, in aquatic ecosystems changes in carbon, pH, temperature, salinity, mixing etc. will all occur at once - and this is without even considering changes in the organisms themselves. For this investigation designed a selection experiment using the model microalga Chlamydomonas reinhardtii, so that the cost (or benefit) of the plastic and evolved response to MEDs could be measured. By using a large number of drivers (up to eight) in different combinations, the effects of the number of environmental drivers (NED) and the identities of the drivers can be disentangled. For example, when only one environmental change occurs, the identity of that change is likely to be very important in determining evolutionary outcomes, and we expect populations evolved under high CO₂ conditions and low phosphate conditions, for example, to differ from each other both in terms of their change in fitness over time and in their phenotypes. However, it is less obvious why populations that evolved in conditions where CO₂, pH, temperature, light, and phosphate levels have all changed simultaneously should differ from populations that have evolved under conditions where the same number but different identity of environmental changes have occurred. Whilst both theoretical and applied research often assume multiple environmental drivers have an additive interaction (Halpern et al., 2007, 2008a; Ban & Alder, 2008; Crain et al., 2008), there is little empirical evidence that the additive model can be explain interactions between multiple environmental drivers (Christensen et al., 2006; Harvey et al., 2013). Many experiments demonstrate that the effect of pairs of environmental drivers are often less than sum of the the single effects (additive), or the effects of the single drivers are reversed when in combination (Crain et al., 2008; Jackson et al., 2015). Therefore, we cannot make inferences on the effect of MEDs based on investigations of single and paired environmental drivers (Folt & Chen, 1999; Christensen et al., 2006). Designing an experiment where there are many environments with the same number of
environmental drivers (NED), but where the identities of the drivers themselves differ, allows me to determine if there is a predictable transition point, where the NED becomes more important than the identity of the environmental drivers. The results from my thesis work will provide guidelines for designing future similar experiments with natural phytoplankton assemblages with strong *a priori* hypotheses.

### 1.1.1 Global change, evolution and MEDs

Species across the globe are experiencing drastic changes in environmental conditions as a result of human activities. Climate change induced by increased greenhouse gas emissions has emerged as a top concern, both scientifically and politically (Kerr 2007; Moss et al. 2010). Consequently, changes to the natural environment are occurring at an unprecedented rate, with many environmental changes occurring simultaneously (Meehl *et al.*, 2007; Gruber, 2011; IPCC, 2013), and increasing evidence that it has already affected wild populations (Parmesan, 2006). The complex nature of global environmental change cannot be represented in laboratory experiments, which has led to the criticisms that such experiments likely have limited predictive power about wild populations (Boyd & Hutchins 2012; Boyd *et al.* 2015a). In particular, the effect of MEDs on natural populations, communities and even in laboratory cultures of model organisms, are not well understood, and we currently lack knowledge on the synergistic effects of the main climate variables on physiological and evolutionary responses (Folt & Chen, 1999; Crain *et al.*, 2008; Darling & Côté, 2008).

Photo-autotrophic microalga are particularly interesting, partly because of their ecological importance as they are responsible for approximately fifty percent of global primary productivity (Myers, 1995; Beardall & Stojkovic, 2006; Ormerod *et al.*, 2010; Reusch & Boyd, 2013). Microalga are themselves drivers of global change, playing a key role in the biological carbon pump (Beardall & Raven, 2004).
addition, the short generation times of microbes (hours to a few days) mean that evolutionary changes can occur on human time scales of months, years, or decades. Global change causes changes to trait evolution in microbes, which has the potential to cause changes in bloom events, primary production, and species composition (Beardall & Stojkovic, 2006; Harley et al., 2006; Collins et al., 2013). Because of their ecological importance, many short-term experiments have been performed to predict the effect of global change on phytoplankton physiology with particular emphasis on the effects of elevated CO$_2$ (Collins & Bell, 2004; Zondervan, 2007; Berge et al., 2010; Low-décarie et al., 2013) and temperature (Falk et al., 1990; Fu et al., 2007; Feng et al., 2009; Thomas et al., 2012; Boyd et al., 2013), on a few model species. It is crucial that we understand evolutionary responses to global change if we are to make informed predictions on how phytoplankton populations will differ (for example, growth rates, cell size, chlorophyll content, population size) in the future (Collins & Bell, 2004).

Using up to eight environmental drivers to investigate the effect of MEDs has never been done before and many models predict a “doom and gloom” scenario of global change and its effects on the natural environment (Houghton, 2001). However, no investigations have quantified the long-term effect of MEDs in terms of adaptation, an important mechanism when overcoming long-term changes to the environment (Gienapp et al., 2008).

### 1.1.2 Environmental drivers

There are many environmental drivers that are predicted to change at varying rates and intensities, at both global and local scales (Meehl et al., 2007). These include atmospheric and oceanic carbon dioxide levels, acidification, rising temperatures and ultraviolet-B radiation (Beardall & Stojkovic, 2006; Gruber, 2011; Hoffmann & Sgrò, 2011; Boyd & Hutchins, 2012). These are some of the big players in global change, but there are also many other environmental changes associated with global
change that have the potential to affect organisms, including reduced light availability, nutrient availability and phosphorus availability (Meehl et al., 2007). There are also indirect effects, such as water column stratification as a result of warming, which causes nutrients to be locked away at lower depths in the water column, making these nutrients inaccessible to non-motile plankton (Gruber, 2011; Bopp et al., 2013). Elevated temperatures also exacerbate eutrophication in freshwater environments (McKee et al., 2003), which has further consequences on grazing, predator prey dynamics and nutrient stoichiometry (De Senerpont Domis et al., 2014). As global temperatures continue to rise, we should expect to see increases in land run-off in aquatic environments due to a rise in the prevalence of unpredictable and more extreme weather events (Easterling et al., 2000; IPCC, 2013). This, together with marine seston and particulate matter in the water column, will drastically lower the amount of light penetrating the water column in marine systems (Harley et al., 2006). In freshwater systems, shading will increase with increasing macrophyte and phytoplankton abundances due to eutrophication (McKee et al., 2003). Alongside temperature-driven effects, anthropogenic activities and increased anthropogenic land use will reduce water quality and increase the concentration of pollutants, such as herbicides, which will have an impact on aquatic microalgae (Abou-Waly & Abou-Setta, 1991; Rioboo et al., 2002; Huertas et al., 2010; Larras et al., 2013). These, and many other changes in aquatic ecosystems will occur simultaneously, yet experimental tests of the impact of large numbers of simultaneous environmental changes remain scarce (see Figure 1.1 for illustration of MEDs in aquatic environment).
Figure 1.1 Schematic diagram illustrates possible MEDs that occur simultaneously in aquatic environments. These include, increased UV radiation, elevated temperature, resulting in increased stratification (indicated by solid horizontal lines). Stratified waters result in nutrients being locked away, inaccessible to non-motile microorganisms that occupy the littoral zone. As atmospheric CO$_2$ increases, dissolved CO$_2$ in the form of carbonic acid results in a reduction in pH. Finally, increasing extreme weather events and land use activities will increase the incidence of land runoff. Note that the eight environmental drivers that are used in the present study are listed in the grey box.
Chlamydomonas reinhardtii is a unicellular freshwater alga and common laboratory model system for experimental evolution. There is a long history of ecologists and evolutionary biologists using model organisms in their research. Srivastava et al. (2004) suggest that this is because model systems have three useful features, tractability, generality and realism, essential for guiding future investigations. The purpose of the present study is to provide a framework which future experiments using ecologically important, non-model, marine and freshwater microalgae, can build upon. Using C. reinhardtii in the present study allows us to use standard methods and measurements so that we can easily compare results between the experiments presented here and those done by others. In addition, C. reinhardtii is more suitable than other model organisms such as Escherichia coli or Pseudomonas fluorescens, traditionally used in experimental evolution, as we can compare changes in cell size, chlorophyll content, and growth rate more easily with non-model microalgae. Marine microalgae cultures are usually grown in low densities, are labour intensive to culture over long time periods, and cannot be preserved for additional analysis following selection (but see Lohbeck et al. (2012)). C. reinhardtii, on the other hand, has well established experimental techniques, available genomic and molecular data, and short generation times that allow evolution experiments to be carried out in the time of a PhD project. This means that experimental variables are easily manipulated, high culture densities and level of replication are easily achieved, and cultures can be preserved for long periods of time so that selected populations can be compared with ancestors and additional analysis can be performed (Elena & Lenski, 2003; Bennett & Hughes, 2009). For these reasons we have the power to generate specific a priori hypotheses of the effects of MEDs on non-model microalgae (Benton et al., 2007).

The drivers in this experiment were chosen because they are studied as single environmental changes (Beardall et al., 2009; Troedsson et al., 2012), occur in many aquatic ecosystems (Gruber, 2011), and are “drivers” (sensu Boyd and Hutchins, 2012) in that they elicit a response in C. reinhardtii (the organism used for this study; see Table 1-1), and many other microbes (the green alga Chlorella pyrenoidosa,
Sorokin and Krauss, 1958; diatoms, Falk et al., 1990; marine phytoplankton communities, Smith et al., 1992, Sinha and Hader, 2002; Larras et al., 2013; the green alga Selenastrum capricornutum, Mayer et al., 1998; E. huxleyi, Engel et al., 2005, Riebesell et al., 2000; diatoms dinoflagellates and rhodophytes, van, Dam et al., 2012). Whenever possible, control and test environments reflect anticipated changes in the natural world. For example, the control environment uses 430ppm CO$_2$, and the test environments uses 2000ppm CO$_2$, in line with the more extreme IPCC predictions (IPCC, 2013). In other cases, the test environment value was chosen using pilot studies. All environmental drivers were investigated alone in order to discover if the response of the single environmental drivers can be used to predict their joint effect, and if the outcome of the joint effect can be predicted from the number of environmental drivers (NED), or if effects are specific to particular combinations of environmental drivers (regimes).

1.1.3 Understanding interactions between MEDs

We can predict with some degree of accuracy the identity of the environmental drivers that will change in the future (Easterling et al., 2000; Meehl et al., 2007), and how they interact physically or chemically outside organisms. For example, antagonistic interactions reduces solubility of CO$_2$, and O$_2$ in the ocean under elevated temperatures (Gruber, 2011; Bopp et al., 2013). Where possible, interactions between environmental drivers are controlled in the present study for example, increasing CO$_2$ has no effect on the pH of the media (another environmental driver). This is so that I can disentangle the number and identity of environmental drivers, without interferences from the antagonistic or synergistic interactions between environmental drivers.

Predicting the outcome (i.e. the resultant net outcome for the organism) of environmental drivers and interactions between MEDs requires further research (Boyd & Hutchins, 2012). There are few empirical studies investigating
physiological outcomes in response to MEDs beyond three environmental drivers (Folt et al., 1999; Crain et al., 2008), and only a handful of evolutionary studies that use two or three environmental drivers (Tatters et al., 2013a; Schlüter et al., 2014). Many argue that inferences made from single-factor experiments are severely limited (Hoffman et al., 2003, Collins and Gardner, 2009), and that responses to individual environmental changes are probably poor predictors of responses to the combination of those same changes (Folt & Chen, 1999; Gruber, 2011; Boyd & Hutchins, 2012; Gao et al., 2012). Meta-analyses demonstrate that interactions between pairs of drivers are not predictable from knowing the effect of the single environmental drivers (Didham et al., 2007; Crain et al., 2008; Darling & Côté, 2008; Harvey et al., 2013; Jackson et al., 2015). For example, the effects of ocean acidification and elevated temperature have been investigated both separately and in combination and often show synergistic effects across a range of ecological measurements (Harvey et al., 2013). Studies that investigate the combined effect of more than two environmental drivers are severely limited and so we currently lack empirical evidence of the interactive effect of MEDs, and if they are predictable from the individual effects of environment drivers in isolation (Vinebrooke et al., 2004). In this study, I measure the outcome of interactions between MEDs (up to eight) on populations of \textit{C. reinhardtii}. The outcome of interactions between environmental drivers may be predictable, using simple models for example, additive, multiplicative, or comparative interactions, which can further be characterised by whether there are antagonistic or synergistic effects. Alternately, interactions between drivers may be unpredictable (models are discussed in more detail in the next section).

### 1.1.4 Predicting the outcome of MEDs using models

Existing models that are used to predict the intensity of chemical mixtures do so based on the effects of each chemical alone and their mode of action. When using the effects of individual drivers, drivers A and B may have an effect on the same target
site and this effect may be similar (increasing the intensity of the effect on the
organism when combined, with no change in intensity when one is substituted at a
constant ratio for any proportion of the other), or dissimilar (A and B act on the same
target site but with different outcomes, their combined effect may be greater or less
than the sum of their individual effects depending on correlations between A and B),
or they may act completely independently from each other on different target sites.
This information can be used to calculate the expected effect of more than one
simultaneous environmental change on organisms (Bliss, 1939; Berenbaum, 1989).
For mixtures where modes of action are the same the **concentration addition**
concept is used. When the modes of action are dissimilar the **independent action**
concept is used (Backhaus & Altenburger, 2000; Faust et al., 2003; Backhaus et al.,
2004). Both concepts predict the intensity of drivers when in combination from the
effects of A and B alone. In addition, the concentration addition concept can predict
the effect of the concentration of MEDs, from knowing the specific concentration of
each driver. Both concepts require concentration–response curves of all individual
drivers (Bliss, 1939; Backhaus & Altenburger, 2000). Bliss (1939) was the first to
apply the independent joint action model (or Bliss model) to biological data. An
equation by Bliss (1939) describes the correlation between drivers A and B:

\[
p_C = p_A + p_B (1-p_A)(1-r)
\]

where \( p_C \) is the proportion mortality by the combination of the individual
effects of drivers \( p_A \), and \( p_B \). when \( p_A > p_B \), and \( r \) is the correlation of effects between
A and B, regardless of the percentage composition or dosage of the mixture.
However, using this equation it is difficult to scale up beyond two environmental
drivers and a revised equation is suggested by Backhaus and Altenburger (2000),
where the concept of independent action remains based on the assumption that the
compounds of a given mixture act on different physiological systems within the
exposed organisms. The mathematical formulation is as follows:

\[
E(cMix) = E(c_1 + ... + cN) = 1 - \prod_{i=1}^{n} [1 - E(ci)]
\]
where $E(c_{Mix})$ denotes the predicted effect (scaled from 0–1) of an n-compound mixture, $c_i$ is again the concentration of the $i$th compound, and $E(c_i)$ is the effect of that concentration if the compound is applied singly (Backhaus & Altenburger, 2000; Backhaus et al., 2004).

Although there is some evidence to suggest that the environmental drivers used here are acting broadly on the same basic physiological processes (Table 1-1), it cannot be inferred from this evidence what the specific modes of action are, or what their specific targets are. For example, in the context of pharmacology where the pharmacodynamics of common antibiotics are well studied on multiple organisms, the concentration addition and the independent action model yield accurate predictions of the joint effect of antibiotics (Faust et al., 2003; Ankomah et al., 2013; Ma et al., 2014). Fischer et al. (2010) applied the independent joint action and the concentration addition model to the joint effects of a chemical (the herbicide atrazine) and an environmental stress (high light) on $C. reinhardtii$. These drivers have the same mode of action in that they both inhibit photosynthesis, but they do not have the same target site. High light causes non-photochemical quenching of excess light energy and adjustment of the photosystem stoichiometry in most photosynthetic organisms including $C. reinhardtii$. As a result, high levels are tolerated without too much cellular damage, but photosynthesis and growth (conventional metrics of stress) are reduced (Mayer et al., 1998; Lin et al., 1999; Fischer et al., 2010). The herbicide atrazine (also used in this investigation) directly blocks the photosynthetic machinery, thus reducing photosynthesis and growth (Fischer et al., 2010; Lagator et al., 2013). Fischer et al. (2010) found that the independent action model predicted the observed effects, but the expected effect varied between the variable end points in cell number-based growth rates and optical density-based growth rates. In addition, whilst the combination of abiotic (high light) and chemical (atrazine) drivers do not fulfil the requirements of the independent action or concentration addition model, as the two stressors neither act fully independently nor do they have exactly the same target site (Faust et al., 2003), both models adequately describe the effect of the joint effect. This is supported by van
Dam et al. (2012) who also applied the independent action model to environmental data on the effects of elevated temperature (abiotic) and herbicides (chemical), and found that this model described their combined effects on photosystem II (PSII) in phytoplankton species.

The independent joint action and the concentration addition models may be useful for understanding the combined effects of MEDs. However, more research is required to uncover the mode of actions and target sites of many environmental drivers. In addition, many key drivers (such as increased temperature) affect a broad range of metabolic processes (Davison, 1991; Tomanek, 2014). Without molecular data to pinpoint the mode of action for each driver, we cannot assume that drivers that produce the same effect on organisms share the same mode of action or target site. For instance, in *C. reinhardtii*, elevated CO$_2$ usually increases growth (decreases cell division times), whilst elevated temperature will decrease growth, but both will act on different target sites (Table 1-1), and this fits the independent joint action model. Other drivers may have the same effect on the study organism for example, elevated temperature and the herbicide atrazine produce the same outcome on growth in *C. reinhardtii* but their mode of action and specific target sites may differ.

The environmental drivers used in the present study cannot be definitively categorised as similar or dissimilar in action. For this reason, traditional models such as, the additive, multiplicative and comparative models are better suited to this study for predicting the outcome of MEDs from the effect of the single environmental drivers alone. The additive model is commonly used to predict the combined effect environmental drivers using the sum of the individual effects (Halpern *et al.*, 2008b). For the multiplicative model, the combined effect is equal to the product of individual effect of the individual environmental drivers. The comparative model describes an effect that is equal to the driver with the largest positive or negative effect alone (dominant environmental driver), and other environmental drivers with smaller effects alone have no additional effect (Folt & Chen, 1999).
1.1.5 Cases where the effect of MEDs cannot be predicted from the effects of the individual environmental drivers alone

A third class of effect (following the independent joint action and the concentration addition models) that cannot be calculated or predicted from knowing the drivers alone are synergistic or antagonistic interactions between drivers (Bliss, 1939). For example, when driver A and B are in mixture, they interact either synergistically (the effects of driver A and B are larger than predicted by an additive model), or antagonistically (the effects of driver A and B are less than predicted by an additive model). In order to best measure the interactive effect of MEDs, the appropriate model must be used that best fits the mechanism in which environmental drivers are acting. Choosing the appropriate model is important to avoid errors in classifying interactions between MEDs. For example, if the additive model was used inappropriately to predict the effect of drivers that are mechanistically explained by the comparative model, then synergistic interactions will be overestimated (Figure 1.2). For example, interactions between nutrients are best described by the comparative model and is synonymous with Liebig’s law of the minimum which states that growth is limited by a single limiting nutrient and when other nutrients in the environment are altered (either up or down) there is no additional effect on growth (de Baar, 1994; Folt & Chen, 1999; Boyd & Brown, 2015).

The information gathered on the single drivers is crucial in order to verify that the correct model is being used. For instance, if the predicted outcome was not expected or if interactions (antagonistic or synergistic) cannot explain the effect of MEDs, the alternative is that an incorrect model is being used, and we do not know enough about how the individual drivers act on the organism. Our ability to determine whether or not the data fit a model depends on the current state of knowledge at the time of observation, and as such interactions between drivers can be reclassified as more is learned about their mode action (Berenbaum, 1989). However, this is a difficult position for those researchers aiming to predict the outcome of MEDs on
ecologically important, threatened or endangered organisms, where conservation efforts and management strategies are supported by information gathered on how organism will respond to future global change (van Dam et al., 2012).

Figure 1.2 Schematic diagram illustrates that choosing the model that best fits the data is important in order to avoid misidentifying synergistic and antagonistic interactions. The observed growth rates (black line) fit the comparative model (red line) and the interactions are described as synergistic as the observed growth rates are lower than expected under the comparative model. If the additive model (blue line) is used, interactions in the observed data are described as antagonistic as the observed growth rates are higher than predicted under the additive model.
1.1.6 Predicting the evolutionary response to MEDs

There are currently no theoretical investigations that describe evolutionary outcomes of adapting to many different simultaneous environmental drivers. The process of adaptation has been studied for many decades; theoretical, modelling and experimental studies have provided detailed insights into adaptation in simple environments (Lenski & Travisano, 1994), temporally and spatially complex environments (Bell, 1997; Bell and Reboud, 1997; Bailey and Kassen, 2012), evolution of complex organisms (Orr, 2000; Wagner & Zhang, 2011), evolution under continuous environmental change (a moving adaptive optimum), including varying rates of environmental change (Collins et al., 2007; Collins & De Meaux, 2009), revealing underlying genetic processes (Koornneef et al., 2004; Hoffmann & Willi, 2008). Experiments investigating adaptation to complex vs. simple environments in the form of multiple vs. single sugar resources found that evolutionary outcomes depend on spatial and temporal structure of resource availability (Bailey and Kassen, 2012). While natural experiments are experiments in complex environments, the effect of this complexity has not been quantitatively compared with evolution in simple environments (Collins and Bell, 2006). Enclosure (mesocosm) experiments are an important step in taking simple laboratory experiments closer to the realism of the natural world, providing important insights into how whole communities might evolve and interact under future global change scenarios (Engel et al., 2005; Scheinin et al., 2015), however currently we cannot disentangle the effects of MEDs within mesocosm. In addition, studies investigating how populations evolved under past climatic changes provide insight into how populations might evolve in the future global climate change (Orsini et al., 2013). However, important questions remain unanswered, such as; What are the evolutionary outcomes of evolving in single environmental drivers vs. MEDs? What impact will adaptation have on organisms as the environment continues to change? How will populations change following evolution under MEDs?
Existing evolutionary theory suggests that increasing organismal complexity increases the cost of adaptation (Fisher, 1930; Orr, 2000). The effect of organismal complexity is simply modelled in Fisher’s (1930) geometric model of adaptation which states that when an organism has a complexity of $n$, beneficial mutations of a fixed size have a reduced effect on the rate of adaptation $r$, and the improvement due to fixation of beneficial mutations (distance travelled towards the optimum) decreases as $n$ increases. However, this theory may be used to guide ecological predictions on the long term response to MEDs. Instead of predicting the effect of complex organisms evolving in simple environments, I want predict the effect of complex environment (with MEDs) on the evolution of simple organisms (C. reinhardtii). I predict that as NED increases, the extent of adaptation over a fixed period of time in simple organisms will decrease, as beneficial mutations will move populations a lower proportion of the distance towards the optimum (Figure 1.3).
Figure 1.3 Schematic diagram illustrates that adapting to two environmental drivers is slower than adaptation to one environmental driver. Adaptive walks can take place in any direction towards the optimum phenotype, however, red arrows indicate where adaptive steps are constrained when adapting to two environmental changes. The rate of adaptation is reduced in populations with two traits under selection as a beneficial mutation of a fixed size will move populations smaller distances toward the optimum (red arrows), than populations with a single trait under selection (green arrow) (Fisher, 1930). Positive pleiotropy will speed up the rate of adaptation, as a mutation that changes trait values of trait 1 and trait 2 in the same direction of selection and will move the population further towards the optimum (green and red arrow). On the other hand, negative pleiotropic interactions will reduce the rate of improvement, due to a mutation that changes one trait value in the same direction of selection but changes the second trait value in the opposite direction of selection, resulting in a smaller step towards the optimum (blue and red arrow).
1.1.7 Experimental evolution

In this thesis, I address the effect of long-term growth under MEDs using the model algae *C. reinhardtii*. This experiment follows from experimental evolution studies that aim to uncover the role of evolution in the response of microbes to future environmental drivers (Collins & Bell, 2004; Lohbeck *et al.*, 2012; Low-décarie *et al.*, 2013; Schaum *et al.*, 2014). Microbial experimental evolution investigations have been traditionally used to deepen our understanding of basic evolutionary theory (Buckling *et al.*, 2009; Collins, 2012). However, more commonly microbial experimental evolution can also be used to gain a better understanding of the properties of microbes of specific interest that may be of ecological, economic or medical importance. For example, identifying the phenotypic outcomes of coccolithophore species evolved at high CO$_2$ (*E. huxleyi*; Lohbeck *et al.*, 2012), due to the impact of ocean acidification on CaCO$_3$ production, and photosynthesis, which in turn feeds back into the carbon pump. A second example of a case where research is motivated by interest in how a specific microbe evolves in a particular environment is the evolution of biofilm development in *P. aeruginosa* (the cause of chronic lung disease in cystic fibrosis patients), which facilitates phenotypic radiation and adaptability in response to antibiotic treatment (Luján *et al.*, 2011). The work in this thesis contributes to gaining a better understanding of basic evolutionary theory and a better understanding of evolution of microbes of interest, as we gain a better understanding of the evolutionary response of simple organisms to MEDs in their environment, which is relevant to understanding how phytoplankton evolve in response to global change.
1.1.8 Goals of the study

In chapter 2, I examine the short-term response to MEDs. This will provide insight into the early stages of the microevolution process, and investigates key differences in the short-term (plastic) responses to single vs. multiple environmental changes. I do this by measuring population growth responses over different NED (0 to 8) to calculate the contributions of the identity of the particular environmental drivers and the NED. Chapter 3 reveals the evolutionary response following 95 transfers in the selection environments with MEDs (1 – 8 environmental drives) and the control environment (no environmental drivers), so that the MED-evolved response can be compared with the evolved control populations to calculate the direct and correlated response to selection. In chapter 4, I investigate the outcome of selection in environments with one or five environmental drivers, in new environments with the same NED, different NED and a novel environment, and compare the responses with the evolved control. This experiment was designed to uncover differences in the outcome of selection in simple environments and environments with MEDs. Finally, in chapter 5, I directly compare the plastic and evolved responses as NED increases including, growth rates, chlorophyll content, cell size. This is an important step in order to provide guidelines for planning future experiments investigating the impact on MEDs on non-model organisms. The experimental design for chapters 2, 3 and 5 is illustrated in Figure 1.4.
Figure 1.4 Schematic diagram illustrates the experimental design of the study. The founding population was split into 96 different environments (environments are represented here by square boxes) containing 1 to 8 NED (6 examples of MED environments are shown here and represented by the different pattern backgrounds), and the benign control environment (white background). (a) Founding populations were inoculated using a single colony of *Chlamydomonas reinhardtii* and as such lack genetic variation. (b) The acclimation response was measured at the very beginning of the selection experiment (T₀) in order to calculate the drop in population fitness when placed suddenly into a new environment with between 1 to 8 NEDs. (c) Populations evolved in each selection environment for 95 transfers. This provides enough time for genetic variation to increase and for adaptive variants within the populations to arise in response to the new environments with MEDs (as a result of fixation of beneficial mutations). (d) The evolved control populations evolved alongside the MED-evolved populations so that evolution to laboratory conditions could be accounted for. At the end of the selection experiment evolved populations were assayed in their selection environment and the control environment in order to calculate the direct and correlated responses to selection.
Table 1-1  Review of current knowledge of the mode of action of environmental drivers used in this study (herbicide, elevated temperature, low light intensity, reduced pH, elevated CO$_2$, general nutrient depletion, UV and reduced phosphate), on *C. reinhardtii*.

<table>
<thead>
<tr>
<th>Environmental driver</th>
<th>Mode of action</th>
<th>Mode of action with other environmental drivers</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herbicide: Atrazine</td>
<td>1. Block the photosynthetic electron transport chain.  2. Photosystem II inhibitor.</td>
<td>a) Toxicity found to decrease with increasing light irradiance.  b) In high light photoinhibition protects photosynthetic organisms from photodamage.</td>
<td>1. (Fischer et al., 2010)  2. (Lagator et al., 2013)  a) (Mayer et al., 1998; Lin et al., 1999)</td>
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<tr>
<td>High Temperature</td>
<td>1. Photodamage: causes reductions in maximum PSII quantum yield. Also bleaching through loss of chlorophyll a (diatoms and dinoflagellates symbionts).  2. Denaturing of proteins and nucleic acids, dissociation of protein complexes, and the destabilisation of membrane structure.</td>
<td>a) Photoinhibition is both temperature and light dependent: At high temperatures (27°C) 50% photoinhibition requires higher light intensities than lower temperatures (12°C) in <em>C. reinhardtii</em>. In addition high temperatures show less efficient recovery of photoinhibition.</td>
<td>1. (Falk et al., 1990; van Dam et al., 2012)  2. (Larras et al., 2013; Kobayashi et al., 2014)  a) (Falk et al., 1990). Also See review by (Zondervan, 2007)</td>
</tr>
<tr>
<td>Low Light Intensity</td>
<td>1. In contrast to high light, low light allows relatively high efficiency of utilization of incident energy without the need to employ protection mechanisms such as, non-photochemical quenching of excess light energy and adjustment of PS stoichiometry.  2. Both chlorophylls a and b increased slightly with decreases in photon flux density in <em>C. reinhardtii</em>.</td>
<td>Low light is beneficial when combined with limiting CO$_2$, nutrient depletion and phosphate depletion, where even a relatively low light is excessive and photoprotective mechanisms are switched on.</td>
<td>1. (Sorokin &amp; Krauss, 1958; Fischer et al., 2010)  2. (Osborne &amp; Raven, 1986)  a) (Harris, 1989; Falk et al., 1990)</td>
</tr>
<tr>
<td>Low pH</td>
<td>1. Decreased growth and photosynthesis at pH 1.5, 4 and 7 are likely a result of internal acidification or alkalinization in acidophilic <em>C. acidophila</em> (optimum growth rates at pH 2.6–3).  2. Intrathylakoid acidification is suggested as a cause of limitation of electron donation to P$<em>{680}$- Evidence for a donor-side limitation of PS II at low pH comes from time-resolved spectroscopic studies of reduction of P’$</em>{680}$.</td>
<td>a) <em>C. acidophila</em> (optimum growth rates at pH 2.6–3), growing at pH 4 in high light (180 µmol m$^{-2}$ ms$^{-1}$) conditions, dark respiration was 71% of gross O$_2$ evolution and growth rates were reduced which indicate extremely stressful conditions.  • Increasing pH to pH 4 causes the</td>
<td>1. (Gerloff-Elias et al., 2005)  2. (Krause &amp; Weis, 1991)  a) (Gerloff-Elias et al., 2005)</td>
</tr>
</tbody>
</table>
| **High CO₂** | 1. Stimulates carbon fixation during photosynthesis  
2. Reduce the need for CCM activity and thereby lower the metabolic costs of inorganic carbon acquisition. | a) *C. reinhardtii* grown in high-CO₂ were less photoinhibited, and showed better recovery in dim light or darkness during the initial period of the recovery process. | 1. (Riebesell, 2004)  
2. (Engel *et al.*, 2005; Collins & Bell, 2006)  
a) (Yang *et al.*, 2001) |
| **Nutrient depletion** | 1. General nutrient depletion has effects on respiration and photosynthesis.  
• Iron-deficient *Chlamydomonas* cells re-adjust metabolism by reducing light delivery to photosystem I (to avoid photodetoxicative damage resulting from compromised FeS clusters).  
• The chloroplast is a major iron-utilizing organelle.  
• Copper-deficient *Chlamydomonas* cells induce a copper assimilation pathway consisting of a cell surface reductase and a Cu⁺ transporter (presumed CTR homologue).  
• Magnesium is abundant in photosynthetic pathways.  
2. Nutrient-limited *Chlamydomonas* cells dissipate energy by nonphotchemical quenching of chlorophyll autofluorescence.  
3. Cessation of growth and regulation of photosynthetic electron transport have been characterised as a general response to adjust metabolism and sustain viability when nutrient levels fall. General nutrient depletion is also characterised by the decline of photosynthetic electron transport in *Chlamydomonas* cells and a transition of the photosynthetic apparatus to state 2, which allows them to more effectively dissipate excess absorbed excitation energy and with a reduction O₂ evolution (reduced by 75%) at subsaturation light levels. | 1. (Bölling & Fiehn, 2005; Merchant *et al.*, 2006)  
2. (Wykoff *et al.*, 1998)  
3. (Grossman, Arthur, Takahashi, 2001) |
| **Ultra Violet-B** | 1. Damage to photosynthetic processes, increase in photoinhibition  
2. UV can induce changes in the molecular structure of the DNA | a) The survival of the bacteriophage T4 exposed to ultraviolet radiation grew | 1. (Smith *et al.*, 1992)  
2. (Sinha & Hader, 2002) |
DNA lesions interfere with DNA transcription and replication and can lead to misreadings of the genetic code causing mutations and death.

UV radiation is known to inhibit the ability of phytoplankton to move and orient within the water column.

with increasing temperatures (from 20°C to 40°C)

a) (Conkling & Drake, 1984)

<table>
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<th>Phosphate depletion</th>
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<tr>
<td>1. Phosphate is a vital macronutrient due to its necessity in the manufacturing of DNA, RNA and lipids and involvement in biosynthetic processes.</td>
</tr>
<tr>
<td>2. Quantitation of the partial reactions of photosynthetic electron transport demonstrated that the light-saturated rate of photosystem (PS) I activity was unaffected by P or S limitation, whereas light-saturated PSII activity was reduced by more than 50%.</td>
</tr>
</tbody>
</table>

1. (Irihimovitch & Yehudai-Resheff, 2008; Hartmann et al., 2011)
2. (Bölling & Fiehn, 2005)
2 Chapter 2 – The plastic response of C. reinhardtii to multiple environmental drivers

2.1 Chapter summary

In this chapter, I focus on understanding the initial response of C. reinhardtii to MEDs. I measured the plastic response of C. reinhardtii in environments with multiple environmental drivers (MEDs). I predicted that the population growth rate of C. reinhardtii would decrease as the number of environmental drivers (NED) increases from one to eight, and this is due to the combined negative effects of the environmental drivers. I explore how the effects of MEDs can be predicted using the NED and/or the effect of the single environmental drivers in the absence of information about interactions between drivers. By virtue of the experimental design the effect of MED and NED can be disentangled; the effect of regimes within the same NED can be compared in order to identify if the NED can be used to predict the outcome of MEDs. The additive, multiplicative and comparative models were used to assess if the response to growing with MEDs can be predicted using responses to growing with the single environmental drivers that make up the MED regimes. While all organisms experience simultaneous environmental changes, I use a eukaryotic photosynthetic microbe as my model system, and so focus on microbial responses throughout my thesis.

2.2 Abstract

I found that the population growth rate of C. reinhardtii decreases as the number of environmental drivers (NED) increases from one to eight. This trend is explained using the comparative model and the growth rates in the presence of the most
dominant environmental driver. Not all environmental drivers have negative effects. Here, elevated CO$_2$ enhances growth rates of C. reinhardtii and I found that when drivers with negative effects on growth are combined with high CO$_2$ the drop in growth rate is smaller than in cases where CO$_2$ is absent, but the general trend of decreasing growth rate with increasing NED is still observed. The data presented in this chapter demonstrate that knowing the effect of the single environmental drivers, especially the dominant environmental driver, is important for making predictions on the effects of the same environmental drivers when in combination.

2.3 Introduction

Studies on how global change will impact photoautotrophic microbes in both freshwater (Folt & Chen, 1999; Christensen et al., 2006) and marine systems (Boyd et al., 2013; Boyd & Brown, 2015) focus on a few key environmental drivers such as changes in temperature, CO$_2$, light levels and pH. In cases where multiple driver experiments are carried out, they rarely exceed three environmental drivers (Crain et al., 2008; Boyd & Hutchins, 2012). This means that even when MED studies are carried out, we still need a way to scale up from few to many drivers. Three types of interactions between drivers are possible; environmental drivers may buffer the effects of additional drivers (antagonistic interaction; Fischer et al., 2010), exacerbate the effects of additional drivers (synergistic interaction; Pörtner et al., 2005), or there may be no additional effect when adding more environmental drivers, and these interactions might depend on the identity, the number, or the intensity of environmental drivers. Here I measure the effect of increasing the number of environmental drivers (up to eight) in a novel environment, in order to determine if there are any trends in the effects when NED increases and if these trends can be predicted.
2.3.1 The effects of MEDs

Physiological responses to environmental drivers associated with global change have been studied reasonably well in isolation in a wide variety of organisms such as freshwater phytoplankton (Rioboo et al., 2002; Christensen et al., 2006), marine phytoplankton (Riebesell, 2004; Boyd et al., 2013), plants (McKay et al., 2003) and invertebrates (Dupont et al., 2008, 2010), (see review by Crain et al. 2008).

Physiological studies are beginning to investigate the effects of MEDs by using two or three environmental changes (Harley et al., 2006; Hutchins et al., 2007; Zondervan, 2007; Crain et al., 2008; Rost et al., 2008; Gruber, 2011; Hoffmann & Sgrò, 2011). That being said, experiments investigating the interactive effects of two or three environmental drivers may have little or no predictive power on the effect of multiple environmental drivers as NED increases (beyond three environmental drivers) because of unexpected synergistic or antagonistic interactions between drivers (Folt & Chen, 1999; Hoffman et al., 2003; Christensen et al., 2006; Ormerod et al., 2010; Gao et al., 2012).

Many of the effects of MEDs on different organisms are rarely predictable and vary greatly between species and life stages even when the same drivers are studied (Byrne & Przeslawski, 2013) and predictions made may not hold when different identities and intensities of environmental drivers are measured in combination (Crain et al., 2008). This has historically placed limits on investigating how large numbers of environmental drivers affect organismal responses, though using scenarios (Boyd et al. 2015b) is one way to get around this, as explained in the next section.
2.3.2 Predicting the effect of MEDs: Scenarios

Depending on the goal of the research and resources available, there are two approaches for designing MED manipulation experiments. One way to predict the action of MEDs is to use scenarios, where interest is on the interaction between environmental drivers that are likely to occur together under future scenarios so that predictions can be made. Scenario based experiments capture regional environmental changes and relationships among the suite of drivers on an organism in order to predict how the study organisms will deal with the future scenario (Boyd et al. 2015b). This is a very powerful tool when we are interested in the effects of specific combinations of drivers and their interactions, on specific organisms. For example, Boyd et al. (2015b), demonstrate that the joint effect of five regional environmental drivers (elevated light intensity/ elevated temperature/ elevated iron/ reduced nutrients/ elevated CO$_2$) had a cumulative effect on a sub-Antarctic diatom, where the positive effects of warming and iron availability buffered the effects of reduced nutrients. Moreover, this study confirms that physiological responses to MEDs are not predictable from the effect of the individual drivers alone due to non-additive interactions between environmental drivers. This type of study relies on understanding the physiological influence of each driver alone on the study organism, so that more targeted manipulation experiments can be designed that have the power to measure interactions between drivers. In this case, temperature was found to be a dominant driver, which allowed the remaining four drivers to be grouped together, removing the need for much larger factorial experimental designs. Scenario based studies are an efficient and powerful tools to quantify the effect of MEDs and their interactions if the purpose of the studies is to predict the effect of a known suite of environmental changes on an organism. The experiment in this chapter complements scenario based experiments, as interactions between drivers are measured with high replication within NED levels, allowing general trends in the effect of increasing NED to be investigated.
2.3.3 Predicting the effect of MEDs: Interactions

An alternative to using scenarios is to model or measure the interaction effect of environmental drivers on organisms. Traditionally, MED studies investigate up to three different environmental drivers where interactive effects are measured and important drivers identified (Folt & Chen, 1999; Crain et al., 2008; Rost et al., 2008; Hoffmann & Sgrò, 2011; Bopp et al., 2013). However, whilst measuring interactions between drivers of interest is often informative (Dupon & Pörtner, 2013), all combinations of environmental drivers must be empirically measured, including all organisms and life stages of interest (Byrne & Przeslawski, 2013; Cross et al., 2015), as the effect of MEDs rarely conform to a single model (additive, multiplicative, comparative). This means that data is time consuming to collect and the size of experiments are often limited.

Here, I measure interactions between environmental drivers when up to eight environmental drivers are present in order to identify if there is a general trend when NED is increased beyond the range usually studied in the lab. In addition, I use the information from the effects of the individual environmental drivers alone in order to see if they conform to a model of interaction (additive, multiplicative, comparative) when they are combined. In all cases, I measure the action of MEDs through their effect on population growth rates.

2.4 Methods and Materials

2.4.1 Experimental design

All populations were founded from a single cell of *C. reinhardtii* (CC-2931, mt-; Chlamydomonas Resource Centre, University of Minnesota), grown in sterile
Sueoka's high salt medium, buffered with Tris HCl (HSMT), under continuous rotation (50 rpm) at 25 °C and constant light at 32 μmol m−2 s−1 photon flux density (Fisher Scientific Traceable dual range light meter), at 420ppm CO₂ (Table 2-1,Table 2-2). These variables were controlled using incubators (Infors AG CH-4103). This strain of *C. reinhardtii* is from a culture collection, and has been grown in the lab for over 7 years – this media, temperature, and light levels represent the usual benign growth conditions for this strain.

### 2.4.2 Culture Environments

Replicate single-genotype populations were grown in replicate novel environments that differed from a benign control environment (430ppm CO₂, pH 7.2, temperature 25 °C, full light and nutrients, no herbicide and no UV) by 1 to 8 of the following parameters: increased CO₂ to 2000ppm, temperature to 26 °C, decreased pH to 6.5, light levels to 18 μmol m⁻² s⁻¹, reduced phosphate to 1.69 mM, general nutrient depletion by 75%, and added 0.5 μM of the herbicide atrazine. In addition, test environments with UV were exposed to a dose 8.1 KJ·m⁻² UV radiation once a week as part of the batch culture protocol (Table 2-1,Table 2-2). There are ninety-six test environments in total in this study and 288 populations (3 independent replicate populations per test environment x 96 test environments, Table 2-3). Cultures were grown in 48-well plates containing 1.6 mL of culture media. Each population was acclimated to its test environment for seven days (three generations), and then transferred to fresh test environment medium for each regime.

Details of how individual drivers were manipulated and the reasoning behind specific manipulations are below. These eight environmental drivers were chosen because they are often studied singly as distinct environmental changes, occur as part of global change, and most are known to affect cellular processes. Four requirements were fulfilled when choosing intensities of each environmental driver: First, the environmental drivers had to be ecologically realistic for microbes in aquatic
systems. Second, it must be possible to use the environmental change in an experiment with *Chlamydomonas*. For instance, nitrogen limitation is ecologically important but cannot be used as nitrogen directly affects *C. reinhardtii* by encouraging sexual reproduction between mating-types (Harris, 2001). Third, the change had to be simple enough to implement that it could be used in a large, long-term experiment. For example, iron is an ecologically important trace element that was omitted due to labour intensive protocols needed to remove trace levels of iron from the environment. Forth, rapid extinctions should be avoided in environments that contained only one driver. Though extinction is one possible outcome of populations being exposed to changes in environments, the goal of this study is to learn how responses to one environmental driver predict responses to multiple environmental drivers and this requires non-zero measures of growth in the single driver environments. Lastly, environmental drivers must produce a change (negative or positive) in growth rate and for this reason some intensities of environmental drivers differ from intensities used in other published investigations.

pH is an example of an environmental change that meets all criteria. However, since *C. reinhardtii* are able to persist in pH levels lower than those predicted under future climate change scenarios, pH was furthered reduced in order for there to be a measurable drop in relative fitness of about ten percent when tested in isolation. Ten percent deterioration in fitness is a strong selection pressure so that natural selection will act for adaptation to occur during the selection experiment. The pH of all Media used in this experiment was buffered using Tris hydrochloride in order to separate the effects of pH and CO₂ when in combination. With the exception of CO₂/pH, I did not attempt to control chemical interactions between drivers; these interactions may contribute to organismal responses and to subsequent patterns of how response scales with the number of drivers. Because this study aimed to understand average biotic responses with increasing numbers of drivers, I designed an experiment that included as many drivers as possible – this necessarily means that we cannot address specific mechanistic interactions amongst drivers.
Temperature: A conductive heat-mat (Exo Terra heat wave substrate heat mat) was placed under the 48-well plates that were used for culturing to increase the temperature of the culture media to 26°C. This did not affect the overall temperature of the incubator and was controlled using a thermostat (Rootit heat mat thermostat). My reasoning is that a 1 °C rise in temperature a) could be produced without affecting the overall temperature of the incubator or causing condensation on the culture vessel lid, b) falls within the range of predicted temperature rises for aquatic ecosystems (Bindoff et al., 2007) and c) produces a change in growth rate in C. reinhardtii and can thus act as a driver, but does not cause mortality (we wanted to avoid large numbers of extinctions during the experiment).

CO₂: Sterile breathable films (AeraSeal breathable sealing film) were used instead of the of the 48-well plate lids that came with the plates. This allows increased CO₂ diffusion into the media. While we did not quantify the precise level of CO₂ in the media, growth in the high-CO₂ conditions was stimulated, indicating that it was acting as a driver, which is all that was needed for the purpose of this study. CO₂ levels in the test environments were chosen based on projected CO₂ levels, and are in line with other experiments investigating responses of microalgae to CO₂ enrichment.

pH: The pH of the culture media was altered by adding 2% HCl. This required one to two drops per litre of HSMT, so the concentration of nutrients was not altered by changes in volume. The pH was measure with a pH meter (Thermo Orion Star A121 pH Portable Meter) and buffered by adding Tris-HCL. Even though this drop in pH (0.7 units) is large relative to changes expected in marine ecosystems (Bindoff et al., 2007) it is well within those experienced in freshwater systems (Harris, 1989). Based on pilot work, this drop reliably affects growth in the C. reinhardtii in the laboratory cultures.
UV: A UV lamp (UVM-57) was used in order to provide a dose of UV radiation at acclimation and at T0 (Appendix 1; Figure 7.1). The breathable films were removed from the culture plates under sterile conditions during UV radiation. The lamp was mounted 5.1 cm from the surface of the culture plates providing an irradiative force of 33.75 W.cm\(^{-2}\). Populations were irradiated for 4 mins and this corresponds to a UV dose of 8.1 KJ.m\(^{-2}\) (Hessen et al., 1995; Häder et al., 2007).

Light intensity: Overall light intensity was reduced by approximately 40% using a neutral density light filter (0.15 Neutral Density filter), designed to reduce the light intensity across all wavelengths equally and attenuate light by absorption with minimal reflection. The filter was secured to the top of the experimental plates allowing sufficient room for air to circulate. My rationale for decreasing rather than increasing light was pragmatic; it is possible to put a filter on some of the culture vessels, but difficult to selectively increase light levels reliably for only a few populations during an experiment of this size. Additionally, increasing light levels for *C. reinhardtii* often leads to bleaching and mortality (Müller et al., 2001; Fischer et al., 2006). We found that with this strain the light intensity used was high enough for growth but limited the amount of bleaching in populations, even in environments that contained many other stressful drivers.

Herbicide: Atrazine was used at a concentration of 50µM in HSMT. Atrazine was then added to the culture media used for this treatment freshly whenever populations were transferred into fresh media. Based on pilot work and published studies (Fischer et al., 2010), this concentration of atrazine reliably affects growth in the *C. reinhardtii* genotype used.

Nutrients: All nutrients within Hunter’s trace elements (HTE) were reduced equally to a concentration factor of 0.25 relative to the control concentration (see Table 2-2 for concentration of each nutrient within HTE). Since laboratory strains are used to growing in rich media such as HSMT, increasing trace nutrients has no measurable
effect on growth. The dilution factor of general nutrients used in this experiment was determined during pilot studies.

*Phosphate:* Phosphate was reduced to a concentration factor of 0.125, relative to the control concentration (Harris, 1989). Salts lost by the removal of dipotassium phosphate (K₂HPO₄) and monopotassium phosphate (KH₂PO₄) were replaced with potassium chloride (KCl). The level of phosphate needed to act as a driver was based on pilot work and previous studies by Collins & De Meaux (2009).

We refer to each unique combination of environmental changes as a “regime”, and the number of environmental changes that make up a regime as NED. For example, a regime with increased temperature and CO₂ has a NED of two, whereas a regime with increased temperature only has NED of one. Each individual environmental driver was tested alone at varying strengths in preliminary trials to ensure that all environmental changes had an effect, and that none of the effects were lethal or near-lethal. Whenever possible, changes in environmental parameters were based on predictions for how these parameters are likely to change in natural systems (Hader, 2000; Wu et al., 2000; Harley et al., 2006; Bindoff et al., 2007; Meehl et al., 2007; Gruber, 2011; Bopp et al., 2013). In choosing regimes at each NED level, where possible environmental drivers should be nested as NED increases from 1 to 8, for example CO₂, CO₂/pH and CO₂/pH/temp, so that the effect of sequentially adding one more environmental driver can be measured. Otherwise, environmental drivers were chosen at random where there were many options of combinations of environmental drivers possible (e.g. NED = 5). For all combinations of environmental drivers (NED 1-8) see Table 2-3.
2.4.3 Population growth

Cell counts were performed every 24 hr for a total of 120 hr using BD FACSCanto II (BD Biosciences, Oxford, UK) flow cytometer calibrated with CS&T beads. The data were acquired with the BD FACSDiva v6 software. Each culture was counted twice. The cell counts were transformed into cells per millimetre and the average rate of divisions per day was calculated for each replicate. Growth rate was calculated as using Equation 2-1.

\[
\text{Rate of Division (day}^{-1}) = \frac{\log_2 \left( \frac{N_t}{N_0} \right)}{(t_f - t_0)}
\]

where \(N_t\) is the cell density (cells/ ml) at time \(t\) (hours) and \(N_0\) is the cell density at time \(t_0\). This calculation was used because different environments produced different shaped growth curves (Appendix 1; Figure 7.3- Figure 7.6), and the usual metric of maximum growth rate was not useful, whereas this measures the average number of divisions per day per population per transfer cycle, and allows the meaningful comparison of populations with different growth strategies (Collins, 2011a). This is appropriate for the experiment – one possible response of population growth to some of the test environments, such as those that are nutrient-deplete, is that maximum population size may decrease (Appendix 1 Figure 7.7), or a lag time may change, or maximum growth rate may change, thus limiting population growth in batch culture. In the present experiment, populations in the control environment were never nutrient-limited (cultures never reached carrying capacity). The metric used allows us to compare of population-level responses across the range of stressful and enriched environments in this experiment.
In addition to the number of divisions \((d^{-1})\), the change in the number of cells over time was calculated using Equation 2-2.

\[
\text{Change in the number of cells } (d^{-1}) = \frac{(N_t - N_0)}{(t - t_0)}
\]

Equation 2-2

In Equation 2-2, log transformations of the data were not performed as was done in Equation 2-1. See appendix 2, section 7.1.1, for further comparisons between analysis of changes in the number of cells and rate of division \((d^{-1})\) (Figure 7.8).

### 2.4.4 Extinction probabilities

The fold increase in the chance of extinction \((P_{\text{ext}})\) relative to the control was calculated as the ratio of 1- the chance of at least one rescue mutation spreading before the population goes extinct from Bell (2013), using Equation 2-3.

\[
P_{\text{ext}(1)} / P_{\text{ext}(0)} = \exp(|r_0| - |r_1|)
\]

Equation 2-3

where the probability that at least one beneficial mutation will spread is given by \(P = 1-\exp(-B)\), such that \(P_{\text{ext}} = \exp(-B)\), and \(B = 2N_0U\phi r_1 / |r_0|\), where \(N_0\) is the initial population size, \(U\) is the population mutation rate, \(\phi\) is the fraction of mutations that are beneficial and \(r_1\) and \(r_0\) are the Malthusian rates of population increase in the changed and control environments, respectively, assuming that the genomic mutation rates \((U)\) and proportion of beneficial mutations \((\phi)\) do not differ between the control and changed environments.
2.4.5 Overlap of NED between regimes

Each regime, by definition, is unique. However, because regimes become more similar as the number of environmental drivers increase, overlap between the drivers making up different regimes for a given NED was calculated as an average pairwise difference between regimes, where each environmental driver is coded as a binary variable (present or absent). Average overlap for each NED is calculated as $1 - (\text{average pairwise distance})$. The degree of overlap for test environments with 0, 8 and one environmental drivers is zero as there is only one control regime, one regime with all eight environmental drivers and in test environments with one driver, all eight changes were assayed alone. The analysis was performed the same way for each subset of the data (including the case study and full dataset less CO$_2$).

2.4.6 Overlap of environmental drivers between regimes

In this experiment, only eight possible environmental drivers were present to choose from, so that as the level of NED increases, the probability of including at least one highly detrimental environmental change increases rapidly, with that probably reaching 1 for NED = 8. Since I found that the population growth rate is largely determined by the single dominant environmental driver, using a finite number of environments should overestimate the average decrease in growth in environments with a high NED. Since using a finite number of environmental drivers is necessary in a laboratory experiment, we verified that the results presented here are robust even if the probability of including at least one highly detrimental environmental change remains constant as the NED increases (Figure 7.9). The effect of sampling from a finite number of possible environmental drivers was explored using a simulation written in R. Using finite (eight) and infinite environmental drivers, with the same distribution of effects on growth rate for single environmental drivers as in the focal experiment, the expected growth rates were simulated at different levels of NED,
while holding the probability of sampling an environment with each growth effect constant. The measured number of cell divisions per day for each of the single environmental drivers were sampled either with (infinite possible environmental drivers) or without (finite possible environmental drivers) replacement to form environments with between 1 and 8 NED, and the growth at each given level of NED calculated as the minimum growth of all effects present. The simulation was run 10000 times.

2.4.7 The comparative, multiplicative and additive models

Expected numbers of division ($N_{exp}$) for each regime were calculated for each of three models (comparative, multiplicative and additive), using the observed number of divisions ($N_{obs}$) measured for NED = 1, where each driver is experienced alone. For the comparative model, $N_{exp}$ is equal to the most dominant individual environmental driver relative to the control ($1 - N_{obs}$). For example, if herbicide causes the largest change in population growth, any other driver present within that regime would have no additional effect. For the additive model, $N_{exp}$ is calculated as the sum effects of all individual drivers when experienced alone (at NED = 1) that make up each regime. For the multiplicative model, $N_{exp}$ is the product of $N_{obs}$ for each of the drivers present in the regime when they are experienced alone (at NED=1). Model fits were compared using the $R^2$ values. The $N_{exp}$ for each model (comparative, multiplicative and additive) was fit against the $N_{obs}$ using a linear model. This was completed in R using the lm function within the R basic package. Deviants were identified by calculating the difference between the observed growth rate and the predicted growth rate (under the comparative, multiplicative and additive model), as $N_{exp} - N_{obs}$. For each model, a threshold for the difference between the $N_{exp}$ and $N_{obs}$ was set; additive $\leq 0.17$ difference, multiplicative $\leq 0.15$ difference, and comparative $\leq 0.1$ difference. These levels were chosen as they explain a minimum of 80% of the variation ($R^2 \geq 0.8$). For each model, deviants were identified as all populations with $R^2 < 0.8$. The number of populations with $R^2 \geq 0.8$ differed for each
model; additive = 92 populations (out of 288 populations), multiplicative = 140 populations and comparative = 155 populations.

2.4.8 Statistical analysis

The effect of the identity and NED on average rate of cell division (d⁻¹) was analysed using a mixed model in R (R Core Team, 2013), using the packages lme4 and lmerTest. NED (0-8) is a fixed factor as is overlap between regimes within each level of NED (measured as the average number of shared drivers between different test environments for a given NED – see above). Regime and replicates within for each regime are random factors. To directly compare the contributions of fixed and random factors to variance, the percent contribution of fixed factors was estimated (Table 7-1) by using Equation 2-4.

Equation 2-4

\[
\text{Percentage of Fixed Effect Variance} = \left( \frac{\sigma_f^2 \times (b^2 - \text{se}^2)}{\sigma_x^2} \right) \times 100
\]

where \( \sigma_f^2 \) is the variance of the fixed effect, \( b \) is the slope of the fixed effect estimated by the mixed effects model, se is the standard error of the fixed effect as estimated by the mixed effects model and \( \sigma_x^2 \) is the variance of the response variable.

A Post hoc mixed model was completed in order to identify any effect caused by the environmental drivers themselves (i.e. CO₂ or temperature). The eight environmental drivers nested within NED were added to the random part of the model in place of regime, as described above, as all environmental drivers that make up each regime are now included in the model.
Table 2-1 A comparison of the control environment and the environmental changes that will be used in the treatment regimes in experiment 1.

<table>
<thead>
<tr>
<th>Environmental drivers</th>
<th>Control</th>
<th>Treatment</th>
<th>pH of Culture Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO₂ (ppm)</td>
<td>420</td>
<td>2000</td>
<td>7.2</td>
</tr>
<tr>
<td>pH</td>
<td>7.2</td>
<td>6.5</td>
<td>6.5</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>25</td>
<td>26</td>
<td>7.2</td>
</tr>
<tr>
<td>Phosphorus (mM)</td>
<td>13.56</td>
<td>1.69</td>
<td>7.2</td>
</tr>
<tr>
<td>Nutrients* (concentration factor)</td>
<td>1</td>
<td>0.25</td>
<td>7.2</td>
</tr>
<tr>
<td>Herbicide (μM)</td>
<td>0</td>
<td>0.5</td>
<td>7.2</td>
</tr>
<tr>
<td>UVB dose (KJ.m⁻²)</td>
<td>0</td>
<td>8.1</td>
<td>7.2</td>
</tr>
<tr>
<td>Light intensity (µmol m⁻² s⁻¹)</td>
<td>32</td>
<td>18</td>
<td>7.2</td>
</tr>
</tbody>
</table>

* see Table 2-2 for concentration of all nutrients.

Table 2-2 Concentration of Hutner’s Trace Elements in 1 litre of HSMT culture media in control and treatment (nutrient depletion) environments.

<table>
<thead>
<tr>
<th>Hutner’s Trace Elements</th>
<th>Control (mM)</th>
<th>Treatment (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂EDTA· 2H₂O</td>
<td>0.134</td>
<td>0.034</td>
</tr>
<tr>
<td>ZnSO₄ · 7H₂O</td>
<td>0.077</td>
<td>0.019</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>0.184</td>
<td>0.046</td>
</tr>
<tr>
<td>MnCl₂ · 4H₂O</td>
<td>0.026</td>
<td>0.006</td>
</tr>
<tr>
<td>FeSO₄ · 7H₂O</td>
<td>0.013</td>
<td>0.003</td>
</tr>
<tr>
<td>CoCl₂ · 6H₂O</td>
<td>0.007</td>
<td>0.002</td>
</tr>
<tr>
<td>CuSO₄· 5H₂O</td>
<td>0.006</td>
<td>0.002</td>
</tr>
<tr>
<td>(NH₄)₆Mo₇O₄· 4H₂O</td>
<td>0.890</td>
<td>0.222</td>
</tr>
</tbody>
</table>
Table 2-3 Environmental changes and their combinations in each unique regime environment.

<table>
<thead>
<tr>
<th>NED</th>
<th>Regimes</th>
<th>No. of Regimes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>CO₂/Temp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CO₂/ LI</td>
<td>CO₂/ pH</td>
</tr>
<tr>
<td></td>
<td>CO₂/ P</td>
<td>CO₂/ Herb</td>
</tr>
<tr>
<td></td>
<td>CO₂/ ND</td>
<td>CO₂/ UV</td>
</tr>
<tr>
<td></td>
<td>Temp/ pH</td>
<td>Temp/ LI</td>
</tr>
<tr>
<td></td>
<td>pH/ UV</td>
<td>P/ LI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Herb/ UV</td>
</tr>
<tr>
<td></td>
<td></td>
<td>UV/ ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>UV/ LI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH/ P</td>
</tr>
<tr>
<td>3</td>
<td>CO₂/ Temp/ pH</td>
<td>CO₂/ Temp/ LI</td>
</tr>
<tr>
<td></td>
<td>CO₂/ pH/ UV</td>
<td>CO₂/ P/ LI</td>
</tr>
<tr>
<td></td>
<td>CO₂/ Herb/ UV</td>
<td>CO₂/ UV/ ND</td>
</tr>
<tr>
<td></td>
<td>CO₂/ UV/ LI</td>
<td>CO₂/ pH/ P</td>
</tr>
<tr>
<td></td>
<td>Temp/ pH/ LI</td>
<td>Temp/ LI/ Herb</td>
</tr>
<tr>
<td></td>
<td>pH/ UV/ ND</td>
<td>P/ LI/ Herb</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Herb/ UV/ ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>UV/ ND/ P</td>
</tr>
<tr>
<td></td>
<td></td>
<td>UV/ LI/ pH</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH/ P/ UV</td>
</tr>
<tr>
<td>4</td>
<td>CO₂/ Temp/ pH/ LI</td>
<td>CO₂/ Temp/ LI/ Herb</td>
</tr>
<tr>
<td></td>
<td>CO₂/ pH/ UV/ ND</td>
<td>CO₂/ LI/ Herb</td>
</tr>
<tr>
<td></td>
<td>CO₂/ Herb/ UV/ ND</td>
<td>CO₂/ UV/ ND/ P</td>
</tr>
<tr>
<td></td>
<td>CO₂/ UV/ LI/ pH</td>
<td>CO₂/ pH/ P/ UV</td>
</tr>
<tr>
<td></td>
<td>Temp/ pH/ LI/ UV</td>
<td>Temp/ LI/ Herb/ pH</td>
</tr>
<tr>
<td></td>
<td>pH/ UV/ ND/ LI</td>
<td>P/ LI/ Herb/ ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Herb/ UV/ ND/ pH</td>
</tr>
<tr>
<td></td>
<td></td>
<td>UV/ ND/ P/ LI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>UV/ LI/ pH/ P</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH/ P/ UV/ Herb</td>
</tr>
<tr>
<td>5</td>
<td>CO₂/ Temp/ pH/ LI/ UV/ ND</td>
<td>CO₂/ Temp/ LI/ Herb/ pH</td>
</tr>
<tr>
<td></td>
<td>CO₂/ pH/ UV/ ND/ LI</td>
<td>CO₂/ P/ LI/ Herb/ ND</td>
</tr>
<tr>
<td></td>
<td>CO₂/ Herb/ UV/ ND/ pH</td>
<td>CO₂/ UV/ ND/ P/ LI</td>
</tr>
<tr>
<td></td>
<td>CO₂/ UV/ LI/ pH/ P</td>
<td>CO₂/ pH/ P/ UV/ Herb</td>
</tr>
<tr>
<td></td>
<td>Temp/ pH/ LI/ UV/ ND</td>
<td>Temp/ LI/ Herb/ pH/ UV</td>
</tr>
<tr>
<td></td>
<td>pH/ UV/ ND/ LI/ Herb</td>
<td>P/ LI/ Herb/ ND/ pH</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Herb/ UV/ ND/ pH/ P</td>
</tr>
<tr>
<td></td>
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<td>UV/ ND/ P/ LI/ Herb</td>
</tr>
<tr>
<td></td>
<td></td>
<td>UV/ LI/ pH/ P/ ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH/ P/ UV/ Herb/ LI</td>
</tr>
<tr>
<td>6</td>
<td>CO₂/ Temp/ pH/ LI/ UV/ ND/ Herb</td>
<td>CO₂/ Temp/ LI/ Herb/ pH/ UV</td>
</tr>
<tr>
<td></td>
<td>CO₂/ pH/ UV/ ND/ LI/ Herb</td>
<td>CO₂/ P/ LI/ Herb/ ND/ pH</td>
</tr>
<tr>
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CO₂, CO₂ enrichment; Temp, elevated temperature; Li, reduced light intensity; pH, reduced pH; P, phosphate starvation; Herb, herbicide; ND, general nutrient depletion; and UV, Ultraviolet-B radiation
2.5 Results

2.5.1 NED drives changes in growth of *C. reinhardtii*.

Population growth rate declines as NED increases (Figure 2.1; $F_{1,93} = 11.18$, $P = 0.001$), apart from NED level two, which shows a slight increase in growth rate (0.52 divisions (d$^{-1}$)). At NED level eight, the growth rate *C. reinhardtii* was 0.2 divisions (d$^{-1}$), which is a decrease of 68% relative to the control (0.68 day$^{-1}$). By virtue of the experimental design there are many replicates of each NED level with the exception of NED one and eight, and the effects of both NED and regime on growth rate can be investigated. Regime and overlap between regimes explains some (32% and 10% respectively) of the decrease in population growth rate. However, the NED itself explains approximately 37% of the decrease in growth rate independently of the particular combination of environmental changes involved.
Figure 2.1 Population growth rate of *C. reinhardtii* under 0 to 8 environmental drivers. Black data points and bars represent mean and standard deviation between regimes within a given level of NED. See Table 2-3 for regimes. Coloured points indicate the mean and variance of individual regimes (96 regimes total, see Figure 7.11 for identity of regimes). The grey dashed line indicates the growth rate of the evolved control in the control environment.
2.5.2 Interactions between environmental drivers

Given that regime explains some of the variation in the response to NED, interactions between environmental drivers within each regime were explored using the comparative, multiplicative and additive models. The best predictor of the drop in fitness is the single dominant environmental driver, so that the effect of increasing NED on population growth is best fit by a comparative model (Figure 2.2A; $R^2 = 0.43; P < .0001$). Using this model, the effect of environmental drivers in combination is determined by the single dominant (positive or negative) environmental driver present. The multiplicative and additive models were worse fits than the comparative model (multiplicative model: Figure 2.2B; $R^2 = 0.33, P < .0001$; additive model: Figure 2.2C; $R^2 = 0.25, P < .0001$). Regimes that do not fit any type of interaction were identified using a threshold of $R^2 > 0.8$. Populations growing in each regime were classified as either ‘deviant’ if they fell below the threshold ($R^2 < 0.8$), ‘comparative’, ‘additive’ or ‘multiplicative’, if they were equal to or higher than the threshold ($R^2 \geq 0.8$; Figure 2.3 - Figure 2.5). As expected from the model fits (Figure 2.2A-C) the majority of populations growing in each regime are comparative, then additive and finally multiplicative. However, a large proportion of populations (36%) are ‘true deviants’ and fail to be categorised into any of the model groups (Figure 2.6). The majority of these true deviant populations have some of the highest growth rates observed and these interactions are not predicted by any model.
Figure 2.2 Population growth rate of *C. reinhardtii* under 0 to 8 environmental drivers (a) is best explained by the comparative model. Population growth rates (mean and standard deviation) predicted by a model (white triangles) alongside observed values (black circles), followed by goodness of fit, for three models. (b) Comparative model ($R^2 = 0.43$, $P < .001$). (c) Multiplicative model ($R^2 = 0.33$, $P < .0001$). (d) Additive model ($R^2 = 0.25$, $P < .0001$); extinction is predicted in environments with >5 changes. Red dashed line indicates population extinction, and shows that under the additive model, populations will go extinct at NED > 4. Coloured points in panel a, indicate the mean and standard deviation of individual regimes (96 regimes total, see Figure 7.11 for identity of regimes).
Figure 2.3 Deviants from the comparative model. Observed population growth rates (open circles) plotted alongside the expected growth rate under the comparative model (solid line). Populations that fit the comparative model ($R^2 \geq 0.8$) are coloured in red. Deviant populations that do not fit the comparative model ($R^2 < 0.8$) are coloured in blue.
Figure 2.4 Deviants from the multiplicative model. Observed population growth rates (open circles) plotted alongside the expected growth rate under the multiplicative model (solid line). Populations that fit the multiplicative model ($R^2 \geq 0.8$) are coloured in red. Deviant populations that do not fit the multiplicative model ($R^2 < 0.8$) are coloured in blue.
Figure 2.5 Deviants from the additive model. Observed population growth rates (open circles) plotted alongside the expected growth rate under the additive model (solid line). Populations that fit the additive model ($R^2 \geq 0.8$) are coloured in red. Deviant populations that do not fit the additive model ($R^2 < 0.8$) are coloured in blue.
Figure 2.6 Observed population growth rates (open circles), under increasing NED (0 – 8).

Populations that fit the comparative model only ($R^2 \geq 0.8$) are coloured in orange.

Populations that fit the additive and multiplicative models ($R^2 \geq 0.8$) are coloured in green.

Populations that fit the comparative and additive models ($R^2 \geq 0.8$) are coloured in purple.

Populations that fit the comparative and multiplicative models ($R^2 \geq 0.8$) are coloured in pink. Populations that fit the comparative, multiplicative and additive models ($R^2 \geq 0.8$) are coloured in red. Deviant populations that do not with any of the models ($R^2 < 0.8$) are coloured in blue.
2.5.3 Case study – Temperature, CO₂ and pH

Most MED studies for photosynthetic microbes focus on three drivers: changes in temperature, pH, and CO₂ (Carpenter, 1992; Gruber, 2011). To understand how these intensively-studied environmental drivers affect growth when they occur in the context of additional environmental drivers, the effect of these three environmental drivers either alone, in pairs, all together, or in various combinations as part of regimes with additional drivers is presented as a case study (see Figure 2.7). When only a single environmental driver is present, populations grow fastest under CO₂ enrichment, slower under lowered pH, and slowest at increased temperature, however these differences in growth rate are not significant due to high variation within within regime. However, when combined as pairs, the effect of CO₂ enrichment counteracts the effect of temperature so that these populations have higher growth rates than when temperature changes alone, whereas the combined effects of high CO₂ and low pH is to lower population growth rates (Figure 2.7). While both lower pH and higher temperature reduce growth rates on their own, populations subjected to both environmental drivers at the same time grow faster than those subjected to either environmental driver alone. Differences between growth rates within environmental driver pairs are not significant due to high variation within regimes. When all three environmental changes occur together, populations grow faster than under any of the single or paired cases.
Figure 2.7 Population growth rates of *C. reinhardtii* in environments containing high CO$_2$, low pH, and high temperature. Each point shows mean and standard deviation for 3 replicate populations. The identity of regimes is indicated by colour. The colour and pattern of lines shows unique patterns of adding NED to environments. In general, population growth drops when environmental changes of larger effect than previously present (herbicide, nutrient depletion and phosphate starvation) are added. Herb, Herbicide; Li, light intensity; Temp, temperature; Co2, CO$_2$; P, phosphate; ND, nutrient depletion; UV, ultra-violet radiation.
Interesting interactions between CO$_2$, pH, temperature and additional environmental drivers are evident as NED increases. When combined with changes in CO$_2$, pH and temperature, reduced light intensity (at NED=4) does not affect growth in *C. reinhardtii*, although light intensity does affect growth on its own. The addition of herbicide, which is one of the strongest individual environmental drivers (decreasing growth rates) in the experiment, at NED=5, significantly reduces growth relative to NED=4. Generally, this trend is followed, where growth drops as NED level increases, largely as a result of a more dominant environmental drivers being more likely to be present. While the interactions between individual environmental changes are somewhat idiosyncratic when we examine this reduced data set, the overall pattern found in the full dataset, where the effect on growth is largely determined by the single dominant environmental change, is still informative. The effect of NED remains significant (Figure 2.8A; $F_{1,16} = 10.142$, $P = 0.006$), regardless of the identity of the environmental changes. The comparative model remains the best fit (Figure 2.8B; $R^2 = 0.36$, $P < .0001$). The multiplicative and additive models are worse fits than the comparative model (multiplicative model: Figure 2.8C, $R^2 = 0.26$; $P < .0001$; additive model: Figure 2.8D, $R^2 = 0.3$, $P < .0001$). The results from the case study suggest that investigations examining the effects of two or three environmental drivers, cannot predict the effect when additional environmental drivers are added, as interactions are idiosyncratic. However, I found when up to 8 environmental drivers are investigated in the case study, interactions become more predictable under the comparative model, albeit with a reduced relationship ($R^2$) between the observed growth rates and the growth rates predicted using the single environmental drivers, and this is due to the smaller sample size.
Figure 2.8 Population growth rates of *C. reinhardtii* in environments containing high CO$_2$, low pH, and high temperature under increasing NED (0 - 8). Only regimes used in the case study displayed. Solid black circles show the observed average population growth rates (± SD) between regimes, at each NED. Coloured points show the average growth rate (± SD) of replicate populations within each regime (18 total) (a). Open triangles show the expected mean population growth rate (± SD) under the comparative model (b), the multiplicative model (c) and the additive model (D). Red dashed line shows population extinction at zero number of divisions.
2.5.4 Post hoc analysis

Post hoc analysis revealed that the effect of NED is important regardless of the identity of the individual environmental drivers that make up each regime (Figure 2.1; \( F_{1,90} = 8.000, P = 0.006 \)). Some of the variation in the model is explained by four environmental drivers; CO\(_2\) (12%), phosphate (11%), herbicide (5%) and UVB (4%). The NED is still important and explains a large proportion of the variance observed in the model (19%).

2.5.5 The effect of removing CO\(_2\) enriched environments

When considering only those populations growing in regimes that include (amongst other drivers) high CO\(_2\), changes in growth rate of C. reinhardtii are driven by NED (Figure 2.9A; \( F_{1,45} = 8.060, P = 0.007 \)), which is consistent with the full data set; as NED increases the number of divisions (d\(^{-1}\)) decrease (high CO\(_2\) dataset intercept = 0.51 and slope = -0.04; full dataset intercept = 0.56 and slope = -0.03; Z-test = 0.28).

The effect of increasing the number of simultaneous environmental changes on growth is still best explained by a comparative model (Figure 2.9B; \( R^2 = 0.58, P < .0001 \)). The multiplicative and additive models were worse fits than the comparative model (multiplicative model: Figure 2.9C, \( R^2 = 0.44, P < .0001 \); and additive model: Figure 2.9D, \( R^2 = 0.33, P < .0001 \)). The effect of the dominant driver is mitigated by high CO\(_2\), such that the average growth at any given level of NED is higher than expected from the comparative model. When environments containing CO\(_2\) enrichment are removed from the dataset, the fit with all three models (Figure 2.9) is improved, mainly because CO\(_2\) enrichment causes an increase in growth when it is part of a complex environmental change.
Figure 2.9 Population growth rate of *C. reinhardtii* under increasing NED (0 – 7), excluding high CO₂. Solid black circles show the observed average population growth rates (± SD) between regimes, at each NED. Coloured points show the average growth rate (± SD) of replicate populations within each regime (48 regimes total, see Figure 7.12 for identity of regimes) (a). Open triangles show the expected mean population growth rate (± SD) under the comparative model (b), the multiplicative model (c) and the additive model (d). Red dashed line shows population extinction at zero number of divisions.
2.5.6 The effect of Environmental Overlap

In the present experiment, as environments become more complex overlap between environmental drivers between regimes (within each NED) increases. The overlap between regimes within NED levels explains 10% of the variation in growth seen here (Figure 2.1; $F_{1,5} = 13.092, P = 0.014$). As such overlap of environmental change has little effect on the amount variation in growth rate between regimes at each level of NED. In addition, I found no significant relationship between NED and the variation in growth rates between regimes (nested within NED) ($R^2 = 0.06, P = 0.53$). No variation is present at NED = 0 and NED = 8, which necessarily contain only a single regime each. Variation between regimes within each NED increases from NED = 1 and is highest at NED = 2 and three. Variation between regimes decreases from NED = 3 to and is lowest at NED = 6. A simulation was run of the same experiment using infinite environments with the same distribution of effects on growth for single environmental changes as in the focal experiment. After 1000 iterations it was found that using a finite number of possible environmental changes (in the present study) slightly underestimates growth rates in regimes with many drivers, but the effect is small (Appendix 1, Figure 7.9), confirming that the increase in overlap between regimes with increasing the number of drivers does not explain the overall pattern of the data.

2.6 Discussion

To date no experiment has had the power to pull apart the effect of the number and the identity of environmental changes. I have shown here that the number of environmental changes can be used to explain population growth responses to MEDs. Whilst changes in growth rates are driven by the single dominant environmental drivers, the drop in growth becomes more predictable as the NED increases. This also highlights an important point that understanding the type of
interaction and using the appropriate model is essential in order to determine if interactions are either synergistic or antagonistic (Folt & Chen, 1999). This is important, because if a model is incorrectly used to explain the interactions between MEDs, any deviations from the model will be defined as either antagonistic or synergic depending on the direction of the effect of the environmental drivers alone (Piggott et al., 2015) (Figure 1.2).

2.6.1 Population growth rate of C. reinhardtii under increasing NED

Population growth rates decrease with increasing NED, regardless of the identity of the environmental drivers (Figure 2.1), and is driven by the dominant environmental driver (Figure 2.2A). Therefore, the relationship between NED and population growth is described best by the comparative model. In the present study, herbicide and phosphate depletion are dominant environmental drivers (Figure 7.2), and so when more drivers are added as NED increases, no additional effect will be observed on the rate of cell division of C. reinhardtii. The relationship between the number of environmental drivers and dominant environmental drivers increases in strength and becomes more predictable as NED increases (Figure 2.7). This means that we can predict the effect of MEDs on organismal growth rate as long as we know the effect of the single drivers alone, or even the NED. The trend presented here is particularly useful if investigators are interested in the outcome of high NED. For example, if the investigator is interested in the outcome of seven environmental drives, and the effect of the dominant environmental driver is known, then predictions on the effects when seven environmental drivers are combined, can be made using the comparative model. However, knowing the dominant environmental driver is not useful for investigators that want to predict the outcome of any three environmental drivers in combination.
2.6.2 The effect of environmental overlap on MEDs

Though overlap between regimes explains very little of the variance in growth (Figure 2.1), using a finite number of environments slightly overestimates the average decrease in growth in environments as NED increases (Appendix 1, Figure 7.9). Environmental changes in the natural environment are inherently complex and we expect many aspects of the environment to change in the future under the framework of global change (Gruber, 2011). Given that few environmental drivers of interest for global change are truly novel or outside the range of current environments (Gienapp et al., 2008), most drivers should have relatively small effects on organismal function. However, a subset of environmental drivers will have large effects on organisms, and the chance of a dominant environmental driver arising increases as NED increases (Appendix 1, Figure 7.10), and this is supported by the simulation data (Appendix 1, Figure 7.9). It is expected that the natural world will lie somewhere in between finite and infinite outputs of the simulation data, with the dominant environmental changes at high NED driving the effects of MEDs. Though expected growth rates from the comparative model are overestimated in the simulation, the prediction that dominant drivers will have large effects on growth as NED increases in natural communities still holds. Possible experimental avenues to correlate findings of laboratory experiments and the natural world are discussed in chapter 6.

2.6.3 The case study shows that single drivers lose predictive power when NED is low

When a few environmental drivers are investigated further, the level of NED becomes more important when forming predictions on the combined effect of environmental drivers. CO₂, temperature and pH were investigated further (Figure 2.7), as they have been studied reasonably well in isolation (Riebesell et al., 2000;
Yang et al., 2001; Yang & Gao, 2003; Riebesell, 2004; Rost et al., 2008; Fulweiler et al., 2011; Boyd et al., 2013), in pairs (Barko et al., 1982; Riebesell, 2004; Schippers et al., 2004; Ventura et al., 2008; Feng et al., 2009), and, rarely, all together (Feng et al., 2008). Note that some methods used in multiple driver experiments investigating the effects of elevated CO\textsubscript{2} do not separate the interactions of CO\textsubscript{2} and acidifying media at the physical-chemical level and are therefore unable to separate the outcome effects of pH and CO\textsubscript{2} at the organismal level. These drivers are also well represented in biogeochemical climate change models (Meehl et al., 2007; Bopp et al., 2013; IPCC, 2013), providing a framework for designing MED and scenario-based experiments in order to predict how these environmental drivers might affect natural communities (Boyd et al. 2015b; Wohlers et al. 2009; Dupont et al. 2010; Pörtner 2012). For this reason, CO\textsubscript{2}, temperature and pH were explored more closely here. Like with the full dataset, population growth rate decreases with increasing NED, independently of the identity of the environmental changes (Figure 7.13). However, the fit of the model is worse, and this is due to having low replication, unlike the full dataset.

2.6.4 The comparative model predicts the outcome of MEDs in the case study

The comparative model is still the best model for predicting the effect of MED when replication levels are low, as changes in the number of divisions are still largely driven by the most dominant environmental change (Figure 2.8). The best example of this is reduced light intensity, which has a small effect on population growth rate when alone and when in combination with other environmental changes (Figure 2.7). The effects of light intensity are masked by more dominant environmental drivers and as such reduced light intensity fits the conditions of the comparative model and violate the conditions of the multiplicative and the additive models. In the complete dataset, light intensity is present in 85% of the regimes where population growth is
driven the dominant environmental drivers and are predicted by the comparative model (Figure 2.6).

Synergistic and antagonistic interactions are also observed in the case study (Figure 2.7). For example, when both phosphate (P) and nutrient depletion (ND) are limited in combination with other environmental changes, the effect on the overall growth rate is greater than that expected under the comparative model - indicating that this is a synergistic interaction (Figure 2.7). Like many other studies, this suggests *C. reinhardtii* is limited by both resources which is not in agreement with the comparative model or the analogous Liebig’s law (de Baar, 1994; Arrigo, 2005). On the other hand, when only one resource is limiting (P or ND) these environmental changes may be interacting with other environmental drivers. For example, at high NED, the addition of nutrient depletion (ND) has very little effect and is masked by the dominant effects of herbicide. ND and herbicide therefore have a comparative interaction, whereas, phosphate limitation and herbicide have an antagonistic interaction (Figure 2.7). This is surprising as both herbicide and phosphate are dominant environmental drivers in most regimes.

The herbicide used here is atrazine which directly blocks the photosynthetic electron transport chain reducing photosynthetic efficiency (Fischer *et al.*, 2010). Phosphate is a limiting factor in many natural environments yet it is a necessary macronutrient that photosynthetic organisms like *C. reinhardtii* require in large amounts (Irihimovitch & Yehudai-Resheff, 2008). Fischer *et al.* (2010) suggests protection mechanisms such as nonphotochemical quenching (NPQ) to explain the antagonistic interaction observed between atrazine and high light in *C. reinhardtii*. NPQ of chlorophyll autofluorescence is an extremely powerful and flexible tool in which photosynthetic organisms can acclimate and tolerate environmental stresses, most notably excess light levels (Müller *et al.*, 2001; Bonente *et al.*, 2012). However, NPQ can be influenced by other environmental factors such as heat, or freeze/thawing (Krause & Weis, 1991), and is likely to be beneficial if photosynthesis-inhibiting
herbicides such as atrazine when present in the environment. Antagonistic interactions between phosphate depletion and other environmental changes have also been documented in a nitrogen fixing species (Trichodesmium; Hutchins et al., 2007). Hutchins et al. (2007) suggest that phosphate limited populations are also CO$_2$ limited and in the presence of high CO$_2$ the population growth rate is elevated. The data presented in this case study agrees in part with the suggestion that elevated CO$_2$ predicted under the framework of global change may have the potential to offset the effects of multiple environmental changes, including phosphate limited environments. However, the presented case study also shows that when nutrients and phosphate are co-limiting growth rate can no longer be recovered by high CO$_2$.

### 2.6.5 Interactions between environmental drivers

Synergistic and additive interactions are common in studies investigating pairs of environmental changes. For example, increased acidification and Ultra violet radiation (Gao et al., 2009), elevated CO$_2$ and increased light exposure (Gao et al., 2012), elevated CO$_2$ and nitrogen limitation (Sciandra et al., 2003; Lefebvre et al., 2012), high light and increased herbicide concentrations (Fischer et al., 2010), warming and elevated CO$_2$ (Schlüter et al., 2014). This trend has also been documented in experiments with three environmental changes such as elevated CO$_2$, reduced O$_2$ and increased temperatures (synergistic effects reviewed by Langenbuch & Michaelidis 2005), warming, drought, and acidification (Christensen et al., 2006) and elevated CO$_2$ and nutrient limitation and high light levels (Leonardos & Geider, 2005). Antagonistic interactions are also demonstrated between antibiotics (Ankomah et al., 2013; Ocampo et al., 2014), pH and temperature (Christensen et al., 2006), herbicides and light intensity (Fischer et al., 2010), pH and increasing salinity (Koprivnikar et al., 2010). In addition, see reviews by Jackson et al. (2015), Crain et al., (2008) and Darling and Côté, (2008).
Interactions between environmental drivers are indeed informative for understanding how organism will respond to MEDs, however the outcome of interactions between drivers is not predictable and so the average interaction cannot be predicted when NED is low. If the goal of an experiment is to understand the outcome of interactions between MEDs then interactions must be measured empirically as inferences cannot be made using the individual effects of the environmental drivers. In addition, the idiosyncratic effects of multiple driver experiments using between two and three drivers cannot be used to make predictions on the effects of these same drivers when NED is increased (Byrne & Przeslawski 2013).

### 2.6.6 The predictive power of scenarios

When NED is low the outcome of MED cannot be predicted, and scenarios must be used (Boyd et al. 2015b). Boyd et al. (2015b) used future climate change projections to predict the effect of changing temperature, nutrients, iron, CO\(_2\) and light intensity, and found that where growth rates are promoted in warming and iron rich environments, the negative effects of the remaining three environmental drivers are mitigated when in combination. Using data in the present study it was possible to recreate a similar scenario, using CO\(_2\), phosphate limitation, general nutrient depletion, reduced light intensity and increased temperature, in order to replicate a five driver experiment (Appendix 1, Figure 7.13). The effects of five environmental drivers produced idiosyncratic patterns and predictions from the single drivers alone could not predict their effect in combination. Though, a conventional factorial design could not be replicated using these five drivers, low replication within NED level greatly limits the predictive power of MEDs.
2.6.7 Elevated CO$_2$ mitigates the negative effects of additional environmental drivers

The data presented here is best described as antagonistic comparative, as CO$_2$ buffers the negative effects of additional drivers. In my study, the models underestimate the growth rate of populations of *C. reinhardtii* under MEDs (Figure 2.9). The comparative model assumes that there are no interactions between non-dominant environmental changes. However, in some cases, non-dominant environmental drivers may mitigate or exacerbate the effects of the dominant driver, a result also observed by Folt & Chen (1999). For example, high CO$_2$ is not the dominant environmental driver in most of the regimes, as the individual effect on growth rate is small, but it does affect growth rates in many regimes. Increasing CO$_2$ enriches the environment and this can be stressful, exacerbating the negative effect of other individual changes (synergistic interaction; see Piggott et al. (2015)), or it can elevate the growth rate even when other environmental changes including the dominant change are present (+ antagonistic if growth rate is less than the effect of CO$_2$ alone, or – antagonistic if growth rates is greater than the effect of CO$_2$ alone; Piggott et al. (2015)) (Schippers et al. 2004). The antagonistic interaction is more common between CO$_2$ and other combinations of environmental changes in my study (Figure 2.7). If we remove all regimes with elevated CO$_2$ from the dataset, the general trend of decreasing growth rate with increasing NED still holds, with an improved fit with all the models as CO$_2$ is no longer elevating the mean fitness at each NED level (Figure 2.9).

CO$_2$ therefore has the potential to mitigate the negative effects of many environmental drivers, so that populations have improved growth rates in response to MEDs when CO$_2$ is present (Figure 2.9, Appendix 1, Figure 7.13), at least at the short-term. This is supported by research on the physiological and biochemical responses to elevated CO$_2$, high temperature and reduced water in *Arabidopsis* by Zinta et al.(2014), who demonstrate the stress-mitigating CO$_2$ effect operates through
up-regulation of antioxidant defence metabolism in addition to reduced photorespiration. However, there are some environmental drivers that have synergistic interactions with high CO$_2$. For example, elevated CO$_2$ and reduced pH have a synergistic interaction in the present study (growth rates are lower than both single environmental drivers alone) (Figure 2.7), which may be a result of limitations in the electron donation in PSII at low pH (see Gerloff-Elias et al. (2005); Krause & Weis (1991)), which is exacerbated by elevated CO$_2$. Another explanation is that when under photosystem stress, the CO$_2$-concentrating mechanisms (CCM) is down-regulated which reduces growth rates (Hopkinson et al., 2011; Gao et al., 2012). The synergistic interaction between elevated CO$_2$ and reduced pH observed here, is similar to the synergistic interaction between high light (which causes cellular stress due to production of reactive oxygen species (ROS)) and elevated CO$_2$, which interact and increase oxidative stress in diatoms (Gao et al., 2012). Although, mechanistic synergistic interactions are reasonable well understood between elevated CO$_2$ and and pH in ocean acidification studies (Boyd & Hutchins, 2012), their individual effect on organisms are rarely disentangled (Gao et al., 2012).

Whilst the mitigating effects of high CO$_2$ makes intuitive sense in photosynthetic green algae, the effects of elevated CO$_2$ are likely to have different effects on a different species, including calcifying phytoplankton. *Emiliania huxleyi* show reduced rates of calcification of their skeleton, due to altered seawater biochemistry as a result of elevated CO$_2$ and increased acidification of seawater (decreasing concentrations of CO$_3^{2-}$) (Riebesell et al., 2000; Beaufort et al., 2011; Schlüter et al., 2014). However, other environmental drivers may be protective and mitigate the effects of dominant environmental drivers in other species. Environmental drivers with mitigating effects may be important in maintaining growth and reproduction levels under high NED predicted in the future.
2.7 Conclusion

Here, I have demonstrated that average changes in population growth in a model microalga are largely predictable from either the number of environmental drivers, or the effect of the single most detrimental driver, in cases where a large number of environmental drivers occur together. Multiple driver experiments and models are growing in number and this paper contributes to this field by providing a method which predicts the general effect of MEDs. Mechanistic understandings on the effect of interactions have been traded in favour of predictive power, and this method is appropriate when there is uncertainty around the identity of environmental drivers that are likely to change, the intensity of these drivers, and organisms likely to be affected, or when responses on organisms of interest cannot be gathered. This method is also useful for designing more ‘realistic’ experiments that include more than one genotype, species or trophic level. As the complexity of MEDs experiments increase more efficient methods such as this (and complimentary scenario based experiments), will be required in order for the experiments to be manageable whilst retaining predictive power.
3 Chapter 3 – The evolutionary response of *C. reinhardtii* to MEDs

3.1 Chapter summary

This experiment follows on from chapter 2 where I measured the plastic response of the founding population to MEDs, and in doing so, quantified the change in fitness before evolution. In this chapter, I explore how the number of environmental drivers affects evolution in an initially isogenic population. I grew replicate populations of *Chlamydomonas reinhardtii* in the same 96 regimes as I used in chapter 2. The regimes contained between one and eight different environmental drivers, including elevated temperature, CO$_2$ and UVB, reduced light intensity and phosphate, acidification, nutrient depletion and the addition of a herbicide for ~350 generations (95 transfers) in batch culture. All populations were founded from a single colony that was grown in the control media for seven days so that initial genetic variation was low and it is likely that all adaptive variants arose through novel heritable variation during the selection experiment. At the end of the selection experiment, I measured the growth rate of the MED-evolved populations in their selection environments and the growth rate of the evolved control populations in the same selection environments, to calculate the direct response to selection. I also measured the fitness of all populations in the control environment to calculate the correlated response to selection (experimental design illustrated in Figure 1.4).

3.2 Abstract

MEDs interactively influence growth rates of groups of organisms such as marine and freshwater phytoplankton, but it is unknown how evolving in response to the MEDs predicted under global change will impact phytoplankton growth. Based on Fisher’s (1930) geometric model of adaptation, which states that the rate of adaptation decreases as the number of independent traits under selection increases, I
hypothesise that the fitness of *C. reinhardtii* will decrease in a predictable way with increasing NED, due to an increase in the number of traits under selection (as NED increases). However, I found that populations evolving in higher NED environments have a larger direct response to selection. I demonstrate that the direct response to selection in environments with MEDs correlates with the strength of selection. As NED increases, the strength of selection increases, and the change in the strength of selection is predictable from knowing the number of environmental drivers. I found that even when adaptation occurs under MED, ancestral growth rates are not restored, and I propose that this is due to the high NED environments being lower quality environments than the control environment. The growth rates of MED-evolved populations decrease with increasing NED, but the majority of the variation in evolved growth rate is explained by the identity of the selection environment. In addition, environmental drivers that cause starvation (reduced phosphate) were found to constrain the evolutionary response, probably by reducing the maximum growth rate of populations. This is consistent with the short-term response where the dominant environmental drivers determine growth rates. Crucially, I found that predictions can be made on the long-term response to MEDs, by knowing the plastic response (the drop in fitness), in the same environments. This is particularly useful since many ecological predications are almost exclusively based on the findings of short-term experiments.

### 3.3 Introduction

The purpose of this study is to extend our understanding of adaptation to include evolution under MEDs. Predicting the long-term effect of MEDs is particularly important for ecologically important organisms such as aquatic phytoplankton, that have short generation times, and can evolve in relatively short timescales (months and years) in response to MEDs predicted under future global change scenarios (Collins *et al.*, 2013; Reusch & Boyd, 2013; Yampolsky *et al.*, 2014). Physiological responses to MEDs associated with global change have been studied reasonably well
in marine (see review by Crain et al., 2008) and freshwater (see review by Folt & Chen 1999) microalgae, and there are an increasing number of investigations into evolutionary outcomes of single environmental drivers (review by Collins et al. 2014). MED studies investigating evolutionary outcomes in the face of many different environmental drivers are limited, rarely investigating more than two environmental drivers (Beardall et al., 2009), and the outcome of interactions between MEDs over micro-evolutionary timescales is largely unknown (Reusch & Boyd, 2013).

3.3.1 Can plastic responses predict the evolutionary response to MEDs?

Physiological responses do not always remain the same over microevolutionary timescales (Godbold & Solan, 2013). For example, a characteristic plastic response of chlorophytes to elevated CO\(_2\) is faster growth rates (Yang & Gao, 2003; Low-Décarie et al., 2011), however after evolution, growth rates can become insensitive to elevated CO\(_2\) (Collins & Bell 2004; Low-décarie et al. 2013). This is in agreement with results obtained from natural microalgae assemblages (three genera: Tetracystis sp., Chloranomala sp. and Chlorococcum sp.), collected from high CO\(_2\) springs (Collins & Bell, 2006). Collins and Bell demonstrated that high CO\(_2\) (1000ppm) adapted C. reinhardtii are no longer able to induce high-affinity CO\(_2\) uptake at ambient CO\(_2\) due to the down regulation of the carbon concentrating mechanism (CCM) (Collins et al., 2006). Support for down regulation of CCM has also been found in the coccolithophore, Gephyrocapsa oceanica. Jin et al. (2013) found that after ~670 generations under high CO\(_2\) (1000 µatm) conditions, chlorophyll a content is reduced in ambient CO\(_2\) suggesting that there is a reduced need for light harvesting associated with reduced activity or inactive CCM (Jin et al., 2013). These investigations demonstrate uncertainties that changes in the regulation of CCM can have on our ability to predict the long-term effect of elevated CO\(_2\) on photosynthetic organisms - long-term experiments are required.
3.3.2 Adaptation to environmental changes

Adaptation in Fisher’s geometric model (1930) is determined by three elements; the distance, $d$, of the current trait from the optimum, the magnitude of change, $r$, of the current trait towards optimum and the number of dimensions or characters, $n$, of the trait(s) under selection. The magnitude of $r$ cannot exceed that of $d$, as this would move the current trait away from the optimum. In addition, mutations with a large effect on $r$ have extremely low probabilities of improving fitness. Conversely, mutations with a small effect on $r$ have a much higher probability of being advantageous, with a probability of 0.5 when $r$ is close to zero (Equation 3-1; Fisher 1930). Thus, the size of the adaptive steps towards the optimum is characterised by an initial rapid rise in fitness, followed by a gradual slowing as the current trait approaches the optimum (Orr 2000). However, Kimura (1983) shows that small mutations must also escape loss via genetic drift. As such, adaptation will most likely involve mutations of an intermediate size (Orr 2005; Orr 2006).

\[
\text{Probability of improving fitness } = \frac{1}{2} \left(1 - \frac{r}{d}\right)
\]

Equation 3-1

Fisher (1930) assumes all beneficial mutations will have an equal effect on all traits under selection, also known as universal pleiotropy (Orr, 2000; Tenaillon, 2014), and as $n$ increases the fitness gain from a beneficial mutation of a fixed size will decrease in a geometric sequence (Fisher, 1930; Orr 2000). Fisher (1930) uses this theory to show that complex organisms adapt more slowly than simple ones, as complex organisms with a large number traits under selection will have a smaller fitness gain from beneficial mutation and populations will move more slowly towards the optimum (Equation 3-2; Fisher, 1930), illustrated in Figure 1.3.
3.3.3 Genetic constraints on adaptation

There are many factors that may constrain responses to natural selection, such as lack of genetic variance and antagonistic correlations among traits (negative pleiotropy) (Czesak et al., 2006; Reed et al., 2011). Negative pleiotropic interactions between multiple traits under selection will result in either a smaller step towards the optimum (Figure 1.3) or if the deleterious effects of new trait values are greater than the beneficial effects (the net effect of the mutation is non-adaptive), then the population must wait for a new beneficial mutation to arise and fix within the population. Etterson and Shaw (2001), demonstrate that despite genetic variance for traits (in this case, leaf number and reproductive stage, and leaf number and leaf thickness) under selection, antagonistic genetic correlations limit adaptive evolution within populations of Chamaecrista fasciculate when placed in warmer and more arid environments, predicated under future global change scenarios. The direction of pleiotropic interactions is important and has fundamental evolutionary consequences. For example, positive pleiotropic interactions among traits under selection can move populations towards the optimum faster, as beneficial mutations increase the fitness of more than one trait under selection (Figure 1.3) (Agrawal & Stinchcombe 2009; Reed et al. 2011).
3.3.4 As NED increases, extreme environmental conditions may prevent adaptation

Environments that are at the edge of a species’ tolerance are often categorised as extreme (Pörtner et al., 2005), and are commonly studied using reaction norms (Chevin et al., 2010a). Here I define extreme environments in the same way. At high NED, extreme environments are those that reduce survival of C. reinhardtii, and as such, may indicate a tipping point where increasing NED any further will increase the risk of extinction (Chevin et al., 2010a). In order to survive extreme environments, resistant genotypes must be able to spread throughout the population and restore growth before populations go extinct (Gomulkiewicz & Holt, 1995), and this is described as evolutionary rescue (Bell & Collins, 2008). In addition to variance in relative fitness, which is required for adaptation, evolutionary rescue requires variation in absolute fitness (Bell, 2013a). However, the probability of evolutionary rescue after environmental change depends upon the initial population size (Gomulkiewicz & Holt, 1995; Bell & Gonzalez, 2009), the rate of environmental change and the supply of genetic variation (Agashe et al., 2011; Lachapelle & Bell, 2012). Maximum fitness may be lower in more extreme environments because of physiological constraints that prevent growth rates from increasing, resulting in new intermediate optimum values (Chevin et al., 2010a). Alternatively, populations growing in very stressful environments (without the ability to disperse) will go extinct if adaptation is insufficient to allow the populations to sustain long-term growth in stressful environments (Gienapp et al., 2008).

3.3.5 The long-term of effect of growth under MEDs

Environmental changes predicted under global change scenarios are unlikely to occur in isolation, and interactions between environmental changes in experimental and natural environments are often synergistic or antagonistic, rather than additive (Folt
When MED experiments have more than two or three drivers (see chapter 2), plastic responses are driven by the dominant environmental driver (chapter 2). However, the long-term effect of MEDs such as those expected under global change, have not been investigated beyond two environmental drivers. Schlüter et al. (2014), investigated the effect of high temperature (26.3 °C) in populations of *Emiliania huxleyi* adapted to ambient (400 µatm), medium (1,100 µatm) and high (2,200 µatm) $p$CO$_2$. *E. huxleyi* populations adapted to elevated temperatures were found to grow significantly better at high temperatures compared with evolved control populations, in all CO$_2$ conditions. However, populations adapted to high temperature suffer reduced growth rates relative to evolved control populations, when grown at ambient temperature (15 °C). Tatters et al. (2013), demonstrate that changes in community composition will occur under MEDs. After 12 months of growing at three levels of CO$_2$ and elevated temperature (ambient + 5 °C), the abundance of species of a mixed diatom community are significantly reduced: the effect of temperature alone significantly reduces species diversity and only *Cylindrotheca fusiformis* remain after selection, regardless of CO$_2$ levels. Although evolutionary responses to the environmental drivers were not detected, Tatters et al. demonstrate that community composition is likely to be altered and communities will look different under elevated CO$_2$ alone and when in combination with elevated temperature. However, the change in community composition was consistent in both short- and long-term experiments, suggesting that predictions can at least sometimes be made from short-term experiments where long-term experiments are not possible.

3.3.6 The evolutionary response of a simple organism to MEDs

My hypothesis that populations evolve less, or more slowly, at high NED is based on more independent traits being under selection as NED increases. It is reasonable to suppose that there is a positive relationship between $n$ and NED if responses to environmental change are not completely general. For instance, when CO$_2$ changes
alone and when CO$_2$ and temperature change together. We might expect that the number of traits under selection increases when both drivers are present if there are some traits that respond only to changes in CO$_2$ levels and/or traits that respond only to changes in temperature, even if some traits respond in the same way to changes in CO$_2$ or changes in temperature. In this case, the number of traits under selection goes up between NED = 1 and NED = 2. Alternatively, when CO$_2$ and temperature change simultaneously and if all traits under selection are the same for both environmental drivers individually, the number of independent traits under selection remains the same as NED increases from 1 to 2.

As the number of independent traits under selection increase, the rate of adaptation is reduced (Figure 1.3). And this is because the new trait value must be favoured by both drivers in order for it to be fixed by natural selection. Otherwise, if new trait values are in the opposite direction of selection for one environmental driver, the new trait value must have a net positive effect on fitness in the selection environment in order to be fixed by natural selection, but will suffer a reduced fitness gain from the beneficial mutation (Figure 1.3). As the number of traits under selection increases with increasing NED the chance that all new trait values will be favoured by all environmental drivers is reduced. Therefore, selection on more independent traits slows adaptation. Pleiotropy will speed up rate of improvement if new trait values are in the same direction of selection for both environmental drivers (positive pleiotropy), but will reduce the speed of evolution if new trait values are against the direction of selection for both environmental drivers (negative pleiotropy).

### 3.4 Methods and Materials

Details of culture environments and measures of population growth rate can be found in chapter 2, section 2.4 Methods and Materials, pages 27 - 37.
3.4.1 Selection experiment

All populations were founded from a single cell of *C. reinhardtii* (CC-2931, mt-; Chlamydomonas Resource Centre, University of Minnesota), grown in sterile Sueoka's high salt medium, buffered with Tris HCl (HSMT; Harris 1989) (Table 2-1, Table 2-2), and subsequently split into 576 populations (6 independent replicate populations per regime x 96 regimes; Table 2-3). Using a single cell to found all populations removes standing genetic variation and ensures that evolution within populations uses *de novo* variation (see Figure 1.4 for schematic diagram of the experimental design for the selection experiment). Environmental drivers (see chapter 2, section 2.4.2 for details of environmental drivers used to measure the initial and evolved response to selection) were implemented in a single step at the beginning of the selection experiment and include 1 to 8 of the following parameters (the control level is shown in brackets): increased CO$_2$ to 2000ppm (420ppm), temperature to 26°C (25°C), decreased pH to 6.5 (7.2 pH), light levels to 18 μmol m$^{-2}$ s$^{-1}$ (32 μmol m$^{-2}$ s$^{-1}$), reduced phosphate to 1.69 mM (13.56 mM), general nutrient depletion by 75% (100% nutrients), and added 0.5 μM of the herbicide atrazine. Test environments with UV were exposed to a dose 8.1 KJ.m$^{-2}$ UV radiation once a week. All populations were grown in 96-well plates containing 250 μL of culture media and were propagated by batch culture (50 μL of growing cells were transferred into 200 μL of fresh media), for 95 transfers in their selection environments, with 3 – 4 days between transfers, for a total of approximately 350 asexual generations. Populations evolved in environments with least one driver are referred to as MED-evolved populations. Populations evolved in the control environment are referred to as control populations. Here, environments that populations were not evolved in are referred to as assay environments and the NED of assay environments are referred to as assay NED.

Some populations went extinct during the selection experiment. Regimes where all populations went extinct during the selection experiment include herbicide (NED =
1) and the regime with all eight drivers (NED = 8). After the initial extinction of all populations within NED = 8 (at transfer number 4, approximately 12 generations into the experiment), the volume of cells transferred during batch culture was increased by 100% in order to prevent further extinctions so that observations in this regime could be continued. However, NED = 8 populations were excluded from the analysis due to differences in transfer regime and because, relative to other populations, they have a fitness of zero because they went extinct. Although evolved NED = 8 populations were not included in the analysis, the response to selection (given that populations could evolve under different transfer regime) to eight environmental drivers is shown in all figures.

3.4.2 Assays – Fitness after evolution

For all calculations of the direct and correlated response to selection, each population was acclimated to the assay environment for 4 days and then transferred to fresh medium at equal cell density (approximately 41,000 cells/ml). Cell counts were performed at 0 and 72 hours of growth in fresh medium using a BD FACSCanto II (BD Biosciences, Oxford, UK) flow cytometer calibrated with CS&T beads. Due to the size of the assays, cell counts were performed in batches. Regimes were randomly assigned to a batch and technical replicates were counted within a single batch. The effect of batch number on growth rates is included in the analysis (see below). The data were acquired with the BD FACSDiva v6 software. Each culture was counted three times. The cell counts were transformed into cells per ml and the average number of divisions per day was calculated using Equation 3-3.
Rate of Division \( (\text{day}^{-1}) = \frac{\log_2 \left( \frac{N_t}{N_0} \right)}{(t_f - t_0)} \)

where \( N_t \) is the cell density (cells/ml) at time \( t \) (hours) and \( N_0 \) is the cell density at time \( t_0 \). This calculation allows the comparison of populations with different growth strategies, and reflects the number of cell division per starting cell, so that the measurement is not sensitive to small differences in the initial number of cells (Collins, 2011a). None of the populations in the experiment reached carrying capacity with the transfer regime and assays used.

The direct response to selection was measured by comparing the growth of an MED-evolved population and a control population in the appropriate MED selection environment. The correlated response to selection in the MED-evolved populations was measured in the control environment relative to the evolved control in the control environment. The plastic response to environmental change (the response in the absence of evolution in that environment) was measured by comparing the growth rate of the control populations in the appropriate MED selection environment with the growth rate of that same control population in the control environment. Evolved controls were used in order to account for the effects of adaptation to general culturing and laboratory conditions. Both the direct and correlated responses to selection were calculated using Equation 3-4, and the differences between MED-evolved populations and evolved control populations are scaled relative to the number of divisions \( (d^{-1}) \) of the control in the relevant the MED environment for the direct response and the control environment for the correlated response.
\[ s = \frac{(E - C)}{C} \]

Equation 3-4

where \( E \) is the number of divisions \( (d^{-1}) \) of MED-evolved populations selected in environments with MEDs, and \( C \) is the number of divisions \( (d^{-1}) \) of evolved control populations selected in the control environment, measured in the appropriate environment. Measurements were carried out at the end of the selection experiment, after 95 transfers in each regime. All populations were acclimated to their assay regimes for one transfer cycle (4 days), before growth measurements were carried out over 72 hours.

### 3.4.3 Statistical Analysis

The effect of the identity and NED on absolute growth rates was analysed using a mixed model in R (R Core Team, 2013), using the packages lme4 and lmerTest. NED (0-8) is a fixed factor, as is overlap between regimes within each level of NED (measured as the average number of shared drivers between different test environments for a given NED; see chapter 2, section 2.4.6). Regime, batch and evolved populations within each regime were taken as random factors. The same model was used for analysis of the direct response to selection, with the strength of selection included as a fixed effect in the mixed model. A post-hoc mixed model was used to identify any variation in the direct response and absolute growth rate explained by identity of each environmental driver (i.e. \( \text{CO}_2 \) or temperature). The eight environmental drivers, nested within NED, were added to the random part of the model in place of regime, as described above. Regimes within NED = 8 were excluded from the analysis due to differences in transfer regime that were necessary to avoid extinctions. In order to determine if NED drives changes in the direct response when the strength of selection does not vary, a subset of data, where the
variation in the direct response to selection is highest (all NED levels are sampled) and the strength of selection is fixed ($2.4 < t < 2.6$), was analysed using a mixed model. The NED and overlap are taken as fixed, and batch, regime and evolved populations are taken as random.

To directly compare the contributions of fixed and random factors to variance, the percent contribution of fixed factors was estimated using Equation 3-5.

$$\text{Percentage of Fixed Effect Variance} = \left( \frac{\sigma_F^2 \times (b^2 - se^2)}{\sigma_X^2} \right) \times 100$$

Equation 3-5

where $\sigma_F^2$ is the variance of the fixed effect, $b$ is the slope of the fixed effect estimated by the mixed effects model, $se$ is the standard error of the fixed effect as estimated by the mixed effects model and $\sigma_X^2$ is the variance of the response variable.

3.5 Results

3.5.1 The evolutionary response of C. reinhardtii to MEDs can be predicted from the strength of selection

As NED increases, the direct response to selection increases (Figure 3.1; the effect of the strength of selection on the direct response of the MED-evolved populations; $F_{1,90} = 14.251, P < 0.0003$). This is probably driven by the strength of selection. The evolved replicate populations within each regime explains the majority of the variation in the direct response of the MED-evolved populations (35%), with the
strength of selection and the identity of the selection environments explaining 18% and 19% respectively. The NED of the selection environment explains less than one percent of the variation of the direct response to selection, and this is not statistically significant (Figure 3.1; the effect of NED on the direct response of the MED-evolved populations; $F_{1,128} = 1.416, P = 0.236$). The the strength of selection is measured using the evolved control populations when grown in each selection environment, relative to the to the growth rate in the control environment (Figure 3.2, light grey boxplots). Growth rates of the evolved control populations decreases with increasing NED (Figure 3.2; the effect of NED on the growth rate of evolved control populations; $F_{1,98} = 11.987, P <0.0001$). However, due to high variation in growth rates between the evolved control populations in the single environmental drivers (Appendix 4, Figure 8.1), predicting the combined effect from the single environmental drivers (using the comparative, additive and multiplicative models) is not useful here (as was done in chapter 2).

The growth rates of the MED-evolved populations follow the trend of decreasing growth rate as NED increases, until NED is greater than 4, where there is little change in growth until NED = 7, and growth rates are lowest (Figure 3.2; the effect of NED on the growth rate of MED-evolved populations; $F_{1,130} = 3.42, P =0.0665$). Note that NED = 8 is not included in this analysis, due to differences in transfer regime (see methods and materials). The evolved replicate populations explain the majority of the variation in growth rates of the MED-evolved populations (42%), with the identity of the selection environment and NED explaining 15% and 5% respectively.
Figure 3.1 Box plots show the direct response to selection measured as number of divisions relative to control populations assayed in the same assay environment. Open circles show the average direct response of evolved populations evolved in each regime within NED 1 - 8. The dashed line indicates that there is no difference between the growth rate of the evolved control and the MED-evolved populations, in the same selection environment.
Figure 3.2 Number of divisions of evolved control populations (red) and MED-evolved populations (blue) assayed in the section regimes containing between 0 and 8 NED. The coloured bands in the middle of the box plot denote the median value, the bottom and top of the red/blue boxes represent the 1st and 3rd quartile of the data, respectively. The ‘whiskers’ extending from the boxes indicate the positions of the lowest and highest values in the data. Coloured points show the average number of divisions of evolved control populations and MED-evolved populations, within each regime. The dashed line shows the average growth rate of the evolved control populations in the control environment (NED = 0). Although evolved NED = 8 populations were not included in the analysis, it is displayed here to show the growth rate of populations they had not gone extinct during the selection experiment.
3.5.2 The strength of selection can be predicted from knowing the NED

The importance of the strength of selection in the evolutionary response to MEDs is supported by a positive correlation between the strength of selection and the direct response (Figure 3.3; $R^2 = 0.23$, $P < 0.0001$). At NED = 1 - 3 the strength of selection is weaker than selection environments with higher NED (greater than 3), resulting in a small direct response (Figure 3.3). The increase in the strength of selection with increasing NED corresponds with an increase in the proportion of significantly positive direct responses (Appendix 2; Figure 8.2). The exception to this is regimes within NED = 7, which have the largest drop in growth rate and the smallest direct responses (Figure 3.4 and Appendix 2; Figure 8.9). This result may indicate that there is a physiological constraint on maximum growth rates of populations growing at high NED, or that adaptation is slower at very high NED. Although, Figure 3.5 shows that there is a physiological limit on growth rate when phosphate is reduced, growth is relatively unaffected by reduced nutrients. Alternatively, this result may indicate that more time is required for beneficial mutations to arise at NED = 7. Note that Appendix 2, Figure 8.3 - Figure 8.9, show the identity of all regimes and r-squared and p-values from linear regression analysis between the average rate of cell division ($d^{-1}$) of the MED-evolved populations and the strength of selection in the selection environments.

When the strength of selection is fixed, and only the strength of selection where the variation in the direct response to selection is highest is analysed ($2.4 < s < 2.6$), there is no relationship between the direct response and NED (all NED levels are sampled within this threshold) (Figure 3.6; $R^2 = 0.016$, $P = 0.041$). This indicates that when the strength of selection is similar across all selection environments NED does not determine the direct response and this is consisted with the full dataset. However, the relationship between NED and the direct response to selection is not consistent between the full dataset and the reduced dataset where the strength of selection is
fixed (2.4 < 1-s <2.6); the direct response increases slightly with increasing NED using the full dataset (intercept = 1.10 and slope = 0.12), but decreases with increasing NED under a fixed strength of selection (intercept = 1.29 and slope = -0.17; Z = 11.84).
Figure 3.3 There is a positive correlation between the strength of selection and the direct response of the MED-evolved populations, assayed in environments with MEDs (1 – 8 NED). Coloured circles show the response in each regime coloured according to NED (1 to 8). Solid line shows the results of the linear regression, and dashed line shows the expected growth rate if the evolved response equals the acclimation response (i.e. control response 1:1).
There is a negative correlation between the strength of selection and the growth rate of the MED-evolved populations, assayed in environments with MEDs. Different colours show the average growth rates between all evolved replicate populations within each regime (± SD), within each level of NED (panels labelled 1 to 7). The dashed line shows the expected growth rate if the evolved response equals the evolved control populations. The dotted horizontal line shows the average rate of cell divisions ($d^{-1}$) of the evolved control populations in the control environment. See Figure 8.3 to Figure 8.9 for identity of regimes within each NED level.
Figure 3.5 Nutritional availability determines the maximum growth rate of MED-evolved populations. Each panel label indicates the nutritional quality of the environment; top left, general nutrient replete and phosphate (P) replete; top right, low nutrients and P replete; bottom left, general nutrients replete and low P; bottom left, low nutrients and low P. Dashed line indicates the growth rate of the evolved control population in the control environment.
Figure 3.6 There is no correlation between the NED of the selection environments and the direct response to selection of MED-evolved populations when the strength of selection is fixed (2.4 < $s < 2.6$). Open circles show the average direct response to selection of each evolved population. Dashed lines indicate where there is no difference in growth rate between the MED-evolved populations and the evolved control population. Solid line indicates the slope and intercept calculated using linear mixed effects model.
3.5.3 The correlated response to selection

There is no relationship between the NED of the selection environment and the correlated response to selection (Figure 3.7; the effect of NED on the correlated response; $F_{1,137} = 0.21, P = 0.64$). Most notable however, is NED = 7, which shows the largest variation between replicate populations growing in each regime when they are assayed in the control environment. This response also highlights that although adaptation is not detectable from the direct response and the growth rates of evolved populations within NED = 7, it is obvious that different adaptive strategies have evolved both between and within regimes, which have different plastic responses when grown in the control environment (Figure 3.7).
Figure 3.7 Correlated response to evolution in selection environments. Box plots show the number of divisions (d⁻¹) of the evolved populations from the selection environment relative to the number of divisions (d⁻¹) of the control populations in the control environment. Open circles show the average correlation response between evolved replicate populations within each regime under 0 to 8 NED.
3.5.4 The effect of each environmental driver

Post hoc analysis reveals that the dominant environmental drivers (identified in chapter 2), reduced phosphate and herbicide, explain most of the variation in the growth rates of the MED-evolved populations (9% and 10% respectively). This is comparable with the short-term response (before evolution), which shows that variation in growth rate is explained by four environmental changes, CO$_2$ (12%), phosphate (11%), herbicide (5%) and UVB (4%) (Chapter 2, section 0). However, the effect of temperature is more important following evolution (3%), and CO$_2$ is less important following evolution (1%). Light intensity, pH, nutrient depletion and UVB do not contribute to the variation in growth rate of the MED-evolved populations. NED contributes approximately 8% to the variation however, the effect of NED is not significant (Figure 3.2; post hoc – the effect of NED and individual drivers on growth rates; $F_{1265} = 2.14$, $P = 0.145$), and the majority of the variation is explained by the evolved replicate populations within each selection environment (39%).

The post hoc analysis on the direct response to selection is consistent with the post hoc analysis on growth rates of MED-evolved populations. The environmental drivers of reduced phosphate and herbicide explains the largest amount of variation relative to the other environmental drives (9% and 5% respectively), with elevated temperature and CO$_2$ explaining some variation (1% each). Light intensity, pH, nutrient depletion and UVB contribute nothing to the variation observed by the selected populations. The strength of selection and the evolved population explain the majority of the variation (27% and 37% respectively) (Figure 3.1; post hoc – the effect of strength of selection and individual drivers on the direct response; $F_{1,265} = 2.12$, $P = 0.145$). See Appendix 2, Table 8-1 – 8-6 for mixed model outputs.
3.6 Discussion

3.6.1 The strength of selection predicts the evolutionary response of *C. reinhardtii* grown under MEDs

I have shown here that in environments with MEDs, the strength of selection, estimated by the plastic response of the evolved control populations in the selection environments, can explain most of the variation in the direct response to selection of *C. reinhardtii* (Figure 3.2), regardless of the identity of environmental drivers. As NED increases, so does the strength of selection (Figure 3.1). The plastic response of the evolved control populations in the selection environments can be predicted from the NED, making acclimation growth rates a good predictor of the magnitude of the evolutionary response to MEDs, at least in terms of growth rate. Previous studies, using the marine alga *Ostreococcus tauri* found that plastic responses of the ancestor predict the extent (though not the direction) of evolution in the face of elevated pCO$_2$ (Schaum & Collins, 2014). This is important because microbes such as marine and freshwater microalgae will evolve over relatively short timescales, due to their short generation times (hours – days) and large population sizes (Collins, 2012). Predicting the effect of MEDs on the evolutionary response is necessary to understand how populations of microalgae will change under future global change scenarios.

3.6.2 The evolutionary response to MEDs cannot be predicted from the NED of the selection environments

I found that the direct response to selection of *C. reinhardtii* increases with NED until it drops again at NED = 7 (Figure 3.1). Based on Fisher’s geometric model of adaptation, I hypothesised that the rate (and thus the extent over a fixed period of
time) of adaptation would decrease as NED increases. However, the direct response
seen here may be explained by the data not fulfilling the three main assumptions of
Fisher’s model. First, it is predicted under Fisher’s (1930) model that as the number
of \( n \) dimensions increase, the number of independent traits under selection will
increase. The number of independent traits under selection is not empirically
measured in the present study however, based on Fisher’s model it was predicted that
the number of independent traits would increase with increasing NED (see Figure
3.8). It is possible that in this study, the traits that were under strongest selection
were the same across many of the regimes, such that the increase in the number of
independent traits under selection with NED was either small or irregular. Second,
Fisher assumes all beneficial mutations will have an equal effect on all traits under
selection (universal pleiotropy), and so a beneficial mutation of a fixed size, will
have a smaller fitness gain as the number of independent traits increase (Fisher,
1930; Orr 2000). However, there is little empirical support for universal pleiotropy
(but see Paaby & Rockman (2013); Hill & Zhang (2012)). Restricted pleiotropy or
modular pleiotropy, is based on the assumption that beneficial mutations effect
functional combinations of phenotypic traits (Wagner & Zhang, 2011), and is a more
widely supported model for the evolution of complex organisms (Chevin et al.,
2010b; Wang et al., 2010). Furthermore, an alternative hypothesis based on restricted
pleiotropy may be useful for understanding evolution under MEDs. If we assume that
many environmental drivers will have similar effects on phenotypic traits (as
indicated in Table 1-1) and pleiotropy is more common when traits are correlated
(Czesak et al., 2006), restricted positive pleiotropy should increase the rate of
adaptation without incurring a cost to other uncorrelated traits (Chevin et al., 2010b;
Wagner & Zhang, 2011).

Third, Fisher assumes that the strength of selection is constant across the number of \( n \)
dimensions (Fisher, 1930). When I consider populations that are evolving under the
same strength of selection (\( 2.4 < 1-s <2.6 \)), where the variation in the direct response
to selection is highest, there is no relationship between the direct response and NED
(Figure 3.6). This is consistent that there is no relationship between NED and the
number of independent traits as predicted in the hypothesis based Fisher’s (1930) model. Alternatively, the relationship between NED and the number of independent traits may not be linear (alternative relationships between NED and the number of independent traits are illustrated in Figure 3.8).
Figure 3.8 Illustration to show three possible outcomes of NED on the number of independent traits under selection. The number of traits under selection will saturate at a certain point shown here by the dashed line. Linear increase shown by the red solid line is hypothesised in the present study. Alternatively, saturation could happen earlier than predicted, such as NED 3-4 as shown here by the green solid line. Another outcome of increasing NED, is a slow increase in the number of traits under selection as NED increases until a tipping point is reached for instance, at NED > 5, where the number of independent traits increases rapidly (blue solid line).
Pleiotropic interactions between traits will directly affect the fitness gain of beneficial mutations. If the assumption that the number of traits under selection increases with NED holds (as illustrated in Figure 3.8), it is predicted that populations selected in high NED will have to wait for mutations to arise that have a net positive effect on all traits under selection, and the chance of a mutation being positive in all traits under selection declines with increasing NED. Cooper et al. (2007) found support for this hypothesis as they found a negative relationship between the number of pleiotropic traits and relative fitness in the yeast *S. cerevisiae*. In contrast, I found that the rate of adaptation increases as NED increases and that the rate of adaptation correlates with the strength of selection. One explanation is that positive pleiotropy is more important when multiple environmental drivers change simultaneously. The rate of adaptation is expected to increase with positive pleiotropic interactions between traits, as only one beneficial mutation is required to improve the fitness of all traits under selection (Figure 1.3).

Negative pleiotropy is the most intuitive outcome of high dimensionality under Fisher’s geometric model, as the chance that a beneficial mutation will have positive effect on orthogonal characters decreases with the increasing *n*. However, in the present study, environmental drivers may be positively correlated increasing the opportunity for beneficial mutations to increase the trait value of correlated traits in the same direction of selection, referred to as reinforcing selection by Etterson & Shaw (2001). However, the effects of positive pleiotropy in response to MEDs may be underestimated in evolutionary theory (Ostrowski et al., 2005), such as evolution of specialisation (Cooper & Lenski, 2000) and constraints on adaptation (Barton & Keightley, 2002). Travisano et al. (1995) found that positive pleiotropy is common when *E. coli* evolved (for 2000 generations) in glucose-limited environments are assayed in novel environments, and demonstrate that the sign of pleiotropy varies consistently with the type of environment. Leiby & Marx (2014) found that whilst both negative and positive pleiotropy determine the growth rate of *E. coli* in novel environments (following evolution in a glucose environment for twenty- and fifty-thousand generations), positive pleiotropy was found to be more important than
expected (based on previous Biolog assays; Cooper & Lenski 2000). In addition, high mutation rates correlate with reduced fitness in novel environments and indicate that mutation accumulation produces specialisation, resulting in negative effects in novel environments and not negative pleiotropy between traits. The effects of pleiotropy when MED-evolved populations of *C. reinhardtii* are assayed in novel environments will be investigated in Chapter 4.

### 3.6.3 Adaptation does not completely reverse the initial drop in growth rates experienced in MED environments

Adaptation to MEDs over hundreds of generations (~350 generations), is not enough to restore growth rates back to that of the evolved control populations in the control environment (Figure 3.2 and Figure 3.4). This is reflected in NED = 4 to 6, which show the highest direct response to selection, but still have low growth rates relative to the evolved control populations in the control environment (Figure 3.2). One explanation for relatively slow growth after 350 generations in the selection environment is limited adaptation at high NED (NED > 3). Populations in high NED environments are smaller than those in low NED environments (Figure 8.10), and small populations sizes have longer waiting times for beneficial mutation to arise due to smaller mutation supply rate (*Nu*). Waiting time was calculated using the total mutation rate of \((3.23 \times 10^{-10};\) Ness et al. 2012) as \(1/(2Nu)\) (Bell, 2008). I found that waiting time is equal across NED levels (Appendix 2, Figure 8.11; \(R^2 = 0.033, P < 0.0001\)), so this is unlikely to be driving these results. The fixation time of beneficial mutations depends on the strength of selection however, in large asexual populations fixation time increases with population size, due to competition between genotypes with beneficial mutations (clonal interference) (Elena & Lenski, 2003; Maddamsetti *et al.*, 2015). As such, it is expected that fixation time decrease as the strength of selection increases and population size decreases (with increasing NED), and this is what I found here (Appendix 2, Figure 8.12; \(R^2 = 0.22, P < 0.0001\)) (Bell, 2008).
Although waiting and fixation time cannot help explain the drop in growth following evolution at high NED. I suggest that, physiological constraints may limit the maximum growth rate in poor (high NED) environments i.e. – there are no beneficial mutations available that would restore growth to ancestral values, even though populations in high NED environments have large direct responses to selection.

### 3.6.4 Genetic constraints on adaptation to MEDs

The dynamics of evolving populations are often discussed in terms of movement on an adaptive landscape, where high fitness is represented as peaks and low fitness as valleys (Colegrave & Collins, 2008). Even with relatively equal waiting times for mutations across different NED environments, pleiotropic interactions may require exceptionally rare beneficial mutations. Negative pleiotropic interactions between traits require beneficial mutations that have a net positive effect on all traits. However, if negative correlations between traits cannot be overcome, rare beneficial mutation(s) that enable populations to effectively cross a valley in the fitness landscape are required. At high NED (NED ≥ 4) where, there is evidence of adaptation, new beneficial mutations may enable populations to climb an alternative fitness peak, allowing populations to reach a new local optimum, resulting in an intermediate level of maximum fitness (Chevin et al., 2010a, 2010b). Alternatively, populations will go extinct before a rare beneficial mutation arises, for instance at NED = 8.

Given the lack of standing genetic variation at the start of the selection experiment, beneficial mutations may take some time to arise. If the experiment were allowed to run for longer, the growth rates of the MED-evolved populations may have been restored giving enough time to allow for the fixation of beneficial mutations (Collins et al., 2013).
3.6.5 Physiological constraints on adaptation to MEDs

The pattern of decreasing growth rate with increasing NED may also be the result of physiological constraints leading to reduced maximum growth rates. Starvation limits the maximum growth rate and the chance of the selection environment containing either reduced nutrients or reduced phosphate (or both) increases with NED. When selection environments containing reduced phosphate are compared to phosphate replete environments it appears that the growth rates are reduced across all NED (1-8) low-phosphate environments, relative to phosphate replete environments (Figure 3.5). In particular, low growth rates across all regimes within NED = 7, may be due to reduced phosphate (in all but one selection environment in NED = 7), producing a new physiological limit, where *C. reinhardtii* cannot increase growth rates, even after evolution. The evolved control populations, assayed in environments with reduced phosphate (after acclimation for one transfer cycle), are able to grow better than the MED-evolved populations. This may be due to a range of mechanisms that allow *C. reinhardtii* to store phosphate over more than one cell division cycle (Irihimovitch & Yehudai-Resheff, 2008). A second possibility is that in the short-term or in otherwise good environments, other resources can be reallocated to increase phosphate uptake. Alternatively, cells may be in worse general condition following hundreds of generations of phosphate starvation than after only a few generations of phosphate starvation. Growth rates are unaffected by general nutrient depletion and differences between limited growth rates in reduced phosphate and general nutrient depletion environments may simply be due to the differences in the amount of each nutrient required by *C. reinhardtii* (for concentrations of all nutrients see chapter 2, Table 2-2). Phosphate is an important resource that *C. reinhardtii* requires in relatively large amounts. In addition, general nutrient depletion experiments (where general nutrients and phosphate are reduced together) are characterised by specific responses to reduced phosphate, including mobilisation and transport of phosphate (Irihimovitch & Yehudai-Resheff, 2008).
3.6.6 Extreme environments prevent adaptation to MEDs

The relationship between the strength of selection and the evolutionary response of the MED-evolved populations breaks down and adaptation is limited when NED is greater than 6 (Figure 3.1 and Figure 8.2). During the selection experiment, all populations selected at NED = 8 were unable to survive the transfer schedule (extinction occurred at transfer 4), and only by increasing the population size (increasing the volume of cells transferred), did these populations persist. Enhanced population sizes allowed populations in NED = 8 to avoid extinction and to adapt. Other empirical studies have similar findings; Bell and Gonzalez (2009), demonstrate that large initial population sizes of *Saccharomyces cerevisiae* are more likely to be rescued when exposed to high salt concentration (125 g L$^{-1}$).

The lack of standing genetic variation of founding populations and small population sizes reduce the probability of evolutionary rescue for all populations under selection within NED = 8 (Figure 3.2). Agashe et al. (2011) found that populations of the beetle *Tribolium castaneum* with high founding genetic variation successfully prevent extinction. Lachapelle and Bell (2012), also indicate the importance of high standing genetic variation and recombination events in the ability of *C. reinhardtii* to survive increasingly stressful environments with progressively higher salt concentrations. The rate of environmental change also plays an important role in determining adaptive potential of the MED-evolved populations. All populations were transferred immediately to each selection environment. Therefore, there was no opportunity to adapt gradually to a steadily changing environment (gradually increasing in NED or intensity of drivers) (Collins & De Meaux, 2009).

The results collected in this chapter support the risk of extinction calculations for the founding populations (Chapter 2, Equation 2-3). From this calculation it was predicted that as NED increased, the risk of extinction would increase (Appendix 1; Figure 7.14). In addition, the limited direct response to selection and high extinction
risks predicted based on the plastic responses of the founding populations suggest that the number of environmental drivers that \textit{C. reinhardtii} can survive saturates at NED = 7, and increasing to NED = 8 causes widespread extinction.

### 3.7 Conclusion

The results from this study suggest that using short-term acclimation investigations and knowing the effect of the single environmental drivers, including identifying the dominant environmental driver, can be used to predict the direct response to selection, at least in terms of population growth rates. The NED and the dominant environmental drivers can predict the evolutionary response to MEDs, without knowing the plastic response of the evolved control populations in each test environment. The ability to predict the outcome of MEDs from the initial response is important as much of the data collected on the effects of MEDs are based on short-term experiments and multi-generational experiments are not always feasible.
Chapter 4 – The outcome of evolution under a single environmental driver vs. five environmental drivers

4.1 Chapter summary

In this chapter, I examine the consequences of the selection history for fitness in new environments. To do this, I measured the growth rate of populations evolved in environments with five environmental drivers, a single environmental driver, and populations evolved in the control environment, when assayed in environments that have the same NED, different NED, and one novel environment. Specifically, I address the following questions:

(1) Is there any difference in the response to new environments after evolving with either 1 or 5 environmental drivers? Do evolved NED = 5 populations grow better in other NED = 5 environments than evolved NED = 1 populations (and vice versa)?

(2) Does evolution at a given NED level (1 or 5) have any effect on environmental variance when assayed in new environments with the same NED or different NED level?

(3) Lastly, is there an advantage to evolving in more stressful environments? For instance, do populations evolved in poor quality environments, indicated by a larger initial drop in fitness have a higher fitness in new environments?

4.2 Abstract

The strength of selection is important for predicting how future of aquatic microalgae, evolved under MEDs, will persist as the environment continues to change. Here, I measure how the number and identity of MEDs affect the ability of evolved populations of C. reinhardtii to grow in new environments. I found that the
strength of selection is useful for predicting the response of evolved populations to new environments. Populations evolved in high NED environments (NED = 5) grow better in their selection environments than new environments, compared with populations evolved low NED environments (NED = 1), and this is due stronger selection in NED = 5 environments than NED = 1 environments. Evolved NED = 1 populations are evolving in comparatively better quality environments and the strength of selection is lower resulting in growth rates that are similar between new environments and the selection environments. In contrast, growth of evolved NED = 5 populations is lowest in NED = 1 environments, and in some cases there is little change in growth rates in alternative NED = 5 environments, suggesting that mutations that improve fitness in evolved NED = 5 selection environments, also improve fitness in the alternative NED = 5 environments, but reduce fitness in NED = 1 environments. The strength of selection and the quality of new environments may provide useful information for making predictions on the responses of evolved populations to continued environmental changes.

4.3 Introduction

An important question that faces biologists today is how organismal phenotypes will respond to continued environmental change (Etterson, 2004a, 2004b; Chevin et al., 2013; Sikkink et al., 2015). One key way that evolutionary biologists address these questions is by studying the extent of local adaptation in different environments. In the absence of gene flow, populations will diversify and beneficial mutations will be selected to improve fitness of the local population in their local environment, regardless of the consequences in alternative environments. However, understanding the consequences of local adaptation in new environments is particularly relevant under future global change scenarios, where many aspects of the environment are expected to change simultaneously, at unprecedented rates and new environments may fall outside the range of environmental conditions normally experienced (Reusch & Boyd, 2013). Here, local adaptation is defined as a drop in growth rate
when population are growing in a new environment that they were not evolved in. Local adaptation will be investigated in the present study by measuring evolved population responses in new environments relative to their growth rates in their local environment.

4.3.1 Selection history and growth in new environments

Reciprocal transplant and common garden experiments are regularly used to understand pleotropic interactions between traits in different environments and uncover patterns of local adaptation (Etterson, 2004a, 2004b). Experiments investigating the effect of selective history and trade-offs in new environments often use a large number of novel of environments in order to detect or measure genotype by environment (GXE) interactions (Bell, 1991, 1992, 1997; Bell & Reboud, 1997). In addition, through the use of biolog assay plates (Cooper & Lenski, 2000; MacLean & Bell, 2002; Bataillon et al., 2011; Melnyk & Kassen, 2011; Hall, 2013), environments can be defined by their carbon sources, and then growth on different carbon sources can be used to measure genetic correlations among novel environments in populations with different selection histories. This has provided researchers with the opportunity to investigate the processes underlying ecological specialisation in microbes (Cooper & Lenski, 2000), the effects of antibiotic resistance (Hall, 2013), the evolution of niche breadth (MacLean & Bell, 2002), and the effect of the adaptive landscape on the repeatability of adaptation evolution (Melnyk & Kassen, 2011).

Investigations that focus on GXE interactions will likely be useful for understanding selection to environmental changes, as they can identify genotypes that can most rapidly respond to environmental change, and genotypes that are vulnerable to future environmental changes predicted under global change scenarios (Reusch & Boyd, 2013). There are many experiments that investigate the distribution of fitness effects (DFE) of beneficial mutations among different environments in order to uncover if
DFE are environment specific. Bank et al. (2014) found that whilst the DFE of beneficial mutations in *S. cerevisiae* was very similar across six environmental conditions (including, salinity and temperature), GXE interactions are important, and so the environment determines fitness effect of individual beneficial mutations. However, other investigations found that DFE is not environment dependent (Kassen & Bataillon, 2006). Bataillon et al. (2011) suggest that experimental estimates of DFE among new environments are conflicting due to limited number genotypes and environmental conditions used. Using 95 different carbon sources and 18 mutants with beneficial mutations the authors found that there is no substantial cost of beneficial mutations in new environments and GXE interactions between different mutants are very similar.

Here, I investigate the outcome of evolution in low NED (NED = 1) and high NED (NED = 5) environments, when evolved populations are grown in new environments with high (NED = 5) and low (NED = 1) NED, and different combinations of environmental drives. In addition, growth will be measured in a novel environment that has not been encountered before, 0.2 µM copper sulphate (CuSO₄).

### 4.3.2 Mutations that are beneficial in one environment may have a different fitness effect in another environment

The direction of pleiotropy is important for understanding how populations will respond to environmental change (Reed *et al*., 2011). New trait values that improve fitness in one environment may be in the opposite direction of selection in a new environment, reducing the fitness of populations and this is known as negative pleiotropy (Paaby & Rockman, 2013). Negative pleiotropy can reduce long-term fitness and may result in population having a narrow niche breadth, and is sometimes referred to as the cost of beneficial mutations (Kassen, 2002; Bataillon *et al*., 2011). Alternatively, changes to trait values (as a result of beneficial mutations) may be in
the same direction as selection in new environments, allowing populations to
diversify into the new environment, increasing the niche breadth of the population
(Buckling et al., 2003). However, Chevin (2013) modelled genetic correlations
between traits under selection in randomly changing environments and demonstrated
that although genetic correlations between traits in the same direction as selection
may result in increased rate of adaptation, the long term-fitness may still be
constrained by genetic correlations depending on the patterns and predictability of
environmental changes.

Negative pleiotropic interactions contribute to maintaining patterns of local
adaptation, as they prevent populations performing well across all environments.
Negative pleiotropy is common after selection in uniform environments, as is
evidenced by fitness decline in other environments. The effect selection history on
the distribution of fitness effects of beneficial mutations has been tackled by
investigating the evolution of growth rates of simple organisms grown with different
carbon sources. Travisano et al., (1995) found that after selection for 2000
generations in glucose-limited environment, populations of *E. coli* were unable to
grow in maltose but were able to grow in lactose (both sugars had not been
encountered for 2000 generations). The authors suggest that this is as a result of
negative pleiotropic interactions in mutations that allow more efficient glucose
uptake at a cost to maltose uptake. In addition, growth in novel environments
revealed genetic diversity between selected populations that was not apparent in the
relative fitness measurements during the selection experiment. Cooper and Lenski
(2000) found that after 20,000 generations of selection in glucose, the majority of
populations of *E. coli* populations showed poorer growth in all 27 different carbon
sources than their ancestor, and in 16 environments they also showed a significant
decay in catabolic functions. This indicated a cost to selection on a single sugar
(glucose media) due to negative pleiotropy, and resulted in ecological specialisation
of the genotypes. Costs of beneficial mutation have also been investigated using
changing temperatures. Bennett and Lenski, (2007) found that trade-offs are not
universal in *E. coli* selected at 20°C for 2,000 generations, when they are
subsequently assayed at 40°C. However, trade-offs as a result of selection at a cooler temperature were apparent in 15 out of 24 populations.

In contrast positive pleiotropy breaks down patterns of local adaptation. Ostrowski et al, (2005) found that that *E. coli* selected in glucose media for 400 generations increased in relative fitness when grown in five novel carbon sources. However, positive pleiotropy can be problematic when considering antibiotic resistance, as resistant genotypes may be both resistant and have higher fitness in other environments (Kassen & Bataillon, 2006). In *P. fluorescens*, antibiotic resistant mutations were beneficial in the ancestral environment and remained beneficial or neutral, across 95 different carbon sources.

### 4.3.3 Generalists versus specialists

Studies investigating the evolution of generalists and specialists disentangle the microevolutionary effects on niche breadth and local adaptation (Barrett *et al.*, 2005; Jasmin & Kassen, 2007a). Specialists are adapted to specific environmental conditions, however growth is reduced when the environment changes. Patterns of local adaptation and narrow niche breadth of specialists are governed by genetic constraints such as negative pleiotropic interactions in new environments and mutation accumulation. Cooper & Lenski (2000), found that negative pleiotropic interactions caused ecological specialisation in *E. coli* selected on a single sugar source. In contrast, generalists often have roughly equal performance across several environmental conditions and as a result have a wider niche breadth. However, this generally comes at a cost to maximum fitness when growth is compared with specialist genotypes in the same environment (Barrett *et al.*, 2005; Chevin *et al.*, 2010a).
Experimental evolution investigations are commonly used to understand the evolution of specialist and generalists genotypes, and the outcome to fitness when the environment changes (Kassen, 2002). Evolution in heterogeneous environments (such as an environment with a mixture of carbon sources), does not necessarily lead to the evolution of perfect or broad generalists i.e. genotypes that are able to take advantage of all patches in the environments. Populations of *P. fluorescens* selected in complex environments with up to eight different carbon sources evolved higher growth rates than their ancestor when assayed in complex environments. In addition, high variation between populations selected in complex environments revealed an increase in niche width, where different genotypes were able to use different sugars in complex environments, with the fittest genotype varying between different sugars. However, no evolved genotype was able to use all of the sugars, with different generalists using different combinations of sugars (i.e. – having different niches). As such, evolution in complex environments is often characterised by many imperfect-overlapping generalists that are neither complete generalists nor complete specialists (Barrett *et al.*, 2005).

Environmental productivity may affect the evolution of specialists as it is expected that specialists will be well adapted to the most productive part of a heterogeneous environment (Jasmin & Kassen, 2007b). Jasmin and Kassen (2007b) found that environmental productivity and the degree of environmental contrast (variance between patches) were important for evolution of specialists, and the response to selection was dominated by adaptation to the most productive patch. The authors support this in part by pointing out that they found that specialists evolved in spatially and temporally heterogeneous environments containing multiple carbon sources. However, specialists adapted to the least productive substrate and this may be due to genetic constraints to evolution in variable environments.
4.3.4 Local adaptation

In the absence other forces, in particular gene flow, local populations will evolve traits that are advantageous in the local environment so long as beneficial variants are accessible. There are two commonly used measurements of local adaptation: (1) home vs. away (HA) comparisons show that performance of the evolved populations is a result of the process of local adaptation by comparing the response of evolved populations in alternative environments. Alternatively, (2) local vs. foreign (LF) comparisons indicate population are locally adapted if evolved populations are the fittest in their selection environment (local environment), compared within foreign populations (with alternative selection environments) (Kaltz & Shykoff, 1998; Kawecki & Ebert, 2004). Four examples of possible outcomes of a reciprocal transplant experiment are given in Figure 4.1, indicating differences between HA and LF measurements of local adaptation.

NED = 1 environments are expected to produce specialists that are locally adapted to the selection environment, but will pay a cost if the environment changes due to negative pleiotropic interactions (i.e. traits that improve fitness in the selection environment reduce fitness in new environments). This is supported by a number of experiments that show that evolution in constant, uniform environments produces specialist genotypes (see review by Kassen 2002). On the other hand, it is expected that evolved NED = 5 populations will be less locally adapted in environments with MEDs. This is due to increased similarity between home and away environments in evolved NED = 5 populations. Similarity may increase the chance of positive pleiotropic interactions (i.e. trait values which improve fitness in the selection environments are beneficial in new environments). This is consistent with studies that demonstrated that evolution in complex environments, i.e. spatially and temporally fluctuating environments, selects for generalists (Kassen, 2002; Jasmin & Kassen, 2007a), constraining local adaptation.
Figure 4.1 Schematic diagram illustrating fitness comparisons between populations evolved in different selection environments. (a) In a reciprocal transplant experiment, local adaptation is evident if each local population (selection environment is the same colour as the filled circles) has higher fitness than any other foreign population in the same environment (local vs. foreign or LF), with a trade-off in fitness when growing in environments that populations are not selected in (home vs. away or HA). (b) Environment 1 supports high fitness regardless of selection history, whereas fitness in environment 2 depends on the selection history of the populations. Only by measuring HA, is local adaptation evident in population A. However, the opposite is true for population B; local adaptation is apparent from LF but not HA measures of local adaptation. (c) Alternatively, populations that have high fitness in their selection environment will have equally high fitness in alternative environments, and populations with reduced fitness in their selection environment will also have reduced fitness in an alternative environment – the genotype is more important than the environment at determining fitness. Local adaptation is evident from LF measures of local adaptation in population A. Population B shows no evidence of local adaptation using both LF and HA measures of local adaptation. (d) In addition, growth rates may be driven by the quality of the environment, and differences in the environments will have the same effect on fitness of populations A and B, regardless of selection history. Local adaptation is evident in population A using HA measures of local adaptation, however
population B shows no evidence of local adaptation using both LF and HA measures of local adaptation.

### 4.3.5 Local adaptation and global change

How does the number of environmental drivers affect local adaptation? Here I have introduced some ideas that have already been tested in the lab using multiple carbon sources (Barrett et al., 2005; Jasmin & Kassen, 2007a) and antibiotics (Ankomah et al., 2013; Ma et al., 2014; Ocampo et al., 2014). Like the present study, these investigations aim to understand how the selection history of evolved populations will affect their ability to grow in new environments. I use populations evolved in NED = 1 and NED = 5 selection environments in order to uncover if NED can predict the growth rates of evolved populations in new environments. One common way to address this is by looking at the degree of local adaptation that evolves. If evolution in MEDs produces generalist populations with large niche breadths, then these populations may be better able to persist as the environment continues to change (Dullinger et al., 2012; Lavergne et al., 2013). This is supported by ecological models, which predict that specialists should decline more quickly than generalists when environmental conditions change (Thuiller et al., 2005).

If the outcome of evolution to MEDs is ecological specialisation, then populations may decline if the environment changes (Slatyer et al., 2013). Ecological models show that following environmental change, generalist genotypes occupy the expanding range and increase in the centre of the shifting range. In contrast, specialist genotypes occupy the retracting margin and are locally maladapted due to reduced population size and founder events, resulting in reduced ability for population persistence under global change (Cobben et al., 2012).
4.4 Methods

4.4.1 Experimental design

Evolved populations from the control, NED = 1 and NED = 5 regimes were used to compare the effects of selection in high NED, low NED, and the control environment. Seven NED=5 regimes were chosen at random, and all NED = 1 regimes with extant populations were used. Populations from the herbicide NED=1 regimes went extinct during the evolution experiment in chapter 3. Three replicate populations evolved in each regime were used for this experiment. The control environment and CuSO₄ environment were also used. This makes eighteen assay environments in total (Table 4-1). CuSO₄ is a novel environment that evolved populations have not encountered before. Based on pilot work, 0.2 μM concentration of CuSO₄ reliably affects growth in the *C. reinhardtii* evolved control populations (Figure 9.1). Details of experimental design, culture environments and measures of population growth can be found in chapter 2, section 2.4 Methods and Materials, (pages 27 - 37). Details of the selection experiment and measure of population growth following 95 transfers in the selection environment (after evolution), can be found in Chapter 3, section 3.4.1 (page 73).
Table 4-1 List of regimes used to measure local adaptation including, the control environment, all NED = 1 assay environments, a subset of NED = 5 environments, all eight environmental drivers and a novel environment, copper sulphate (0.2 µM)

<table>
<thead>
<tr>
<th>Regimes</th>
<th>NED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>CO₂</td>
<td>1</td>
</tr>
<tr>
<td>Herb</td>
<td>1</td>
</tr>
<tr>
<td>LI</td>
<td>1</td>
</tr>
<tr>
<td>ND</td>
<td>1</td>
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<td>P</td>
<td>1</td>
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<tr>
<td>pH</td>
<td>1</td>
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<tr>
<td>Temp</td>
<td>1</td>
</tr>
<tr>
<td>UV</td>
<td>1</td>
</tr>
<tr>
<td>CO₂/pH/P/UV/Herb</td>
<td>5</td>
</tr>
<tr>
<td>CO₂/pH/UV/ND/LI</td>
<td>5</td>
</tr>
<tr>
<td>CO₂/Temp/LI/Herb/pH</td>
<td>5</td>
</tr>
<tr>
<td>CO₂/UV/ND/P/LI</td>
<td>5</td>
</tr>
<tr>
<td>pH/UV/ND/LI/Herb</td>
<td>5</td>
</tr>
<tr>
<td>Temp/pH/LI/UV/ND</td>
<td>5</td>
</tr>
<tr>
<td>UV/LI/pH/P/ND</td>
<td>5</td>
</tr>
<tr>
<td>CO₂/Temp/pH/LI/UV/ND/Herb/P</td>
<td>8</td>
</tr>
<tr>
<td>Copper</td>
<td>novel</td>
</tr>
</tbody>
</table>

CO₂, elevated CO₂; Temp, elevated temperature; LI, reduced light intensity; ND, nutrient depletion; Herb, herbicide; P, phosphate depletion; pH, reduced pH; UV, UV-B radiation, Copper, novel environment copper sulphate (concentration)
4.4.2 Population growth

Each population was acclimated to the assay environment for 4 days and then transferred to fresh media at equal cell density (approximately 41,000 cells/ml). Each assay was completed two times. The number of divisions per day was calculated as using Equation 2-1 (Chapter 2; page 33). Cell counts were performed at time zero and after 120 hours using a BD FACSCanto II (BD Biosciences, Oxford, UK) flow cytometer calibrated with CS&T beads. Due to the size of the assays, cell counts were performed in batches. Regimes were randomly assigned to a batch and technical replicates were counted within a batch. For reciprocal transplants, each population was assayed in all environments in a single batch (including technical replicates), with populations randomly assigned to a batch. The effect of batch number on growth rates is included in the analysis (see below). The data were acquired with the BD FACSDiva v6 software. Each culture was counted twice. The cell counts were transformed into cells per millimetre and the average rate of divisions per day was calculated for each technical replicate.

The direct and correlated responses to selection were calculated for each evolved population (15 total) using Equation 3-4 (Chapter 3; page 76 ). In order to measure the degree of local adaptation following evolution, the ‘home vs. away’ (HA) definition of local adaptation was calculated as the mean growth rate of each population in the selection environment (home), minus the mean growth rate in all environments that MED-evolved populations were not selected in (away) (Blanquart et al., 2013). However, to account for the effect of environmental quality on growth rates of the MED-evolved populations, the difference in growth rates between the MED-evolved populations and control populations in each selection environment and new environments was calculated. Thus, if growth rates in the home environment (direct response) are greater than growth rates in foreign environments (MED-evolved population growth rate – control population growth rate) populations are locally adapted to the home environment. In addition, patterns of local adaptation
were uncovered by ranking each MED-evolved population by growth rate in each environment, defined as the local vs. foreign (LF) measure of local adaptation (Blanquart et al., 2013) (14 environments were used here). Local adaptation is apparent if the local population is ranked higher than foreign populations.

To account for the effect of shared environmental drivers between home and away environments used in the reciprocal transplant, on the growth rates of evolved populations, the degree of similarity was calculated. Similarity was calculated as an average pairwise difference between regimes, where each environmental driver is coded as a binary variable (present or absent). Average similarity between each home and away environment is calculated as 1 – average pairwise distance. A value of zero indicates that no environmental drivers between the home environment and away environment are shared and a value of 1 indicates that all environmental drivers between the home and away environment are shared. Similarity was included in the mixed model analysis (see below).

### 4.4.3 Statistical analysis

The effect of the selection NED, selection environment, assay NED, assay environment and their interactions on absolute growth rate and the correlated response was analysed using a mixed model in R (R Core Team, 2013), using the packages lme4 and lmerTest. In forming the random and fixed groups of the mixed effect model, assay NED, selection NED, the interaction between assay and selection NED, and similarity have been taken as fixed effects, while selection environment, assay environment, the interaction between selection and assay environment, batch number, evolved populations and the interaction between evolved population (nested within regime and NED) and the assay regime (nested within assay NED) have been taken as random effects. The justification is that the specific populations used in the assay were chosen randomly from the subset of populations that had been evolved within the selection environment (Chapter 3). In addition, each selection and assay
environment was randomly chosen from subset of 15 NED = 5 environments (all NED = 1 environments were used). To directly compare the contributions of fixed and random factors to variance, the percent contribution of fixed factors was estimated using Equation 3-5 (chapter 3, page 77).

Patterns of LF local adaptation were uncovered by ranking each evolved population by growth rate in each assay environment (14 environments that have a local population were used). Local adaptation is apparent if the local population is ranked higher than foreign evolved populations. A Chi-squared goodness of fit test was used to test the null hypothesis that there is no significant difference between the distribution of average ranked fitness of the evolved control population (ranked out of 15) across 14 different assay environments and the observed distribution of average ranked fitness of evolved NED = 1 and NED = 5 populations grown in the same assay environments. The expected frequencies are calculated using the frequencies of the ranked fitness of the evolved control populations (control.freq), relative to the sum of the frequencies of ranked fitness of the evolved control populations (expected frequencies = control.freq / sum of control.freq). The expected rank order of NED = 1 and NED = 5 populations was then calculated using Chi-squared goodness of fit test and compared with observed ranked fitness of NED = 1 and NED = 5 populations (Table.9-6). This was performed in the using the base R package, within R.
4.5 Results

4.5.1 Interactions between selection and assay environments are important for determining growth rates of evolved populations

The main question that this experiment was designed to answer, is how the number of environmental drivers in each selection environment effects the growth rate of evolved populations in new environments. I find that there is a significant interaction between selection NED and Assay NED (Figure 4.2; the effect of the interaction between selection NED and assay NED on the growth rate of evolved populations; F\(_{1,210}\) = 12.57, P = 0.002). The interaction between selection environments and assay environments is not influenced by similarity between the drivers within the selection and assay environments, which explains almost none of the variation (<1%) (Figure 4.3; the effect of similarity on the growth rate of evolved control populations; F\(_{1,228}\) = 0.003, P = 0.064). See Table 9-1, Table 9-2 for model output. Figure 4.2 shows that overall growth rates of the evolved control, evolved NED = 1 and evolved NED = 5 populations are higher when assayed in NED = 1 environments, decreasing as NED of the assay environments increases from one to five (explaining 17% of the variance in growth rate), and this trend is significant (Figure 4.2; the effect of the assay NED on the growth rate of evolved populations; F\(_{1,19}\) = 10.30, P = 0.001). However, there is a lot of variation in this pattern and this is due to the interaction between the evolved populations and assay environments which explains 31% of the variation the in growth rates. In addition, the evolved populations explain some of variation (10%), and the assay environment explains 28% of the variation in growth (Figure 9.2).
Figure 4.2 Rate of cell division (d\(^{-1}\)) of evolved populations in assay environments with different numbers of environmental drivers (NED = 1, 5 and 8) and the control environment. Coloured boxplots show the growth rate of the evolved populations selected in NED = 1 (green), NED = 5 (blue) and the control (red) environment. Each panel label indicates the number of environmental drivers in each assay environment. Dashed lines show the growth rate of the evolved control in the control environment.
Figure 4.3 There is no relationship between the average rate of cell division (d⁻¹) of evolved populations and similarity between the selection environments and the assay environments. Similarity ranges from zero (no environmental drivers are shared between the selection and assay environments) to one (all environmental drivers are the same between the selection and assay environments). Coloured circles show the growth rate of the evolved populations selected in NED = 1 (green), NED = 5 (blue) and the control (red) environment.
4.5.2 Response of evolved NED = 1 and evolved NED = 5 populations to new environments

The majority of populations show adaptation in their selection environment, as measured by a positive direct response to selection (Figure 4.4). However, evolved populations with reduced growth rate relative to the evolved control populations in their selection environment (indicative by a negative direct response to selection) are able to persist in more stressful environments and this is examined in the discussion (Figure 4.2). Evolved NED = 5 populations within each selection environment show more variation in the direct response to selection when compared with evolved NED = 1 populations (Figure 4.4). This may indicate that there are more evolutionary trajectories available when evolving with five environmental drivers than with a single driver.

Absolute growth rates are highest in the control environment for all evolved populations, with the slowest growth rates in the most stressful environments (herbicide, copper sulphate and NED = 8) (Figure 9.2). There are marginal differences observed between the growth rates of the evolved control, evolved NED = 1 and evolved NED = 5 populations when they are growing in non-selected environments (Figure 4.5). As a result, the correlated responses vary around zero (indicating growth is equal to the control). In addition, neither evolved NED = 1 or evolved NED = 5 populations have the highest growth rates across all new environments (Figure 4.5). However, the rank order of growth rates of evolved populations across all environments, may indicate that evolved NED = 1 populations grow better in better quality environments (as indicated by the rank order of evolved control population) and evolved NED = 5 populations perform better in poor quality environments (Figure 4.8). However, it should be noted that variation in growth rates between evolved populations grown under the same selection environment is high and overall differences between the average growth of populations selected in difference environments is not significant (Figure 9.4).
Figure 4.4 Box plots showing the direct response to selection of populations MED-evolved populations (colour indicates selection environment) with one and five environmental drivers. Dashed line indicates no difference between the growth rate of the MED-evolved populations and the evolved control populations in the same environment.
Figure 4.5 Correlated response of evolved NED = 1 (red boxplot) and evolved NED = 5 (blue boxplot) populations grown in assay environments with different numbers of environmental drivers (NED = 1, 5 and 8) and the control environment. Each panel label indicates the number of environmental drivers in each assay environment. Dashed line indicates no difference between the growth rate of the MED-evolved populations and the evolved control populations.
4.5.3 Local adaptation is more common in evolved NED = 5 populations than evolved NED = 1 populations

There is evidence of local adaptation in some evolved NED = 1 and evolved NED = 5 populations (shown by the stars in Figure 4.6). However, when the NED of the assay environment are taken into consideration, patterns of local adaptation are more common in evolved NED = 5 populations (shown by the stars in Figure 4.7; the effect of the interaction between NED of selection environment and NED of the assay environment on the correlated response of evolved populations; $F_{1,209} = 12.86$, $P = 0.001$). Variation in the correlated response to selection is explained largely by the interaction between evolved populations within the selection environments (nested within selection NED) and the assay environment (nested within assay NED), explaining 43%. The evolved populations and the assay environment each explains 15% of the variation in growth rate (see Table 9-3 and Table 9-4 for mixed model output). Local adaptation was calculated as the difference between the direct response in the home environment and the growth rate in new environment (minus the average growth rate of the evolved control populations in the same environment) (Figure 4.6, Figure 4.7). A direct response that is greater than the growth rate in new environments indicates that there is ecological specialisation within the selection environment, with reduced growth when the population is growing in new environments.
Figure 4.6 The average response to selection of evolved NED 1 and evolved NED 5 populations assayed in 17 different new environments. Response to selection is calculated as the difference between the direct response and the growth rate in new environments (minus the growth rate of the evolved control in the same environment). If growth rates in the home environment (direct response) are greater than growth rates in the new environments (MED-evolved population growth rate – control population growth rate), populations are locally adapted to the selection environment (indicated by a star). Each circle shows the mean response to selection of each evolved replicate population (± SD) across all assay environments. Dashed line indicates no difference between the growth in new environments and the home environment (data points above the dashed line show higher growth in home environment than new environments, and data points below the dashed line show lower growth in the home environment than new environments). Each panel is labelled with the NED of the selection environment.
When the correlated responses for each NED assay environment is considered separately, it becomes clear that evolved NED = 1 and evolved NED = 5 populations deal with the number of environmental drivers differently (Figure 4.7). Some populations selected in NED = 5 environments only show patterns of local adaptation when grown in NED = 1 environments i.e. growth in local environment is higher compared foreign NED = 1 environments (single star, Figure 4.7). In many cases, evolved NED = 5 populations grow better in foreign NED = 5 environments (i.e. growth rates are higher in NED = 5 environments than the local environment and alternative NED = 1 environments). This indicates that evolved populations are not responding to improved environmental quality in NED = 1 environments (indicated by the decline in growth rate of the evolved control populations as NED increases), and may indicate that NED of the assay environment is important following selection in high NED environments (NED =5). In contrast, evolved NED = 1 populations perform similarly well in foreign NED = 1 and NED = 5 environments and the local environment. In addition, patterns of local adaptation are stronger in populations that have a positive direct response to selection (for example, populations evolved in elevated UV (NED =1), and pH/UV/ND/LI/Herb in (Ned = 5) (Figure 4.4).
Figure 4.7 The average response to selection of evolved NED 1 and evolved NED 5 populations assayed in 17 different new environments. Coloured points indicate the response to selection in NED = 1 (green) and NED = 5 (blue) assay environments. Response to selection is calculated as the difference between the direct response and the growth rate in new environments (minus the growth rate of the evolved control in the same environment). If growth rates in the home environment (direct response) are greater than growth rates in the new environments (MED-evolved population growth rate – control population growth rate), populations are locally adapted to the selection environment (indicated by a star). Each circle shows the mean response of the evolved replicate populations (± SD). Dashed line indicates no difference between the growth in new environments and the home environment (data points above the dashed line show higher growth in home environment than new environments, and data points below the dashed line show lower growth in the home environment than new environments). Each panel is labelled with the NED of the selection environment.
Patterns of local adaptation were also uncovered using LF measures of local adaptation. Overall, the local populations grow faster in their selection environment than the majority of foreign populations (evolved in alternative selection environments), but are not always ranked first (Figure 4.8). The exception is local populations evolved at elevated CO$_2$ only. Here, the evolved populations under elevated CO$_2$ are ranked last. One explanation is that selection at high CO$_2$ reduces the need for CCM activity, thereby lowering the metabolic costs of inorganic carbon acquisition, and this is consistent with what other studies have found when photosynthetic algae are evolved at elevated CO$_2$ (C. reinhardtii, Collins & Bell (2004)). Evidence of local adaptation is stronger in evolved NED = 5 populations than in evolved NED = 1 populations, when comparing the average rank fitness in the local environment of the evolved populations (average rank of evolved NED = 1 populations is 5.6 and the average rank of evolved NED = 5 populations is 4.5). In addition, the fitness rank of evolved populations in each assay environment deviates from the distribution of ranked fitness of the evolved control populations in the same environments. Chi-squared goodness of fit test shows that the expected distribution of the evolved NED = 1 and evolved NED = 5 populations are significantly different from a from the expected frequencies of the evolved control population (Figure 4.8; $\chi^2 = 26.44$, df = 14, $P < 0.023$). See Table.9-6 for observed and expected distributions of average rank fitness of NED = 1 and NED = 5.
Figure 4.8 The rank order of the average growth rate of evolved replicate populations growing in each assay environment. Gold-filled squares show the rank position of local populations (evolved in the assay environment), and dark grey-filled squares show the rank position of foreign NED = 1 populations, light grey squares show the rank position of foreign NED = 5 populations and black filled squares show the rank position of the evolved control populations. Each panel is labelled with the NED of the assay environment.
Overall, HA measures of local adaptation reveal that the evolved NED = 5 populations are locally adapted to their selection environment, with a trade-off in growth when evolved NED = 5 populations are grown in NED = 1 assay environments, and in some cases no trade-off in growth when grown in alternative NED = 5 assay environments (Figure 4.7). This is supported by LF measures of local adaptation, as evolved NED = 5 populations have lower ranked fitness in the NED = 1 assay environments (Figure 4.8). There are more examples of local adaptation in evolved NED = 1 populations using LF measures of local adaptation than with HA measures of local adaptation. Although many evolved NED = 1 populations are ranked highly in LF measures of local adaptation, growth rates between local and foreign populations in each selection environment are very similar for both evolved NED = 1 and evolved NED = 5 populations (Figure 9.3). In addition, HA measures of local adaptation are more accurate in this study because calculations use the growth rate of evolved populations in the home environment and new environments, minus the growth rate of the average evolved control population in the same environments, which accounts for the quality of the assay environments. Traditional HA measures of local adaptation (Figure 9.4) measure the growth rate of the evolved populations in the local environment and in alternative environments, and it is unclear if a population grows better in an alternative environment due to positive pleiotropic interactions between home and away environments, or because the away environment is of better quality and therefore supports higher growth rates (see Kawecki and Ebert, 2004; Blanquart et al., 2013 for detailed description of measurements of local adaptation).

4.5.4 Selection NED and Assay NED have no effect on variance of growth rates

All populations were assayed in new environments that have either the same or different NED from their selection environment. However, assay NED has no significant effect on the variance in growth rate between evolved populations,
growing in different regimes, within each selection NED (Figure 9.2; the effect of assay NED on the variance in growth of populations with in each NED; F_{1,17} = 0.27, P = 0.41). This result does not support the hypothesis that variance between populations evolved at a given NED would be lower when assayed in environments with the same NED than variance when grown in environments with different NED.

4.5.5 MED-evolved populations grow faster than evolved control populations in novel environments

When populations were assayed in the CuSO₄ environment (which has not been encountered before by the evolved populations), evolved NED = 1 populations (mean of 7 regimes = 0.98 cell divisions (d⁻¹), ± 0.44 cell divisions (d⁻¹); average ± SD) and evolved NED = 5 populations (mean of 6 regimes = 0.77 cell divisions (d⁻¹), ± 0.22 cell divisions (d⁻¹)) are able to maintain higher growth rates than the evolved control populations (single control regime with three biological replicates = 0.80 cell divisions (d⁻¹), ± 0.72 cell divisions (d⁻¹)) (Figure 4.9). Whilst selection NED explains 55% of the variation in the growth rate of evolved populations in the CuSO₄ environment, the effect of selection NED on growth rate is not significant effect (Figure 4.9; F_{1,9} = 0.04, P = 0.84), and this may be explained by the small sample size of control populations, as there is only one selection NED = 0 environment (control). In addition, there are small differences in growth rates between evolved NED = 1 and evolved NED = 5 populations, and evolved populations (nested within each selection environment and NED) explains 26% of the variance in growth rates in the mixed model.
Figure 4.9 Average rate of cell division (d\(^{-1}\)) of evolved populations (NED = 0, 1 and 5) grown in copper sulphate (0.2 µM). Coloured boxplots show selection environments of evolved populations. Dashed lines shows the growth rate of the evolved control populations in the control environment.
4.6 Discussion

4.6.1 Interactions between selection history and assay environment affect *C. reinhardtii* growth in new environments

This study was designed to determine if the number of environmental drivers during recent evolution affected growth in new environments. New environments differed from selection environments both in terms of the identity of the drivers involved, but also in the number of drivers present (Figure 4.2). I find that the NED of the selection environment, although significant, only explains a small amount of variation in growth rate, and that interactions between evolved populations and assay environment (GXE) explain the majority of the variation in growth across all environments. Thus, the selection history of each evolved populations is important for determining how well they grow in new environments, but the magnitude of the effect is environment specific.

4.6.2 Local adaptation cannot be predicted using similarity between selection and assay environments

There are many studies that demonstrate that fluctuations in environmental variables select for generalists as a result of pre-adaptation. For example, *S. marcescens*, exposed to fluctuating temperatures (daily variation in temperature between 24°C and 38°C, mean of 31°C), were able to outperform strains selected at 31°C (all strains originally maintained at 25°C). The generalist grown in fluctuating temperatures are pre-adapted for growth at high temperatures, due to exposure at very high temperatures during the selection experiment. Physiologists describe organisms with an the ability to persist under future environmental changes due to their past
environmental experiences as pre-adapted (Dupont et al., 2010). For example, sea urchins that inhabit fluctuating pH conditions in polar seas become pre-adapted to future pH conditions when exposed to reduced pH in the lab (Clark et al., 2009). Similarly, evolved population of C. reinhardtii evolved in environments with five environmental drivers may be pre-adapted to growing in new environments that share some environmental drivers with the selection environment, due to positive correlations between traits values that improve fitness in the selection environment, and new environments (Etterson, 2004a).

Based on the above, I predicted that increased similarity between assay environments and selection environments with five environmental drivers (i.e. more environmental drivers are shared between the assay and selection environments), would break down patterns of local adaptation in evolved NED = 5 populations, and as a result growth rates in the selection environments and assay environments would be similar. I predicted that evolved NED = 1 populations would show evidence of local adaptation, with reduced growth rates in new environments and this is due to low similarity (little or no shared environmental drivers) between the assay and selection environments. The results presented here do not fall in line with these predictions, and I found that there are more instances of local adaptation in evolved NED = 5 populations (6/12) than in evolved NED = 1 populations (2/14) (Figure 4.6), and that the pattern of local adaptation varies with assay NED in evolved NED = 5 populations (Figure 4.7). LF calculations revealed similar patterns in evolved NED = 1 and evolved NED = 5 populations, and indicate that local populations are more fit in their local environment than expected by chance (Figure 4.8). HA measures of local adaptation show that there is little local adaptation evident in populations evolved in NED = 1 environments, with a few exceptions. Populations evolved in elevated temperature show evidence of local adaptation based on their growth in NED = 5 assay environments, and populations evolved under UV appear locally adapted when grown in NED = 1 assay environments. In addition, PCA results show that evolved NED = 5 populations are more specialised in their response to high NED environments, environments that contain more dominant environmental drivers.
such as herbicide (identified as a dominant environmental driver in chapter 2 and chapter 3), and to copper sulphate (a poor quality environment, indicated by the evolved control, Figure 4.9). Populations evolved in NED = 1 environments show no obvious clustering, which indicates consistent growth across all environments (Figure 9.5 and Figure 9.6).

**4.6.3 The strength of selection effects the outcome of evolution**

Populations that show more evidence of local adaptation measured by HA also have the largest direct response to selection (Figure 4.4 and Figure 4.7). In chapter 3, I demonstrated that the response to selection is correlated with the strength of selection. This means that the distance travelled towards the optimum is greater simply because the optimum phenotype is further away from the ancestral phenotype (MacLean & Bell, 2002). Beneficial mutations that improve fitness in the local selection environment may be deleterious in an alternative environment due to negative pleiotropic interactions, and the chance that a beneficial mutation is deleterious in a new environment is positively correlated with the size of beneficial mutation (Eyre-Walker & Keightley, 2007). As the fitness of populations with a direct response to selection improves, trait divergence increases, and evolved populations become locally adapted to the selection environment. This was seen by Buckling et al., (2003) who found that as fitness increased in *P. fluorescens* the ability to diversify was reduced. This is consistent with studies that found increased trait divergence in poor quality environments, where the strength of selection is presumably high (see review by Litchman and Klausmeier, 2008).

Based on this, it is plausible that in my study the reduced ability for growth in new environments is caused by negative pleiotropy. This would result in local adaptation in environments where the strength of selection is high, which is what I see. In addition, the cost of mutations with large effect on fitness in new environments may explain why evolved populations with the largest direct response show more
evidence of local adaptation. This is consistent with Barrett et al., (2005), who found that populations evolved in poor quality environments were more locally adapted.

4.6.4 The NED of the selection environment partially explains patterns of local adaptation

A specific question that this chapter set out to answer is how the number of environmental drivers affects local adaptation. Interestingly, in some cases, there is no evidence of local adaptation when some evolved NED = 5 populations are assayed in alternative NED = 5 assay environments (blue circles fall below the dashed line in Figure 4.7). This could indicate that evolved NED = 5 populations are responding to the NED of the assay environments and traits that improve fitness the local environment with five environmental drivers are positively correlated with alternative NED = 5 assay environments. One possible explanation is that the identity of environmental drivers is less important as NED increases (NED = 5), and the NED is more important. This would enable evolved NED = 5 populations to have equal growth rates across NED = 5 assay environments, regardless of the identity of environmental drivers. This pattern of improved growth in NED = 5 environments is observed in half the evolve NED = 5 environments (3/5). It is less clear how populations that have evolved a general response to five environmental drivers would deal with NED = 1 assay environments, however, I found that in the majority of cases, evolved NED = 5 populations grow poorly in NED = 1 environments. This suggests that traits that improve fitness in NED = 5 environments reduce fitness in NED = 1 environments.

This explanation of local adaptation in evolved NED = 5 populations suggests that the identity of single environmental drivers is more important. However, there is no evidence of increased specialisation in the single environmental drivers (apart from those drivers identified with large direct responses). In many cases evolved NED = 1...
population have improved growth rates when assayed in new environments, with little differences in response to NED = 1 and NED = 5 assay environments. Similar growth across new environments indicates that evolved NED = 1 populations are more generalist than NED = 5 evolved populations. However, the strength of selection is low in these environments, and hence the direct response to selection is reduced in the majority of evolved NED = 1 populations, and the correlated response to selection is not predictable.

4.6.5 Local adaptation and environmental quality

The evolution of specialists and generalists has been previously investigated using single sugar resources, where the metabolic pathways are well defined and the outcome of selection can be understood by considering the effects of metabolic changes (MacLean & Bell, 2002). In contrast, while we know that the single driver environments are more simple than the NED = 5 environments, we have little information on metabolic responses to each driver (Chapter 1; Table 1-1) or on how differences or similarities in metabolic responses to drivers might contribute to generalists and specialist genotypes. Here, I interpret my results partly as responses to environmental quality. This approach was also taken by Bell and Reboud, (1997), who found that population of C. reinhardtii evolved in the better quality light environments showed greater environmental variance than that those populations evolved in the more stressful dark environment. A review by Falconer (1990) also suggests that selection in good quality environments leads to increased environmental variance, and that generalists are more likely to evolve in uniform environments. Similar results to selection in uniform environments have been found in investigations of antibiotic resistance. Hall (2013) found that evolved resistant genotypes of E. coli are able to grow as well as the ancestor in some assay environments (31 single-carbon-source environments on Biolog Plates), and that GXE interactions observed may be a result of indirect effects to selection that contribute to the changes in growth across antibiotic-free environments. Similar
results were reported by Bataillon et al., (2011), who found that antibiotic resistant are able to grow in a wide range of alternative environments.

### 4.6.6 Response to a novel environment

Evolved populations were grown in copper sulphate, a novel stressful environment, in order to determine if there is an advantage to evolving in poor quality environments when confronted with a new stressful environment. There is some evidence that evolving in more stressful environments results in selection for general stress tolerance traits (Sikkink et al., 2015). Therefore, it is expected that as environments becomes more stressful with increasing NED, that populations will favour traits characteristic of stress tolerance, such as slow growth (Stanton et al., 2000). Here, I found that growth rates of evolved NED = 1 and NED = 5 populations are very similar, with NED = 1 populations growing on average slightly faster than NED = 5 populations (Figure 4.9). However, growth rates of evolved NED = 1 and NED = 5 populations are much greater than the evolved control populations, which failed to grow when exposed to copper sulphate. This supports the assumption that characteristics that favour stress tolerance may be selected for in more stressful environments, but it does not show that stress tolerance is affected by NED.

In addition, slow growth may explain why evolved NED = 5 populations grown in foreign NED = 5 assay environments have, on average, slower growth rates than both evolved NED = 1 populations and control populations. Evolved NED = 5 populations may have been under selection for stress tolerance traits, which can allow populations to persist in stressful or toxic environments. Slow growth is a well-characterised stress tolerance trait, due to a reduction in metabolism and uptake of poisons (Rangel, 2011). This has been empirically shown by Ketola et al., (2013) who selected *S. marcescens* for slow growth in order to increase overall growth among novel environments. Growing slow may then also be beneficial when assayed in stressful novel environments such as copper sulphate or herbicide
4.6.7 Conclusion

There is some evidence that the strength of selection in the local environment is important for determining the extent of local adaptation in evolved populations. The results from this chapter suggest that organisms evolved in poor quality environments (where the strength of selection is high) will be more locally adapted than populations that inhabit better quality environments (where the strength of selection is low). However, I also demonstrate that interactions between NED of the selection environment and new environment may be important in explaining the growth of evolved NED = 5 populations in new environments. In some cases, growth rates of evolved NED = 5 populations are improved when assayed in alternative environments with the same NED. This is not the case for evolved NED = 1 populations, which show no consistent trend in growth in new environments. This may indicate that we may be able to predict the response of populations that inhabit poor quality environments, when exposed to new environments, given some information on whether the environments are of comparable quality. Future experiments should build on these results by including more selection histories with different NED and different environmental drivers, in order to establish if predictions can be made by knowing the NED of the selection environments.
Chapter 5 - Phenotypic consequences of multiple environmental changes before and after evolution

5.1 Chapter summary

Predictions on the long-term response of natural organisms to global change are almost exclusively based on short-term experiments. For this reason, I compared the short- and long-term response of *C. reinhardtii* to MEDs, to link plastic and evolutionary responses to MEDs. I compared the growth rates, the proportion of chlorophyll positive cells, chlorophyll autofluorescence per cell volume (1/µm$^3$), and cell size, before evolution, and following 95 transfers in each regime. Because chlorophyll autofluorescence is an easily measured trait in the lab and in the field, I further analysed subpopulations with different chlorophyll autofluorescence properties to measure the relationship between chlorophyll autofluorescence and growth rate.

5.1.1 Abstract

How does the response of *C. reinhardtii* to MEDs differ before and after evolution? This is an important question if we are interested in using the wealth of physiology studies available to predict long-term responses of organisms to MEDs. Following evolution, physiological measurements (such as cell size and chlorophyll content) that were once correlated with growth rates (before evolution), no longer scale with NED following evolution, and evolved populations converge on the same phenotypes. In addition, in chapter 3 I demonstrated that the strength of selection (measured using the evolved control populations) can predict evolutionary changes in growth rates of *C. reinhardtii*, growing under increasing NED. The data presented here confirm that the plastic response collected using the founder populations can
predict the change in growth rates of the evolved populations, enabling predictions to be made without running long-term selection experiments.

5.2 Introduction

MED experiments are rarely carried out over hundreds of generations and there only a handful of investigations that directly compare the plastic and evolutionary response to MEDs (Benincà et al., 2008; Tatters et al., 2013a, 2013b). However, the question of how short- and long-term responses to MEDs are related to each other is important in order to meet the challenge of applying the results of manipulation experiments in the lab to predict the outcome of global change in natural populations (Boyd & Hutchins, 2012; Collins et al., 2013; Petchey et al., 2015), where short-term manipulations are often used to predict long-term responses (Tatters et al. 2013). In order to answer this question, many phenotypic responses, including growth rate, chlorophyll autofluorescence, cell size, were measured at the beginning and at the end of the selection experiment, so that the plastic and evolved responses to MEDs can be compared.

5.2.1 Physiological changes in natural communities

Variations in physiology in response to MEDs has important ramifications, not only for the organisms under investigation, but for natural freshwater and marine communities. Marine and freshwater phytoplankton are the base of aquatic food webs, and physiological changes will have consequences for higher trophic levels (Boyd & Hutchins 2012; Beardall & Stojkovic 2006). For example, reduction in cell size may result in grazers that size-select food, not being able to physically consume larger cells (Christaki et al., 1999; Cuevas, 2006), potentially altering food web structure (Carpenter et al., 1987). The reduction of food quality available to higher trophic levels, indicated by a reduction in fatty acid composition, has been measured
experimentally in response to single drivers such as UVB, where impaired energy transfer to the next trophic level was observed (De Lange & Van Reeuwijk, 2003; Tank et al., 2013). A reduction in primary productivity, as a result of changes in photosynthetic carbon fixation (Falkowski, 1998), will reduce energy transfer to higher trophic levels (Wohlers et al., 2009). Typically, the plastic response to elevated CO₂, results in an increase in growth and photosynthesis (Low-Décarie et al., 2014) however, how this response scales up as more environmental drivers are added (Gao et al., 2012; Todgham & Stillman, 2013), and over evolutionary times scales (Collins & Bell, 2004) remains unknown.

5.2.2 The effect of MEDs is revealed by phenotypic measurements

Physiological changes in cell size and chlorophyll content are commonly measured in investigations which aim to characterise the physiological responses of Chlamydomonas spp. and other photosynthetic eukaryotes to environmental drivers (Prado et al., 2011, 2012; Herrero et al., 2012). For example, Rioboo et al. (2009) demonstrated that in response to increasing concentrations of the photosynthesis-inhibiting herbicide terbutryn, Chlorella vulgaris show a decrease in chlorophyll autofluorescence. In addition, C. moewusii cultures exposed to increasing paraquat concentrations also show a decrease in chlorophyll autofluorescence (Prado et al., 2011). Some investigations also found a relationship between chlorophyll bleaching and reduced cell viability, alongside reduced cell size (Prado et al., 2011). Cell size has been found to change in response to stressful environments. For example, the cell size of C. vulgaris decreases in a dose dependent manner in response to terbutryn, though the response was reversed when length of exposure increased to 48 h (Rioboo et al., 2009). Cell size of photosynthetic microalgae has also been found to increase in response to the heavy metal cadmium (Jamers et al., 2009), and herbicide exposures (Rioboo et al., 2002; Prado et al., 2011; Herrero et al., 2012).
The loss of chlorophyll autofluorescence can be related to the irreversible damage of photosystem II (PSII), the main source of \textit{in vivo} chlorophyll autofluorescence (Herrero \textit{et al.}, 2012). In addition, reactive oxygen species (ROS), produced in response to photosystem interfering herbicides such as atrazine (used here), can also cause chlorophyll bleaching (Pouneva, 1997; Prado \textit{et al.}, 2011). Many of the environmental drivers used here have been shown to interfere with PSII in \textit{C. reinhardtii} (Table 1-1). In this study \textit{C. reinhardtii} is grown in carbon free media, so that carbon must be acquired through photosynthesis, which in turn requires functional chlorophyll. Therefore, there should be strong selection pressure to maintain chlorophyll to ensure survival, even in poor quality environments. This close link between acquiring a necessary nutrient (carbon) and growth suggests that chlorophyll content, which is easy to measure even in the field or in samples that cannot be cultured, is potentially a good indicator of population fitness (Molina-Montenegro \textit{et al.}, 2013). Cell size is an essential characteristic of all unicellular organisms, and in \textit{C. reinhardtii} cell size is also related to population growth through its effect on cell division, as a critical threshold size must be reached before cells divide (Harris, 2001; Matsumura \textit{et al.}, 2003; Machado & Soares, 2014). These investigations provide support for the usefulness of chlorophyll autofluorescence and cell size, and their relationship with the physiological status of cells growing in different regimes.

\textbf{5.2.3 Flow cytometry measures physiological change in \textit{C. reinhardtii} grown in MEDs}

Flow cytometry provides detailed information of the physiological status of the individual cells (Veldhuis & Kraay, 2000). With this technique, autofluorescence may be used to distinguish between different populations or subpopulations. For example, the morphology of non-viable bleached cells and viable chlorophyll positive cells have been investigated separately in \textit{C. moewusii} (Prado \textit{et al.}, 2011). In addition, a fluorescent dye may be used to specifically label the cell population of
interest or monitor organelle function (Dubelaar & Jonker, 2000; Kay et al., 2013). Fluorescent cells can also be sorted into separate tubes, based on specific cell properties (such as cell size, autofluorescence intensity) by fluorescence-activated cell sorting (FACS) (Cooper et al., 2010). This provides further opportunities to separately analyse the phenotypic differences between subpopulations, such as differences in the proteome between populations and changes in protein abundances depending on environmental conditions (Han et al., 2008; Perrineau et al., 2014).

5.3 Methods and Materials

This chapter focuses on phenotypic changes in response to MEDs during the initial response and evolved response to selection in, chapter 2 and 3 respectively. Details of experimental design and culture environments can be found in Chapter 2, section 2.4 Methods and Materials, pages 27 - 37. Details of the selection experiment and measure of population growth following 95 transfers in the selection environment (after evolution), can be found in Chapter 3, section 3.4.1, page 73.

5.3.1 Flow cytometric analysis of physiological parameters

A FACS CANTO was used to determine red autofluorescence (chlorophyll) and orange autofluorescence (putative chlorophyll breakdown products, PE channel, see Appendix 10.1.1), event number (cell density) and forward scatter (related to cell size) (Jamers et al., 2009; Herrero et al., 2012; Prado et al., 2012; Schaum & Collins, 2014). Relative chlorophyll autofluorescence intensity was detected in the PerCP-Cy5.5 channel (Ex-Max 488 nm/Em-Max long pass (LP) 670 – 725 nm). Samples were run on the CANTO from 200µl cultures in 96 well plates, and the average well was read for 30 seconds at average flow rates of 1µl/second.
5.3.2 Determination of cell size and chlorophyll autofluorescence

Variations in cellular volume were determined by flow cytometry after 120h of acclimation in the treatment and control environments. Forward scatter was used since forward light scatter (FSC) signal is routinely correlated with an increase in cell size (Rioboo et al., 2002; Prado et al., 2011, 2012); larger cells take more time to pass through the laser and thus produce larger FSC scatter signals. The FSC was calibrated with size calibration beads (Bang Laboratories, Inc.; Schwartz et al. 1983) and FSC was converted to μm (Figure 10.1). Relative chlorophyll autofluorescence per cell volume (1/μm³) was calculated assuming that each cell is spherical (Machado & Soares, 2014). Cell volume was calculated using Equation 5-1.

\[ V = \frac{3}{4} \pi r^3 \]

Equation 5-1

Cells classified as dead (not included in the analysis), were outside the range of normal cells size (arbitrary units before size calibration). This is associated with a decrease in the forward (FS) and side (SS) scatter (Darzynkiewicz et al., 1992; Prado et al., 2011). Chlorophyll autofluorescence was analysed downstream of this gate.

5.3.3 Chlorophyll positive and chlorophyll negative cells

Two distinct subpopulations were consistently identified using C. reinhardtii autofluorescence in some of the cultures: chlorophyll positive cells (CP) and chlorophyll negative (CN) cells. Using the FACS sorter (BD Aria I), a small sub-sample of four populations growing in different MED environments (pH/UV/ND/LI, CO₂/pH/UV/ND/LI, UV/ND/P, and CO₂/UV/ND), were sorted into CP and CN groups (2 sorted groups x 4 populations = 8 sorted populations). Sorted populations were re-suspended into six (three replicates for each sorted population) culturing
tubes containing 1 ml phosphate buffered saline (PBS) (Appendix Figure 10.2). Cells were gently centrifuged (settings: at 3220 g, for 5 mins at 21°C) until a pellet was formed. The supernatant was removed and the pellets were immediately resuspended into fresh culture media from the appropriate selection environment and grown for 14 days (with a second transfer at day 7). Cell counts were performed using the flow cytometer and the rate of cell division (d⁻¹) for sorted groups was measured at time 0 and 72 h, and again after transferring cells into fresh media at time 0 and 48 h. For each sorted population (CP and CN), the proportion of chlorophyll positive and chlorophyll negative cells was identified and using DIVA software, using the same gates of chlorophyll positive cells and chlorophyll negative cells that was used for sorting populations into CP and CN groups. In addition, the rate of cells division (d⁻¹) of chlorophyll negative and chlorophyll positive subpopulations within sorted groups was calculated. A portion of the cell pellet was transferred to liquid nitrogen for further analysis using mass spectrometry (Appendix 4, section, 10.1.2, page 234).

5.3.1 Statistical Analysis

For both time points, before evolution and after evolution, the effect of NED on changes in cells size (µm), the proportion of chlorophyll positive cells and chlorophyll autofluorescence per cell volume (1/µm³) was analysed using a mixed model in R (R Core Team, 2013), using the packages lme4 and lmerTest. NED (0-8) is a fixed factor, as is overlap between regimes within each level of NED (measured as the average number of shared drivers between different test environments for a given NED; see chapter 2, section 2.4.6). Regime, batch and evolved populations within each regime were taken as random factors. In order to investigate the effect of the number of transfers in each selection environment on changes in cells size (µm), the proportion of chlorophyll positive cells and chlorophyll autofluorescence per cell volume (1/µm³), the initial response and evolved response datasets were combined. The same mixed model, as described above, was used to analysis the combined
datasets and the effect of transfer number was included as a fixed factor in this analysis. A linear regression model was used to measure the relationship between cell size and the rate of cell division (d\(^{-1}\)), the relationship between the proportion of chlorophyll positive cells and the rate of cell division (d\(^{-1}\)), and the relationship between chlorophyll autofluorescence per cell volume (1/\(\mu\)m\(^3\)) and cell size (\(\mu\)m) (before and after evolution), using the lm function within R.

In order to test the effect of chlorophyll content on the growth rate of sorted populations (CP and CN), a mixed model was performed using the lmer function in R. Growth rates of each subpopulation of chlorophyll negative and chlorophyll positive cells, within sorted groups (CP and CN) were measured using flow cytometry. In forming the fixed and random factors in this model, the chlorophyll content (chlorophyll positive or chlorophyll negative cells), and the identify of the sorted groups that each population was grown from (either CP or CN) were taken as fixed factors. The effect of the identify of evolved populations and transfer number were taken as random factors.

### 5.4 Results

#### 5.4.1 Variation in plastic response to MEDs explains variation in the evolutionary impact of MEDs

In this chapter, I address how the plastic and evolved responses to MEDs differ. I found that there are significant differences between phenotypic data collected at the beginning (before evolution) and end (after evolution) of the selection experiment. The most notable change between the plastic and evolved response is the increase in the growth rates, where, in some cases, the growth rate doubles following 95 transfers in the selection environment (Figure 5.1; the effect of the number of transfers in each selection environment on growth rate, \(F_{1,5} = 54.351, P = 0.033\)).
However, the trend of decreasing growth rate as NED increases, is remarkably similar between non-evolved and evolved populations (Figure 5.2; lower panels; the effect of NED on the growth rate relative to the respective control populations, $F_{1,83} = 10.599, P = 0.002$), with transfer number (0 or 95), explaining none of the variation in relative fitness (Figure 5.2; the effect of transfer number on the growth rate relative to the respective control populations, $F_{1,9} = 0.069, P = 0.798$). The plastic and evolved growth rates in response to MEDs were presented in chapter 2 and 3. To avoid repetition these results will not be discussed at length here, however population growth rate can be a good indicator of fitness (Collins, 2011a), and will therefore be used to understand the relationships between physiological measurements, such as chlorophyll autofluorescence, and fitness.
Figure 5.1 Population growth rate of *C. reinhardtii* under 0 to 8 environmental drivers. Black data points and bars represent the mean and standard deviation between regimes for each NED. Coloured points indicate the average growth rate between replicate population within each regime (± SD) (96 regimes in total, see Figure 7.11 for identity of regimes). Panel labels indicate the plastic response (“Before Evolution”) and evolved response (“After Evolution”) to MEDs. Dashed line shows the average growth rate of the control populations in the control environment. Upper and lower panels show the same data, however the lower panels have a different y-axis so that the relationship between NED and growth rate is clear in both panels.
Figure 5.2 Population relative fitness when growing in environments with 0 to 8 environmental drivers. Fitness is calculated relative to the average growth rate of the control populations in the control environment, before and after evolution. Black data points and bars represent the mean and standard deviation between regimes for each NED. Coloured points indicate the average growth rate between replicate population within each regime (± SD) (96 regimes in total, see Figure 7.11 for identity of regimes). Panel labels indicate the plastic response and evolved response to MEDs. Dashed line shows the mean relative fitness of the control populations in the control environment.
5.4.2 Populations converge on the same cell size during evolution regardless of NED

Before evolution, the cell size of *C. reinhardtii* declines as NED increases from 3 µm to 8 µm, with very little change between NED 0 and 2 (Figure 5.3a). The largest cells are 16 µm (NED = 1; elevated temperature), and the smallest cells are 7 µm (NED = 7; CO₂/ Temp/pH/LI/P/ND/UV). The cell size in the control environment is 12.5 µm (± 0.3 µm; average ± SD), which is typical for *C. reinhardtii* (Harris, 1989). Before evolution, cell size declines with increasing NED regardless of the identity of the environmental drivers (Figure 5.3a; $F_{1,94} = 54.351, P < 0.0001$). I suggest that this is because, as environmental quality decreases with NED, cell size also decreases. This is consistent with the positive correlation that exists between cell size and the rate of cell division before evolution (Figure 5.4a; $R^2 = 0.33, P < 0.0001$). This may indicate that healthier cells are larger and faster growing than unhealthy cells.

Following evolution, NED explains less than two percent of the variation in cell size (Figure 5.3b; $F_{1,60} = 0.356, P = 0.553$). Cell size fluctuates around 15.5 µm (± 2.33 µm). The majority of variation in changes to cell size is explained by the evolved populations (replicated within each regime) (28%) and the identity of the environmental drivers (13%). Interestingly, there is no longer a relationship between cell size and the rate of cell division after evolution (Figure 5.4b; $R^2 = 0.02, P = 0.006$). There is a significant difference in the relationship between cell size and NED between the plastic and evolved populations (Figure 5.3; *the effect of the number of transfers in each selection environment on growth rate, $F_{1,5} = 65.582, P = 0.0001$*). This is because cell correlates with NED before evolution, but not after evolution.
Figure 5.3 Changes in cell size of *C. reinhardtii* under 0 to 8 environmental drivers. Black data points and bars represent means and standard deviation between regimes for each NED. Coloured points indicate the average growth rate between replicate population within each regime (± SD) (96 regimes in total, see Figure 7.11 for identity of regimes). Panel labels indicate the plastic response and evolved response to MEDs. Dashed line shows the average cell size of the control populations in the control environment.
Figure 5.4 Correlation between cell size and the rate of cell division before (a) and after evolution (b). Colour of points is scaled from red (low NED) to blue (high NED).
5.4.3 NED drives changes in the proportion of chlorophyll positive and negative cells in populations before evolution, with no relationship between NED and proportion of chlorophyll positive and negative cells after evolution

Before evolution, the proportion of chlorophyll positive cells per population of *C. reinhardtii* declines as NED increases (Figure 5.5a; $F_{1,94} = 39.874, \ P < .001$). The proportion of chlorophyll positive cells is 90% in the control environment, which steadily declines as NED increases to reach 5% chlorophyll positive cells in a NED = 7 environment (CO₂/Temp/pH/LI/P/ND/UV). As expected for photosynthetic cells, there is a positive correlation between the proportion of chlorophyll positive cells and the rate of population growth (Figure 5.6b; $R^2 = 0.47, \ P = <0.0001$) and this remains following evolution (Figure 5.6a; $R^2 = 0.24, \ P = <0.0001$). The proportion of chlorophyll negative cells increases with NED (Figure 5.7a; $F_{1,94} = 10.332, \ P = 0.002$) and like a threshold response the proportion of chlorophyll negative cells increases from the lowest proportion of chlorophyll negative cells growing in the control environment (6 %), until NED = 4, where the proportion of chlorophyll negative cells levels off at 40 % (Figure 5.7a).

The proportion of cells lacking chlorophyll changes substantially after evolution. Following evolution, the proportion of chlorophyll positive cells decreases with increasing NED until NED = 4, where there is little change until a drop at NED = 7. The average proportion of chlorophyll positive cells across all evolved populations (87 % ± 17.06 %) is not significantly different from the proportion of chlorophyll positive cells in the evolved control populations (98 % ± 1 %) (Figure 5.5), and there is no significant effect of NED on the proportion of chlorophyll positive cells after evolution (Figure 5.5b; $F_{1,58} = 0.800, \ P = 0.375$). Changes in the proportion of chlorophyll positive cells after evolution are explained by the selection environment (27%) and the overlap within each NED level (13%). There is general trend of
increasing proportion of chlorophyll negative cells with increasing NED, from the lowest proportion of chlorophyll negative cells within the evolved control population growing in the control environment (2 %), to the largest proportion found in evolved populations growing under NED = 7 (89 %). However, variation between regimes is large after evolution (average proportion of chlorophyll negative cells across all evolved populations is 12 % ± 16.72 %), and as result the relationship between NED and proportion of chlorophyll negative cell is not significant (Figure 5.7b; $F_{1,53} = 0.805, P = 0.374$).

There is very little difference between the control populations before and after evolution (indicated by dashed lines), demonstrating that cells growing in the benign environment, with some of the highest growth rates, do not lose their chlorophyll, and changes in the proportion of chlorophyll positive cells is probably associated with evolutionary responses to the drivers in the selection environments. Differences observed between the plastic and evolved response are significant for both the proportion of chlorophyll positive cells (Figure 5.5; the effect of the number of transfers in each selection environment on the proportion of chlorophyll positive cells, $F_{1,6}= 118.656, P < 0.0001$) and the proportion of chlorophyll negative cells (Figure 5.7; the effect of the number of transfers in each selection environment on the proportion of chlorophyll positive cells, $F_{1,6}= 59.874, P = 0.0002$).
Figure 5.5 Proportion of chlorophyll positive cells in populations of *C. reinhardtii* growing in environments with 0 to 8 environmental drivers. Black data points and bars represent the mean and standard deviation between regimes for each NED. Coloured points indicate the average growth rate between replicate population within each regime (± SD) (96 regimes in total, see Figure 7.11 for identity of regimes). Panel labels indicate the plastic response and evolved response to MEDs. Dashed line shows the proportion of chlorophyll positive cells of the control populations growing in the control environment.
Figure 5.6 Correlation between proportion of chlorophyll positive cells and the rate of cell division before (a) and after evolution (b). Colour of points is scaled from red (low NED) to blue (high NED).
Figure 5.7 Proportion of chlorophyll negative cells in populations of *C. reinhardtii* growing in environments with 0 to 8 environmental drivers. Black data points and bars represent the mean and standard deviation between regimes for each NED. Coloured points indicate the average growth rate between replicate population within each regime (± SD) (96 regimes in total, see Figure 7.11 for identity of regimes). Panel labels indicate the plastic response and evolved response to MEDs. Dashed line shows the Proportion of chlorophyll negative cells of the control populations growing in the control environment.
5.4.4 NED explains variation in chlorophyll autofluorescence per cell volume before evolution but cannot explain variation in chlorophyll autofluorescence per cell volume following evolution

While having chlorophyll is necessary for photosynthesis, the amount of chlorophyll per cell also varies, and can affect photosynthesis. The intensity of chlorophyll autofluorescence indicates the total amount of chlorophyll (chlorophyll a and b) in a cell (Aguilera et al., 2008), and can vary between populations regardless of the proportion of chlorophyll positive cells. Unsurprisingly, there is a positive relationship between chlorophyll autofluorescence and cells size, both before evolution (Figure 10.3a; $R^2 = 0.19$, $P < 0.0001$), and after evolution (Figure 10.3b; $R^2 = 0.37$, $P < 0.0001$), indicating that larger cells can contain more chlorophyll than smaller cells. Assuming that each cell is a sphere I have calculated the chlorophyll autofluorescence per cell volume ($1/\mu m^3$), thus removing the effect cell size from the analysis.

Before evolution, chlorophyll autofluorescence per cell volume ($1/\mu m^3$) increases with NED between NED = 3 and 8, with little change between NED 0 to 3. Surprisingly, the lowest chlorophyll autofluorescence per cell volume ($1/\mu m^3$) is found in populations growing in NED =1 and NED = 2 environments, with the highest chlorophyll autofluorescence per cell volume ($1/\mu m^3$) found in some of the smallest cells at NED = 8, and this effect is driven by NED (Figure 5.8a; $F_{1,23} = 32.393$, $P < 0.0001$). However, it was expected that populations growing in poor quality environments at high NED (with lower growth rates), would produce cells with lower chlorophyll autofluorescence intensity, since chlorophyll autofluorescence is related to chlorophyll content of the cell (Aguilera et al., 2008), and previous studies have found a negative relationship between cell viability and the loss of chlorophyll (Prado et al., 2011; Herrero et al., 2012).
In contrast, after evolution, there is no trend between NED and chlorophyll autofluorescence per cell volume (1/µm³) (Figure 5.8b; $F_{1,64} = 0.058, P = 0.811$), and chlorophyll autofluorescence per cell volume (1/µm³) in populations evolved under MEDs are not significantly different from the evolved control populations in the control environment (12.15 chlorophyll autofluorescence per cell volume (1/µm³) (±3.07 chlorophyll autofluorescence per cell volume (1/µm³)), mean of all population is 9.24 chlorophyll autofluorescence per cell volume (1/µm³) (±3.84 chlorophyll autofluorescence per cell volume (1/µm³)). This is consistent with populations having adapted to their environments, as they no longer show a standard sign of stress. For example, after evolution NED = 7 and NED = 8 populations have the lowest chlorophyll autofluorescence per cell volume (1/µm³), which is the opposite trend seen in the plastic response (Figure 5.8). Overall, the change in chlorophyll autofluorescence per cell volume (1/µm³) before and after evolution is significant (Figure 5.8; $F_{1,6}= 14.219, P = 0.01$).
Figure 5.8 Chlorophyll autofluorescence per cell volume (1/µm³) in populations of *C. reinhardtii* growing in environments with 0 to 8 environmental drivers. Black data points and bars represent the mean and standard deviation between regimes for each NED. Coloured points indicate the average growth rate between replicate population within each regime (± SD) (96 regimes in total, see Figure 7.11 for identity of regimes). Panel labels indicate the plastic response and evolved response to MEDs. Dashed line shows the proportion of chlorophyll autofluorescence per cell volume (1/µm³) of the control populations growing in the control environment.
5.4.5 Chlorophyll negative cells are capable of division

The growth rates of chlorophyll positive and chlorophyll negative cells were measured in order to better understand the relationship between chlorophyll content and population growth rate. I found that growth rates, following sorting of CP and CN populations (sorted by FACS), are not significantly different (Figure 5.9; \( F_{1, 53} = 0.47, P = 0.495 \)). However, growth rates of chlorophyll positive and chlorophyll negative cells within each sorted group are significantly different (Figure 5.9; \( F_{1, 53} = 3.53, P = 0.0008 \)), which is consistent with a positive relationship between chlorophyll content and the rate of cell division (Figure 5.6). Interestingly, growth rates of chlorophyll negative cells are positive. However, since there is no organic carbon source in the growth media, only cells capable of photosynthesising can go through several cell divisions. While stored nutrients may be sufficient for some growth, cells that do not eventually photosynthesis are not expected to survive in this experiment, and I do indeed find a negative relationship between the proportion of chlorophyll negative cells in a population and population growth rate before evolution (Figure 5.10a; \( R^2 = 0.25, P = < 0.0001 \)), and after evolution (Figure 5.10b; \( R^2 = 0.24, P = < 0.0001 \)). The results presented in Figure 5.9 demonstrate that lacking chlorophyll is not a heritable trait, since chlorophyll positive cells and chlorophyll negative cells reappear during population growth in both sorted groups.

5.5 Discussion

5.5.1 Cell size and proportion of chlorophyll positive cells are a good indication of adaptation to MEDs

Changes in cell size and chlorophyll content are commonly measured in order to quantify the impact of environmental change on microalgae (Prado et al., 2011,
2012; Schaum & Collins, 2014), and the loss of chlorophyll autofluorescence has been related to irreversible damage to cells (Pouneva, 1997; Prado et al., 2011). I found a positive relationship between the portion of chlorophyll positive cells and growth rate (Figure 5.6), and cell size and growth rate, before evolution. In this experiment, as NED increases the quality of the environment decreases, which appears to result in damage to *C. reinhardtii* cells, indicated by a decline in both cell size and proportion of chlorophyll positive cells as NED increases during the plastic response. After evolution, populations converge on the same proportion of chlorophyll positive cells and cell size, which is consistent with adaptive evolution to increase fitness in poor-quality environments by avoiding or repairing cellular damage (Figure 5.3, Figure 5.5).

Interestingly, although cell size decreases with increasing NED (Figure 5.3), chlorophyll autofluorescence per cell volume (1/µm³) increases with NED during the plastic response (Figure 5.8). As such, larger cells do not explain the increase in chlorophyll autofluorescence, as smaller cells in NED = 8 have more chlorophyll autofluorescence per cell volume (1/µm³) than the largest cells at NED levels 0 – 2. This may be due to larger cells avoiding self shading (Dubelaar & Jonker, 2000). Avoiding self-shading, known as the packaging effect, has been demonstrated by comparing different species of phytoplankton cells, where species with larger cells produce less chlorophyll per cell volume (Dubelaar & Jonker, 2000). It is not possible to infer from my data if cell size affects chlorophyll autofluorescence. However, no reports of self shading have been documented for *C. reinhardtii* (Rioboo et al. 2002, Prado et al. 2011). In addition, differences in cell size between phytoplankton species are greater than differences of cells size reported here. Sosik et al. (1989) consider the packaging effect to be a potential factor contributing to differences in the absorptive properties of pigments between the phytoplankton *Amphidinium carteri* and *Thalassiosira weissflogii*, which differ greatly in cell volume (350 – 1600 µm³, a 4.6 fold change). In addition, Collins & Bell (2004) describe a syndrome which evolved in some cells of *C. reinhardtii* after 1000 generations of growth at high CO₂, involving high rates of photosynthesis and
respiration, combined with higher chlorophyll content and reduced cell size. However, that was an evolutionary response to long-term growth in a high CO₂ environment, whereas I see this phenotype in the plastic response when NED is greater than 5 (Figure 5.8a). Following evolution, the relationship between chlorophyll autofluorescence (µm³) and NED breaks down (Figure 5.8b). However, similar to the findings of Collins & Bell (2004), cells evolved at elevated CO₂ show a decrease in chlorophyll autofluorescence per cell volume (1/µm³) following evolution (from 14.55 to 8.81 chlorophyll autofluorescence per cell volume (1/µm³)).
Figure 5.9 Boxplots show the growth rates subpopulations of *C. reinhardtii*, sorted using FACS flow cytometry (sorted groups labelled on the x axis). Growth rates of chlorophyll positive cells (grey filled boxplot) and chlorophyll negative cells (white filled boxplot) were calculated over two weeks (labelled at the top of each panel). At the end of week 1 the cultures were transferred to fresh media.
Figure 5.10 Correlation between proportion of chlorophyll negative cells and the rate of cell division before (a) and after evolution (b). Colour of points is scaled from red (low NED) to blue (high NED).
5.5.2 Uncoupling of growth rates and chlorophyll production following evolution

One explanation for the differences before and after evolution in cells size, chlorophyll content and the proportion of chlorophyll positive cells, is that mutations that improve the ability to survive long-term growth in poor quality environments have been fixed in the populations. In addition, this may lead to previously correlated traits becoming uncoupled after evolution and changes to traits associated with reducing cellular damage (cell growth and chlorophyll content) may be at the expense of improved growth rates. For example, the positive correlation between cell size and growth rates (Figure 5.4), and the positive correlation between the proportion of chlorophyll positive cells and growth rates (Figure 5.6), become uncoupled during the evolutionary response. Whereas the evolved populations in poor quality environments did not recover growth rates back to the growth rates of the evolved control (Figure 5.1, see also chapter 3), the proportion of chlorophyll positive cells and cell size of evolved populations have returned to a similar size and proportion of chlorophyll positive cells of the evolved control populations (Figure 5.3, Figure 5.5). This suggests that in poor quality environments populations evolve so as to make a larger investment in maintaining chlorophyll content and cell size, and these may improve the populations ability to survive long-term growth in poor quality environments. If *C. reinhardtii* must grow by photosynthesis in poor quality environments, then it is reasonable to suppose that evolving mechanisms to maintain functional chlorophyll would be under selection. This is consistent with data from Rioboo et al. (2002), who found that growth rates of *C. vulgaris* cells were lowest, but cell volume, dry weight and pigment content were highest, under the highest concentrations of herbicides isoproturon and terbutryn. The authors suggest that reproductive processes and cell size are uncoupled at high concentrations of herbicide. Uncoupling was detected following 96 hours of growth however, in the present study uncoupling appears to occur only after evolution to MEDs, suggesting
that there is a benefit to the breaking down the relationship between growth rates and chlorophyll content and cell size after evolution.

In addition, where adaptation is not obvious (due to small or negative direct responses to selection, Chapter 3; Figure 3.1), for example at NED = 7, this may be due to fixation of mutations that improve cell growth (size) and chlorophyll content in order to improve survival in a poor quality environment, whereas growth rates cannot improve to the same extent (Figure 5.1). This may be due to trade-offs associated with improving these traits and simultaneously decreasing cell division times, which from an energy and resource allocation standpoint, seems likely (Bennett & Lenski, 2007). These results suggest that growth rate measurements may be complemented or supported by additional physiological measurements that have a clear relationship with fitness because of known links with survival or key metabolic functions, such as photosynthesis. It is clear from the correlated responses to selection (Chapter 3; Figure 3.7), that populations evolving at high NED, including NED = 7, have evolved, however, this is not always apparent from the growth rates. This is supported by Bell (2013b) who suggests that correlated responses might reflect precise adaptation to ways of life that are only slightly different.

5.5.3 Chlorophyll negative cells are still viable

It was assumed that because chlorophyll negative cells cannot photosynthesis in the carbon free media, that the presence of chlorophyll negative cells is a result of cells coming to the end of the cell cycle. If this assumption is accurate, it would be expected that when sorted populations are transferred into fresh culture media, chlorophyll negative populations would be unable to grow and the number of populations should decline. Chlorophyll positive cells, on the other hand, are expected to grow, divide, and increase cell numbers in fresh culture media, and cells at the end of the life cycle would once again lose chlorophyll and the chlorophyll negative population will over time recover. Instead, I found that all sorted
populations were able to grow, with little difference in growth rate between sorted populations (Figure 5.9). In the present study, culture conditions support only photoautotrophic growth. However, it is clear that organic carbon is being released into the media, perhaps product of cell death, or compromised cell walls of *C. reinhardtii* cells. Blifernez-Klassen et al. (2012) found that under certain environmental conditions, such as CO₂-limiting conditions in the light, *C. reinhardtii* are able to digest exogenous cellulose. This is possible through the secretion of endo-β-1,4-glucanases. Thus, if evolved populations of *C. reinhardtii* are growing heterotrophically under stressful conditions, there is no need maintain costly chlorophyll pigments, and so cells become bleached. Although bleached cells are commonly associated with dead or dying cells (Prado *et al.*, 2011, 2012), bleached *C. reinhardtii* cells have also previously been described as an incidental response to selection (i.e. there is no relationship between chlorophyll bleaching and population fitness) in *C. reinhardtii* cultured as heterotrophs in the dark (Bell, 2013c), although I did not test the incidental response of phenotypes measured here.

### 5.5.4 Bet-hedging for long term growth

One possible explaining for a rise in viable, chlorophyll negative populations with increasing NED, is due to bet hedging. When growing in poor quality environments, dividing cells may produce higher quality cells by supplying a fraction of the cells with old cellular components and other fraction of cells with healthy components needed to survive (see appendix Figure 10.2) (WAVE *et al.*, 2006). This way the healthy cells have a greater chance of surviving in the poor quality environment (Zhang & Rainey, 2010; Bonduriansky *et al.*, 2012). Zhang & Rainey (2010) found that when grown in starvation conditions, the soil bacterium *Sinorhizobium meliloti* show bet-hedging strategies by dividing into two types of daughter cell, one suited to short-term and the other to long-term starvation. This is due to asymmetric allocation of the lipid-like poly-3-hydroxybutyrate (PHB), which is stored by *S. meliloti* and can be used to survive long-term starvation. Similarly, *C. reinhardtii* in the present
study are growing in carbon free media, in poor quality environments and so asymmetric allocation of chloroplast may allow a proportion of the cells to survive long-term growth with MEDs. This is supported by the plastic response before evolution (Figure 5.7), demonstrating that the proportion of chlorophyll negative cells increases with increasing NED, which may be associated with a greater chance of having either reduced phosphate, general nutrients or both in the regime. Interestingly, when NED is greater than 4, the proportion of chlorophyll negative cells reaches a threshold with no additional effect of NED, and this may due to similarity between environments, i.e. more environments have reduced phosphate and nutrients as NED increases.

5.5.5 Future work

The presence of chlorophyll negative subpopulations may be more complicated than simple bet hedging. Preliminary data collected from the mass spectrometer of the sorted chlorophyll positive and chlorophyll negative samples demonstrates significant up-regulation of proteins in chlorophyll negative cells (Appendix 4, section 10.1.2, page 234). One example is the up regulation of ribosomal proteins in chlorophyll negative populations that allow organisms to respond rapidly changing environmental conditions (Condon et al., 1995; Elena & Lenski, 2003). This is just one example, and preliminary data suggest that mass spectrometry proteomics is a fruitful avenue to explore the expression of proteins of subpopulation growing in a given environment, at a given time point. In addition, mass spectrometry is widely used to describe protein interactions and pathways, and measure changes in protein abundances in different environmental conditions (Han et al. 2008). Future work should build on preliminary mass spectrometry analysis in order to determine the molecular underpinnings of the differences between chlorophyll negative populations. This has been completed successfully to understand the evolution of salt tolerance in C. reinhardtii (Perrineau et al., 2014). Specifically, identification of the pigment responsible for PE autofluorescence would be valuable as all chlorophyll
negative populations are autofluorescent in the PE channel (Appendix 4; 10.1.1; Figure 10.4), and as such this pigment may be useful for understanding and predicting the outcome of evolution of photosynthetic algae (such as C. reinhardtii) under MEDs.

5.6 Conclusions

The similarities between the plastic and evolved response to MEDs suggests that although the molecular underpinnings in the response to MEDs may be complex, there remains a general order to how phenotype changes in C. reinhardtii before and after evolution. There is a general trend in how chlorophyll content and cell size change with NED, before and after evolution. However, understanding the physiological responses to MEDs is complicated, and the data presented here suggest that trade-offs between improving growth rates or repairing cellular damage such as the loss of chlorophyll are important. In this case, phenotypic changes under MEDs must be studied empirically. I suggest that future experiments should make use of proteomic methods in order to better characterise phenotypic changes in response to MEDs. The ability to predict the physiological changes of ecologically important organisms will allow ecologists to gain a better picture of how MEDs may indirectly effect the whole community.
Chapter 6 – General discussion and future directions

6.1.1 Significance statement

This is a novel contribution to the field of multiple driver experiments, in that it is the first experiment that, first, can disentangle the effects of the number of drivers and their identity, and second, that directly compares plastic and evolutionary responses to growth in MEDs. Quantifying the effects of MEDs is central to our understanding of the impact of future global change, as many aspects of the environment are predicted to change simultaneously. The importance of MEDs research is evident in the rise of MED investigations, yet few studies have been designed to disentangle the effects of MEDs (Ormerod et al., 2010), and at present no other study has investigated the short- or long-term effect of more than three environmental drivers. In the present study, a model microalga was used in order to explore the effects of NED on the short- and long-term growth response, and disentangle the effects between the number and identity of environmental drivers. Through the use of a simple model, I show that knowing the effect of individual environmental drivers, especially the dominant environmental driver with the largest effect, explains the plastic response in growth to multiple environmental drivers (chapter 2). I found that the plastic response to MEDs is able to predict the strength of selection, and thus the magnitude of the direct response to selection in the same environments (chapter 3). In addition, changes in chlorophyll content and cell size demonstrate that following evolution in MED environments, populations are able to restore the phenotypes measured here back to phenotypes of the control populations (chapter 5). Finally, taken together, the data show that the number of drivers present in an environment affects patterns of local adaptation (chapter 4). The data presented in this thesis provide unique insight into the long-term consequences of MEDs on microbial populations.
6.1.2 Are organismal responses to MEDs predictable?

The data collected in chapter 2 demonstrate that single environmental drivers are informative for predicting the plastic effects on growth when in combination. In particular the dominant environmental driver with the single largest effect alone largely drives changes in growth rates between multiple driver environments and a control environment (Brennan & Collins 2015). NED is a good estimate of the expected growth rate when NED is high, and this is due to high NED environments having an increased chance of containing at least one dominant environmental driver. Thus, when the individual effect of environmental drivers is unknown, the outcome of growth is still predictable from the NED. In addition, an important contribution of this investigation is that it highlights the utility of studies investigating the outcome of single environmental drivers. Results from chapter 3 and chapter 5 confirm that short-term acclimation responses to MEDs can provide predictions on the magnitude of the direct response to selection, by allowing us to use the plastic response as an estimate of the strength of selection.

Predicting how ecological systems will be impacted by MEDs under future global change is arguably one of the most important goals in ecological research (Clark et al., 2001; Evans et al., 2012; Harvey et al., 2013). Predictions on how MEDs effect communities or populations of interest are commonly based on ecological models (Boyd, 2002; Woodward et al., 2010; Evans, 2012). However, the best way to verify the assumptions that underlie models and theoretical discussions is to test them in the lab (Benton, 2012; Evans, 2012). Both marine and freshwater MEDs investigations typically use two or three environmental drivers in MEDs experiments, which is informative for understanding precise physiological mechanisms involved in the response to MEDs, and specific interactions between environmental drivers (Dupon & Pörtner, 2013), but lack predictive power for how organisms will respond when even more drivers co-occur. The case study presented in chapter 2 (Figure 2.7), demonstrates that interactions are idiosyncratic when NED is low, and the effects of
two and three environmental drivers must be empirically measured. This is consistent with meta-analyses which demonstrate that interactions between pairs are not simply additive; Jackson et al. (2015) found that in freshwater systems, the majority of paired driver interactions are antagonistic and many were reversible (a reversal occurs when the net impact of two stressors is in the opposite direction). Similarly, in marine systems, Crain et al. (2008) found that the majority of interactions were antagonistic or synergistic. Generally, the extent to which synergistic and antagonistic interactions deviate from additive effect cannot be predicted, making predictions about organismal responses to MEDs difficult. There is an urgent need for general tests of the assumptions underlying theories and models of organismal responses to MEDs (Benton et al., 2007).

Measuring and understanding the outcome and the mechanisms in organismal responses to MEDs is demanding and even if complex experiments with many drivers are designed, the assumption that all interactions are unpredictable implies that unless we are able to know the drivers present in real environments with high certainty, we cannot design useful experiments. In addition, it is not practical to carry out MED experiments for the many species of phytoplankton or microbes that have the potential to respond to global change. These considerations highlight the need for a general framework to predict, at some level, the effects of MEDs on key groups of organisms.

6.1.3 Are long-term MEDs investigations necessary to understand organismal responses to MEDs?

Understanding the relationship between adaptation and the plastic response to poor quality environments is important for understanding how populations evolve in multiple driver environments (Ghalambor et al., 2007; Chevin et al., 2010a). Several selection experiments have demonstrated that the short-term effect of environmental
changes on organisms cannot explain the evolutionary response to those same environmental changes (Collins & Bell 2004; Mueller et al. 2010; Crawfurd et al. 2011; Lohbeck et al. 2012a). Yet, scaling up MEDs studies in time is rare (but see, Schlüter et al. (2014); Tatters et al. (2013)). However many argue that organismal responses to MEDs on timescales that are relevant for human activities cannot be predicted without considering the effect of evolution (Bell & Collins, 2008; Gienapp et al., 2008; Collins et al., 2013).

The focal experiment involved 95 transfers (~350 generations). I found that there are striking similarities between the plastic and evolved growth responses and much of the variation in the evolved growth response to selection is explained by the short-term response. This is important, as selection experiments are demanding, time consuming and sometimes impossible for many non-model organisms (Elena & Lenski, 2003). Given that the present experiment demonstrates that short-term MEDs experiments are a good indication of the trend in the long-term response, long-term experiments are not necessary for predicting the outcome of long-term growth under MEDs. However, they are informative for understanding evolutionary processes. In particular, further examination can be carried out to understand the molecular underpinnings of evolutionary processes involved in adapting to MEDs that may not be predictable from the plastic response.

6.1.4 Can adaptation keep up with the MEDs organisms are experiencing under global change?

In chapter 3, I demonstrated that as the strength of selection increases with NED, the response to selection increases, until NED = 7, where the response to selection drops. At NED = 8, all populations went extinct under the usual transfer conditions, and only persist when number of cells transferred (population size) is increased by the experimenter. This indicates that populations are unable to persist when NED is
greater than seven and population sizes are low. In addition, I found that growth rates do not recover fully. Populations evolved in MED environments still grow more slowly in their selection environments than the evolved control populations in the control environment. However, is it clear that populations have evolved and this is evident from both the direct (in high NED environments) and correlated (in all environments) responses to selection. In chapter 4, I found that populations evolved in NED =1 and NED = 5 show differences in the degree of local adaptation is consistent with populations in NED = 5 environments being under stronger selection. Growth rates of evolved NED = 1 and evolved NED = 5 populations are significantly higher than the control environment in a novel stressful environment that is lethal to control populations, suggesting that future populations that evolve under MEDs will be better able to tolerate future environmental changes than populations that have evolved in an unchanging environment.

The data collected here indicates that when population sizes are low, populations are more vulnerable (Willi et al., 2006) and these population may be more severely impacted when the NED is high and environmental quality is poor. Further work on sensitive species, such as small populations, may aid in the identification of populations that cannot adapt and in understanding the reasons why this is the case (Lynch & Gabriel, 1987).

6.1.5 The future of MEDs experiments

The aim of my thesis is to uncover the effects of increasing NED on plastic and evolutionary changes to growth and other key traits for primary producers. Following evolution, I found that the short-term changes in chlorophyll content and cell size are largely reversed, so that the evolved populations from the MED environments phenotypically resemble the evolved control population in the control environment, at least for these traits. This may indicate that populations growing under MEDs may be able to maintain somewhat normal functioning, but with reduced growth rates.
Changes in growth rate, chlorophyll content and cell size are important to consider as phytoplankton are the base of the aquatic food web and phenotypic changes may have consequences to higher trophic levels in marine and freshwater systems (Karentz & Bosch, 2001; Davies et al., 2011). This investigation is a good first step in understanding the outcome of short and long term growth under MEDs. Like many models, this experiment is designed to be generalisable at some level, and although I wholly expect that different organisms and/or environmental drivers will have different specific responses (from the responses observed in this investigation), the general pattern of reduced overall fitness and an increase in the strength of selection as NED increases is expected. Predictive power could be further increased by integrating other studies aimed at gaining a mechanistic understanding of interactions between specific drivers.

Figure 6.1 illustrates the current position of research that aims to predict biotic responses to future global change. There are many studies that investigate the effects of up to three environmental drivers over short timescales (Crain et al., 2008), and fewer long-term studies (Collins et al., 2013). There are also investigations that tackle the effects of single environmental drivers on whole communities over evolutionary time scales (Kim et al., 2012). The ultimate goal is to investigate the impact of a realistic number of simultaneous environmental drivers, on whole communities and over evolutionary timescales.
Figure 6.1 Illustration of current state of knowledge and future goals of global change research. Coloured circles show the current position of research investigating the impacts of single environmental drivers on single genotypes/species (green), single environmental drivers on multiple genotypes/species (purple) and MEDs on single genotypes (blue). Dashed arrow and faded filled circles indicates that there are some examples of long-term studies, on evolutionary relevant time-scales. Red closed circle indicates the position of the present study investigating both the evolved and plastic response to up to eight environmental drivers. Star indicates the future goals of global change research, which is to investigate the impact of a realistic number of simultaneous environmental drivers, on whole communities and over evolutionary timescales.
6.1.6 Scaling up to whole communities

Predicting which aspects of the environment might change and what effect this will have on organisms is informative. However, it does nothing to tackle questions such as which organisms will make up these communities and how will they interact following alterations to the environment (Low-Décarie et al., 2011). For instance, Winder & Schindler (2004), found that elevated temperatures disrupt the trophic interactions between phytoplankton and zooplankton due to differences in thermal tolerance between species. Kim et al. (2012) found that peatland bacterial and methanogen communities release significantly more carbon when the temperature is increased by 3°C for three growing seasons (3 years). In addition, interactions between soil depth and temperature indicate that changes in temperature will likely alter community structure and function in peatlands. However, laboratory experiments with multiple genotypes or species are arduous and time-consuming, and are normally studied over short time-scales (but see Lawrence et al. 2012), and indeed long-term experiments are on-going (D. Lawrence and S. Collins, personal communication). Nevertheless, evolutionary responses are required to fully appreciate how community dynamics will change following evolution under MEDs.

I propose that the next step in understanding the evolutionary responses to MEDs is to use microcosm experiments, where the short- and long-term effects of MEDs are investigated using multiple genotypes or species – and this can be conducted on small spatial scales in the laboratory using multi-well plates (Lawrence et al., 2012) or on larger spatial scales using artificial lakes (Moss et al., 2003; Hansson et al., 2012). Microcosm experiments have been used to study general concepts in population biology, community ecology and evolutionary biology such as, predator–prey interactions, behavioural responses, physiological responses, evolutionary responses, population dynamics, competition and succession (see review by Altermatt et al. 2015). Short-term microcosm experiments are already being conducted in freshwater systems in order to examine the relationships between the
effect of MEDs (up to three simultaneous environmental drivers), and species invasion rates (Griffiths et al., 2015), and the effect on trophic interactions (Hansson et al., 2012). Although artificial, microcosm experiments can uncover how MEDs will effect single organisms, or a group of organisms, in a more realistic scenarios than the laboratory (Altermatt et al. 2015). MED microcosm experiments will enable investigators to increase the number of taxa in a community so that more ecologically realistic responses to MEDs can be studied.

The next logical step in linking the results obtained in the lab and the natural world that includes competitors and grazers, is to study the effect of MEDs in situ. Mesocosm experiments offer a large degree of control over environmental drivers (such as elevated CO$_2$), but also allow investigators to unravel the effects of environmental drivers on complex communities in more natural habitats than can be built in the lab (Engel et al., 2005; Ventura et al., 2008; Troedsson et al., 2012; Riebesell et al., 2013). Though, mesocosm experiments are essentially MED experiments, as organisms are exposed to natural environmental conditions (in a closed system), it must be possible to measure and disentangle the effects of MEDs in order for predictions to be made on the effects of increasing NED on focal organisms. Only in the past year have evolutionary responses been measured using a marine diatom in a mesocosm experiment (see Scheinin et al. 2015), and with the replication required, it might be some years until we are able to accurately measure the long-term effects of MEDs, with a practical number of environmental divers required to make predictions. However, it is easy to imagine, as techniques are improved that there will be more scope to investigate the outcome of MEDs in situ.

### 6.1.7 How realistic do MED experiments have to be?

There is some argument that laboratory investigations are too artificial to be compared with the natural world (see review by Jessup et al. 2004). Carpenter (1996) cites problems such as large differences in spatial scale of artificial experiments
relative to the ecological system that they aim to represent, and inconsistencies between results gathered in the lab and results collected in the field. However, I agree with researchers who argue that there is a need for model systems to unravel the complexities of the natural world (Fraser & Keddy, 1997; Elena & Lenski, 2003; Drake & Kramer, 2012). For example, they provide insights into hidden worlds such as soil food webs (Daehler & Strong, 1996), allow us to directly study key processes like adaptation (Buckling et al., 2009), and test ecological and evolutionary theory with replicate populations (Benton et al., 2007). Jessup et al. (2005) sums up the benefits of microbial experimental systems for providing a necessary link between theoretical models and the natural world:

“These advantages allow ecologists to dissect the complexity of nature into its component parts, analyzing each part’s role in isolation and then in combination.”

Arguably, the same qualities that make theoretical models so valuable in ecological and evolutionary research are also beneficial to experimental systems. It is suggested that the best models are general so that they can be applied to many populations and scenarios, realistic so that they can be applied to real biological systems (as opposed to mathematical objects), and accurate so that the evidence can be relied on to guide future investigations (Evans, 2012). For example, Fisher’s geometric model of adaptation captures the essence of the process of adaptation and by virtue of its simplicity it allows assumptions to be applied to a wide range of real-life biological systems or environmental conditions. However, it forces concrete biological understanding of these systems in order for predictions to be precise and outcomes realistic. It is a testament to the model that so many researchers reference this piece of work in their research today. Manipulation experiments and experimental evolution are simple compared to the natural world however, experiments are powerful in that are able to produce generalisable results that disentangle the effects of environmental drivers, and link them directly to responses (Elena & Lenski, 2003; Scheinin et al., 2015).
There is hopeful evidence indicating that mesocosm experiments accurately capture the effects of large scale field investigations (Tran et al., 2015). To date there are no publications that compare the results of mesocosm experiments to the results gathered from lab experiments, but work is currently ongoing (Lawrence and Collins, personal communication). Investigations such as these are important to test how the results obtained from laboratory experiments compare with more realistic experiments. If the outcome is that we cannot extrapolate from laboratory experiments, investigators must change focus away from artificial laboratory experiments to more natural experiments such as natural microcosms (Srivastava et al., 2004) and mesocosm experiments. However, a trade-off must be made, as the closer to the natural world we get, the more confidence we have that our results apply to natural organisms, but the more difficult it becomes to interpret the results, and disentangle the effects of environmental variables (Srivastava et al., 2004; Evans et al., 2012).

6.1.8 Communication between freshwater and marine biologists

There is very little communication between freshwater and marine biology, yet both have set out similar goals for the future of multiple driver research in both marine (Andersson et al., 2015), and freshwater (Hering et al., 2014) ecosystems. Marine and freshwater manipulations often focus on phytoplankton as they are well-suited to manipulation experiments due to the small amount of space required, short generation times (Collins, 2012), manageable number of well-defined traits (Litchman & Klausmeier, 2008), and are ecologically important in aquatic ecosystems (Häder et al., 1998; Beardall & Raven, 2004). Environmental drivers predicted under future global change will effect both marine and freshwater ecosystems and there are urgent calls for investigators to not only measure the effect of MEDs, but to disentangle their effects (Benton et al., 2007; Ormerod et al., 2010). There is a need for a general framework that is applicable to both marine and
freshwater phytoplankton. These facts suggest that more dialogue between the two disciplines would be beneficial for a collaborative effort to fill in the knowledge gaps of multiple driver research (Hering et al., 2014; Andersson et al., 2015).

There is a long history of MED research in freshwater biology (Carpenter et al., 1987; Schindler et al., 1996; Yan et al., 1996) and as a result, most theory of the effects of multiple environmental drivers on aquatic systems was founded in freshwater biology (Boyd & Hutchins, 2012). Despite similarities in organismal function between freshwater and marine phytoplankton (and presumably other taxa), there are few links between freshwater and marine multiple driver studies. Boyd & Hutchins (2012) suggest that communication between freshwater and marine biologists would benefit marine investigations. For example, one of the most developed areas of multiple driver research in marine biology is the impact of ocean acidification on marine phytoplankton. This has been studied in the lab (Riebesell et al., 2007) and in situ (Engel et al., 2005), and is one of the first examples of investigating the evolutionary outcomes of environmental drivers predicted under future global changes (Lohbeck et al., 2012). However, these experiments do not have the power to disentangle the effects of reduced pH and elevated CO₂. Predictions on the effects of combinations of environmental drivers can only be made by knowing their effect individually, and this is supported by traditional models used to predict the outcome of chemical mixtures (Bliss, 1939), scenario experiments (Boyd et al. 2015b), and here in the present study (Brennan & Collins 2015).

More recently, marine biologists and oceanographers have begun to respond to the need to study the impact of environmental drivers over evolutionary relevant timescales (see review by Reusch & Boyd (2013) and Collins et al. 2014)). Yet freshwater investigations of single and multiple environmental drivers are concerned with the short-term acclimation responses (apart from studies that use freshwater model organisms such as Chlamydomonas reinhardtii to better understand
evolutionary processes). However, understanding how evolution under MEDs will shape populations, will improve predictions on the impact of global change on natural populations, and result in better-informed theory and, hopefully, policy. There is clearly something to gain from better communication between both disciplines and there are methods and tools in place for aquatic ecology to move closer to the future goals outlined in Figure 6.1.

### 6.1.9 Future directions

Although much of this discussion is framed in the context of aquatic ecology, the effect of multiple environmental changes in the context of global change is a challenge faced by many biologists. Several experimental approaches could be considered depending on the question at hand. If the aim of a study is to understand the effect of MEDs and attempt to disentangle the effects of the number of environmental changes and the identity of the environmental changes, then this requires a high level of replication using different combinations of environmental changes (as presented here). However, without the use of model organisms, the level of replication within each level of NED achieved here would not be possible. As a consequence, many experiments are often limited to a combination of a few different drivers and whilst these are useful for understanding the specific effects of multiple drivers tested, predictions cannot be made on the effect of these changes at higher levels of NED.

My work suggests that studies aimed at predicting population responses to global change that are limited by space and time, should measure responses to many individual environmental drivers alone, sampling from a wide distribution of drivers and driver intensities in order to identify dominant drivers. Unlike other models (additive, multiplicative) where each environmental driver will contribute equally to the combined effect on the organism, the comparative model only requires that the dominant environmental drivers be identified in the investigation in order for
predictions to be made (Folt & Chen, 1999; Jackson et al., 2015). This provides encouraging evidence that continuing to build our understanding of how single drivers affect population growth is a useful way to proceed, especially in cases where large experiments are impossible. This point of view is supported by investigations using scenario based experimental designs (Boyd et al. 2015b).

On the other hand, studies that are interested in particular environmental changes and their interactions should continue to measure these interactions empirically, as the combined effect of a few environmental drivers cannot be predicted (Byrne & Przeslawski, 2013; Hering et al., 2014; Jackson et al., 2015). In addition, proteomics will reveal the mode of action of different environmental drivers by measuring changes in gene expression in response to environmental drivers. Proteomics will enable the identification of pathways and target sites of environmental drivers and this will allow the investigator to distinguish between driver interactions that effect different target sites and pathways, and driver interactions that increase the intensity of the effect by acting on the same target site. This is certainly a method that could be used to better understand the effects of MEDs on the ancestor and the evolved populations in the present study, and may help explain the remainder of the variation in the plastic growth responses that cannot be explained by comparative model (36% of the variation in growth is unexplained by the comparative, additive and multiplicative model). Using proteomic data, up or down regulated proteins are recorded in response to each regime, including biological pathways and protein interactions so that the mode of action in response to each driver can be explored in more detail (Aebersold & Mann, 2003; Hoffmann & Willi, 2008; Vaudel et al., 2014). Using time-of-flight mass spectrometry a metabolic profile was produced for C. reinhardtii in response to different nutrient limited environments (iron, phosphorus), and numerous changes in metabolite levels were measured (Bölling & Fiehn, 2005). This type of detailed analysis is very powerful for filling in gaps in the knowledge of an organism’s response to environmental drivers and combinations of environmental drivers.
7 Appendix 1

Appendix for Chapter 2

7.1.1 NED drives changes in the number of cells (d⁻¹)

In the present experiment the rate of cell division (d⁻¹) was used to investigate the effect of NED on growth rate. However, we can draw the same conclusions from the data when the same analysis is performed on the change in the number of cells (d⁻¹) (using Equation 2-2); NED has a significant effect of the change in the number of cells (d⁻¹) (Figure 7.8; $F_{1,94} = 41.034, P < 0.000$). The fastest growing populations (including populations growing in the control environment) achieved a population cell density of $4 \times 10^6$ cells per millimetre following 120 h of growth. The average population cell density was $1.6 \times 10^6$. The rate of cell division (d⁻¹) was used in the present study as this metric accounts for the differences in initial population size in each culture, and reports a biologically meaningful measure that can be compared across test environments, despite differences in the shape of growth curves.
Figure 7.1 The wavelength of the emissions from the UV lamp (UVM-57) used.
Figure 7.2 The average rate of cell division of *C. reinhardtii* growing in the single environmental drivers. Solid circles show the average value (± standard deviation) over all regimes for each NED.
Continues in Figure 7.4, 7.5 and 7.6.

Figure 7.3  Population growth curves of C. reinhardtii under 0 to 8 environmental drivers. Cell counts were measured every 24 h for 120 h in each regime. Coloured points and lines indicate average of each technical replicate (± standard deviation) for each regime. Each panel is labelled with the number and identity of the environmental drivers for each regime respectively. Figure is split into four sections so that panels are large enough to read and continues in Figure 7.4, 7.5 and 7.6.
Figure 7.4: Population growth curves of *C. reinhardtii* under 0 to 8 environmental drivers.
Figure 7.5: Population growth curves of *C. reinhardtii* under 0 to 8 environmental drivers.
Figure 7.6: Population growth curves of *C. reinhardtii* under 0 to 8 environmental drivers.
Figure 7.7 Cell density of *C. reinhardtii* after 120h of growth in regimes containing 0 to 8 environmental drivers. Box plots show cell densities over three replicates for each regime. The solid bands denote the median value, the bottom and top of the each box represent the 1st and 3rd quartile of the data respectively. Whiskers indicate the positions of the lowest and highest value that is within 1.5 times the interquartile range. Dashed lines show the highest and lowest cell density between all 96 regimes. NED is shown here by the colour of each box plot and the identity of the environmental drivers within each regime is labelled on the x axis. Drivers are: CO$_2$, CO$_2$ enrichment; Temp, elevated temperature; LI, reduced light intensity; pH, reduced pH; P, phosphate starvation; Herb, herbicide; ND, general nutrient depletion; UV, UV radiation.
Figure 7.8 Population growth of *C. reinhardtii* under 0 to 8 environmental drivers.

Population growth is measured as the change in number of cells (d−1) (a) Black data points and bars represent the mean and standard deviation between regimes for each NED. See Table 2-3 for regimes. Coloured points indicate the average growth rates among replicate population grown in the same regimes (± SD) (96 regimes in total, see Figure 7.11 for identity of regimes). (b)-(d) Population growth rates (mean and standard deviation) predicted by a model (white triangles) alongside measured values (black circles), followed by goodness-of fit, for three models. (b) Comparative model (R² = 0.61, P < .0001). (c) Multiplicative model (R² = 0.60, P < .0001). (d) Additive model (R² = 0.41, P < .0001); extinction is predicted in environments with >3 changes (red dashed line).
Figure 7.9 Effect of sampling from a finite number of environments. Blue circles show the results of simulating the expected population growth rate (± standard deviation) at each number of environmental drivers given a comparative model and the growth effects of single drivers measured in the experiment, where a finite number of possible drivers (8) exist to choose from. In these cases, each driver can only be chosen once per regime, such that each possible growth effect can only be sampled once per regime, and environments thus become more similar as the NED increases. Orange circles show the results of simulating the expected population growth rate (± standard deviation) for increasing numbers of environmental drivers given a comparative model and the growth effects of single drivers measured in the experiment, where an infinite number of possible drivers exist to choose from. In these cases, the same growth effect can be sampled multiple times at a given NED and environments are not constrained to become more similar as the NED increases. All points are the results of 10000 iterations of the simulation.
Figure 7.10 Cartoon of the effects of multiple drivers on organismal function using an environmental tolerance curve. Some aspect of organismal function, such as growth, is plotted as a function of the environment experienced by the organism, with the value of “Environment” being determined by multiple environmental drivers. Initially, organismal function is high (solid black circle). When single drivers change, organismal function changes (patterned filled circles). While the effect of each driver may be unknown, as more and more drivers occur, the likelihood of at least one driver or driver interaction having a large detrimental effect on organismal function increases. This thought experiment does not require that the population be in its optimal environment, just that among the environments sampled, the control environment be one where organismal function is high. Figure 2.1 shows that this is the case here, since the control environment is among the “best” environments available in this experiment. Note that this cartoon is meant to illustrate the thought process, and not to indicate the quantitative effects of the specific environments used in this experiment. Please refer Figure 2.1 for quantitative data.
Figure 7.11 The identity of regimes within each NED level shown in figure 2.1, figure 2.2a, figure 5.1, figure 5.2, figure 5.3, figure 5.5, figure 5.7, figure 5.8, figure 7.8a, figure 7.14 and figure 10.5.
Figure 7.12 The identity of regimes (without CO$_2$) within each NED level shown in figure 2.9.
Figure 7.13 The growth rate of *C. reinhardtii* after 120h of growth in regimes containing 0 to 5 environmental drivers, similar to those environmental drivers in Boyd et al. (2015b). The identity of the environmental drivers within each regime is indicted by each shape and identified within the label. Drivers are: CO2, CO2 enrichment; Temp, elevated temperature; LI, reduced light intensity; pH, reduced pH; UV, UV radiation.
Figure 7.14 Population extinction risks of *C. reinhardtii* at different numbers of environmental drivers. Ratio of extinction risks of populations relative to the risk of extinction in the control environment (NED = 0). Solid circles show the average value (± standard deviation) over all regimes for each NED. Coloured points represent mean (± standard deviation) ratio of extinction risks for 3 replicate populations for each regime for a given number of environmental drivers (96 regimes total, see Figure 7.11 for identity of regimes).
Table 7-1 A comparison of the percentage variation explained by each source within the mixed effects model

<table>
<thead>
<tr>
<th>Source</th>
<th>Variation (%)</th>
<th>Mixed Effects Model Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate</td>
<td>1.39</td>
<td>Random</td>
</tr>
<tr>
<td>Regime</td>
<td>32.04</td>
<td>Random</td>
</tr>
<tr>
<td>Within replicate</td>
<td>19.64</td>
<td>Random</td>
</tr>
<tr>
<td>Overlap</td>
<td>10.34</td>
<td>Fixed</td>
</tr>
<tr>
<td>NED</td>
<td>36.59 **</td>
<td>Fixed</td>
</tr>
</tbody>
</table>

Number of observations: 576, groups: replicate, 288; regime, 96; Overlap, 52; NED, 9
8 Appendix 2

Appendix for Chapter 3

8.1.1 Specific responses of regimes within NED levels

At low NED, environments with NED = 1 to 2 have a strong positive correlation between the average population growth rate of the MED-evolved populations and the strength of selection, explaining 32% of the variation (Figure 8.3; \( NED = 1 \); \( R^2 = 0.32, P < 0.0001 \)), and 36% of the variation respectively (Figure 8.4; \( NED = 2 \); \( R^2 = 0.36, P < 0.0001 \)). The trend of a higher strength of selection producing a larger direct response in the MED-evolved populations is more apparent as NED increases. At NED = 3 the correlation between the growth rate of the MED-evolved populations and the evolved control populations is weaker (Figure 8.5; \( NED = 3 \); \( R^2 = 0.15, P < 0.0001 \)).

The correlation between the growth rates of the MED-evolved populations and the strength of selection remains strong at NED = 4 (Figure 8.6; \( NED = 4 \); \( R^2 = 0.61, P < 0.0001 \)) and NED = 5 (Figure 8.7; \( NED = 5 \); \( R^2 = 0.43, P < 0.0001 \)). This is due to two clusters of regimes - one cluster showing high growth rates that are the result of evolving in high-quality environments, and so have small direct responses to selection, and a second cluster with high growth rates as a result of a large positive direct responses due to stronger selection caused by evolving in low-quality environments. Although the correlation between the strength of selection and the growth rates of the MED-evolved populations is high at NED = 6, this is caused by a single regime with a low strength of selection and high growth rates (\( CO_2/Temp/pH/LI/UV/ND \)). The strength of selection in all other regimes is high but the direct response varies between regimes (Figure 8.8; \( NED = 6 \); \( R^2 = 0.45, P < 0.0001 \)). The strength of selection is high in all regimes at NED = 7, but adaptation and growth rates are limited. Growth rates are not significantly higher than the
evolved control populations in the majority of regimes within NED = 7 (Figure 8.9; $NED = 7$; $R^2 = 0.022$, $P < 0.0001$).

There are negative direct responses in some regimes, which is consistent with weak or no selection pressure at low NED (1-3). However, negative direct responses also occur in some regimes where the strength of selection is high. In NED = 4, on average all regimes of the MED-evolved populations grow better, or just as well as the evolved control populations in the same environment, with two exceptions (CO$_2$/pH/UV/ND) and (CO$_2$/Temp/pH/LI). At NED = 5, two MED-evolved populations have lower growth rates than the evolved control populations in the selection environment (CO$_2$/pH/UV/ND/LI) and (pH/UV/ND/LI/herb). Finally, at NED = 6 all regimes grow as well or better than the control in the selection environments with the exception of (CO$_2$/P/LI/herb/ND/pH). These environments have general nutrient depletion and reduced phosphate in common, which may limit maximum growth rate possible in this selection environment.
Figure 8.1 The average rate of cell division (d⁻¹) of the evolved control replicate populations (black fill circles) in the single environmental drivers (± SD).
Figure 8.2 The proportion regimes (1 to 7 NED), where the MED-evolved populations have a significant direct response to selection. Significance is calculated as the proportion of regimes where the direct response of each evolved population, is greater than the third quartile of the direct response of the MED-evolved populations growing NED = 1 (see Figure 3.1).
Figure 8.3 There is a negative correlation between the strength of selection of the selection environments and the growth rate of evolved populations. Coloured filled circles show the average growth rate between evolved replicate populations within each regime ($\pm 1$ standard deviation), within NED = 1. The 1:1 dashed line indicates the expected growth rate if the MED-evolved populations growth rates were equal to the growth rates of the evolved control. The dotted line indicates the growth rate of the evolved control populations in the control environment.
Figure 8.4 There is a negative correlation between the strength of selection of the selection environments and the growth rate of evolved populations. Coloured filled circles show the average growth rate between evolved replicate populations within each regime (± 1 standard deviation), within NED = 2. The 1:1 dashed line indicates the expected growth rate if the MED-evolved populations growth rates were equal to the growth rates of the evolved control. The dotted line indicates the growth rate of the evolved control populations in the control environment.
Figure 8.5 There is a negative correlation between the strength of selection of the selection environments and the growth rate of evolved populations. Coloured filled circles show the average growth rate between evolved replicate populations within each regime (± 1 standard deviation), within NED = 3. The 1:1 dashed line indicates the expected growth rate if the MED-evolved populations growth rates were equal to the growth rates of the evolved control. The dotted line indicates the growth rate of the evolved control populations in the control environment.
Figure 8.6 There is a negative correlation between the strength of selection of the selection environments and the growth rate of evolved populations. Coloured filled circles show the average growth rate between evolved replicate populations within each regime (± 1 standard deviation), within NED = 4. The 1:1 dashed line indicates the expected growth rate if the MED-evolved populations growth rates were equal to the growth rates of the evolved control. The dotted line indicates the growth rate of the evolved control populations in the control environment.
Figure 8.7 There is a negative correlation between the strength of selection of the selection environments and the growth rate of evolved populations. Coloured filled circles show the average growth rate between evolved replicate populations within each regime (± 1 standard deviation), within NED = 5. The 1:1 dashed line indicates the expected growth rate if the MED-evolved populations growth rates were equal to the growth rates of the evolved control. The dotted line indicates the growth rate of the evolved control populations in the control environment.
Figure 8.8 There is a negative correlation between the strength of selection of the selection environments and the growth rate of evolved populations. Coloured filled circles show the average growth rate between evolved replicate populations within each regime (± 1 standard deviation), within NED = 6. The 1:1 dashed line indicates the expected growth rate if the MED-evolved populations growth rates were equal to the growth rates of the evolved control. The dotted line indicates the growth rate of the evolved control populations in the control environment.
Figure 8.9 There is a negative correlation between the strength of selection of the selection environments and the growth rate of evolved populations. Coloured filled circles show the average growth rate between evolved replicate populations within each regime (± 1 standard deviation), within NED = 7. The 1:1 dashed line indicates the expected growth rate if the MED-evolved populations growth rates were equal to the growth rates of the evolved control. The dotted line indicates the growth rate of the evolved control populations in the control environment.
Figure 8.10 Number of cells per millimetre of evolved control populations (red) and MED-evolved populations (blue) after 72 h of growth in selection environments containing between 0 and 8 NED. Coloured point points show the average number of divisions between regimes from evolved control populations (red) and MED-evolved populations (blue). The average of the evolved control populations, assayed in the control environment (NED = 0) is shown here by a dashed line.
Figure 8.11 The waiting time for a mutation to arise within the populations selected in environments with between 1 and 8 environmental drivers. The black bands denote the median value, the bottom and top of each box represent the 1st and 3rd quartile of the data respectively. The ‘whiskers’ extending from the boxes indicate the positions of the lowest and highest values of populations within each NED and solid black circles show outliers within the data. Text gives the slope and intercept (calculated using a linear regression model) of the relationship between NED and waiting time, as shown by the solid line.
Figure 8.12 The fixation time for a mutation to arise within the populations selected in environments with between 1 and 8 environmental drivers. The black bands denote the median value, the bottom and top of each box represent the 1st and 3rd quartile of the data respectively. The ‘whiskers’ extending from the boxes indicate the positions of the lowest and highest values of populations within each NED and solid black circles show outliers within the data. Text gives the slope and intercept (calculated using a linear regression model) of the relationship between NED and waiting time, as shown by the solid line.
Table 8-1 ANOVA of the effect NED selection of selection and overlap on the **average rate of cell division** \((d^{-1})\) of *C. reinhardtii*.

<table>
<thead>
<tr>
<th>Source</th>
<th>Mean Squares</th>
<th>df</th>
<th>Denominator df</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>NED</td>
<td>3.4529</td>
<td>1</td>
<td>130.69</td>
<td>3.4239</td>
<td>0.067</td>
</tr>
<tr>
<td>Overlap</td>
<td>0.0017</td>
<td>1</td>
<td>132.68</td>
<td>0.0121</td>
<td>0.913</td>
</tr>
</tbody>
</table>

Table 8-2 A comparison of the percentage variation of the **average rate of cell division** \((d^{-1})\) explained by each group within the mixed effects model.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Variance (%)</th>
<th>Mixed Effects Model Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate</td>
<td>42.43</td>
<td>Random</td>
</tr>
<tr>
<td>Regime</td>
<td>15.35</td>
<td>Random</td>
</tr>
<tr>
<td>Batch</td>
<td>15.10</td>
<td>Random</td>
</tr>
<tr>
<td>Within replicate</td>
<td>22.12</td>
<td>Random</td>
</tr>
<tr>
<td>NED</td>
<td>5.01</td>
<td>Fixed</td>
</tr>
<tr>
<td>Overlap</td>
<td>0.00</td>
<td>Fixed</td>
</tr>
</tbody>
</table>

Number of observations: 1158, groups: ID, 343; regime, 92; Batch, 8
Table 8-3 ANOVA of the effect NED selection of selection and overlap on the **direct** response of *C. reinhardtii*.

<table>
<thead>
<tr>
<th>Source</th>
<th>Mean Squares</th>
<th>df</th>
<th>Denominator df</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>NED</td>
<td>0.67</td>
<td>1</td>
<td>72.20</td>
<td>0.63</td>
<td>0.429</td>
</tr>
<tr>
<td>Overlap</td>
<td>0.75</td>
<td>1</td>
<td>72.21</td>
<td>2.25</td>
<td>0.138</td>
</tr>
<tr>
<td>Strength of Selection</td>
<td>3.22</td>
<td>1</td>
<td>76.56</td>
<td>10.21</td>
<td>0.002</td>
</tr>
<tr>
<td>NED x Strength of Selection</td>
<td>0.01</td>
<td>1</td>
<td>70.53</td>
<td>0.07</td>
<td>0.799</td>
</tr>
</tbody>
</table>

Table 8-4 A comparison of the percentage variation of the average **direct response** explained by each source within the mixed effects model.

<table>
<thead>
<tr>
<th>Source</th>
<th>Variance (%)</th>
<th>Mixed Effects Model Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate</td>
<td>34.52</td>
<td>Random</td>
</tr>
<tr>
<td>Regime</td>
<td>19.40</td>
<td>Random</td>
</tr>
<tr>
<td>Batch</td>
<td>8.32</td>
<td>Random</td>
</tr>
<tr>
<td>Within replicate</td>
<td>18.63</td>
<td>Random</td>
</tr>
<tr>
<td>NED</td>
<td>0.93</td>
<td>Fixed</td>
</tr>
<tr>
<td>Overlap</td>
<td>0</td>
<td>Fixed</td>
</tr>
<tr>
<td>Strength of Selection</td>
<td>18.20 **</td>
<td>Fixed</td>
</tr>
</tbody>
</table>

Number of observations: 1158, groups: ID, 343; regime, 92; Batch, 8
Table 8-5 A comparison of the percentage variation of the **average rate of cell division** ($d^4$) explained by each source within the post hoc mixed effects model

<table>
<thead>
<tr>
<th>Source</th>
<th>Variance (%)</th>
<th>Mixed Effects Model Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate</td>
<td>39.13</td>
<td>Random</td>
</tr>
<tr>
<td>Batch</td>
<td>16.39</td>
<td>Random</td>
</tr>
<tr>
<td>Light intensity</td>
<td>0.00</td>
<td>Random</td>
</tr>
<tr>
<td>Nutrient depletion</td>
<td>0.00</td>
<td>Random</td>
</tr>
<tr>
<td>UVB</td>
<td>0.00</td>
<td>Random</td>
</tr>
<tr>
<td>Herbicide</td>
<td>10.15</td>
<td>Random</td>
</tr>
<tr>
<td>Phosphate depletion</td>
<td>8.26</td>
<td>Random</td>
</tr>
<tr>
<td>pH</td>
<td>0.40</td>
<td>Random</td>
</tr>
<tr>
<td>Temperature</td>
<td>2.98</td>
<td>Random</td>
</tr>
<tr>
<td>$CO_2$</td>
<td>0.94</td>
<td>Random</td>
</tr>
<tr>
<td>Within replicate</td>
<td>18.33</td>
<td>Random</td>
</tr>
<tr>
<td>NED</td>
<td>1.56</td>
<td>Fixed</td>
</tr>
<tr>
<td>Overlap</td>
<td>1.87</td>
<td>Fixed</td>
</tr>
</tbody>
</table>

Number of observations: 1158, groups: ID, 343; Batch, 8; LI, 2; ND, 2; UV, 2; Herb, 2; P, 2; pH, 2; Temp, 2; CO2, 2
Table 8.6 A comparison of the percentage variation of the direct response explained by each source within the post hoc mixed effects model

<table>
<thead>
<tr>
<th>Source</th>
<th>Variance (%</th>
<th>Mixed Effects Model Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate</td>
<td>36.93</td>
<td>Random</td>
</tr>
<tr>
<td>Batch</td>
<td>6.43</td>
<td>Random</td>
</tr>
<tr>
<td>Light intensity</td>
<td>0.00</td>
<td>Random</td>
</tr>
<tr>
<td>Nutrient depletion</td>
<td>0.00</td>
<td>Random</td>
</tr>
<tr>
<td>UVB</td>
<td>0.00</td>
<td>Random</td>
</tr>
<tr>
<td>Herbicide</td>
<td>4.51</td>
<td>Random</td>
</tr>
<tr>
<td>Phosphate depletion</td>
<td>8.94</td>
<td>Random</td>
</tr>
<tr>
<td>pH</td>
<td>0.00</td>
<td>Random</td>
</tr>
<tr>
<td>Temperature</td>
<td>1.11</td>
<td>Random</td>
</tr>
<tr>
<td>CO₂</td>
<td>1.44</td>
<td>Random</td>
</tr>
<tr>
<td>Within replicate</td>
<td>14.07</td>
<td>Random</td>
</tr>
<tr>
<td>NED</td>
<td>0.00</td>
<td>Fixed</td>
</tr>
<tr>
<td>Overlap</td>
<td>0.00</td>
<td>Fixed</td>
</tr>
<tr>
<td>Strength of selection</td>
<td>26.56</td>
<td>Fixed</td>
</tr>
</tbody>
</table>

Number of observations: 1158, groups: ID, 343; Batch, 8; LI, 2; ND, 2; UV, 2; Herb, 2; P, 2; pH, 2; Temp, 2; CO₂, 2
9 Appendix 3

Appendix for Chapter 4

9.1.1 PCA analysis

PCA analysis was performed using the average growth rate of evolved populations in all assay environments. This was performed in R and plotted using the package ggbioplot. Evolved plastic response of populations evolved in NED = 1 and NED = 5 environments were analysed with respect to the first two principal components. This was explored by summarising variation in the ability of evolved populations ability to grow in the control environment (NED = 0), in environments with one environmental driver (NED =1), five environmental drivers (NED =5), eight environmental drivers (NED=8) and a novel environment copper sulphate (0.2 µM). In addition, variation in evolved population growth was summarised across all 18 assay environments.
Figure 9.1 Pilot data showing the response of the evolved control populations to increasing copper sulphate concentration. Open circles show the average response of three replicate populations (± SD).
Figure 9.2 Rate of cell division \((d^{-1})\) of evolved populations selected in 18 different assay environments. Coloured boxplots show growth rate of the evolved populations selected in the one of 15 environments (indicated by the legend), with 0, 1 or 5 environmental drivers. Each panel label indicates the assay environments that each evolved populations were grown in. Dashed lines show the growth rate of the evolved control.
Figure 9.3 Home vs. away (HA) local adaptation is calculated as the mean fitness of the population at home minus the average mean fitness of the population when transplanted in all other habitats. This measurement of local adaptation does not take into consideration the quality of the environment. Circles show the average response per evolved replicate within each regime with NED = 0 (red), NED =1 (green) and NED = 5 (blue).
Figure 9.4 Local vs. foreign (LF) local adaptation is calculated as the mean fitness of a local population at home minus the average mean fitness of all other populations when transplanted into each local habitat. Circles show the average response (± SD) per evolved replicate within each regime with NED =1 (red) and NED = 5 (blue).
Figure 9.5 Plastic response of populations evolved in environments with one environmental driver (solid green points) and with five environmental driver (solid blue points) with respect to the first two principal components summarizing variation in their ability to grow in environments with either no environmental drivers (control), one environmental drivers (NED =1), five environmental drivers (NED =5), eight environmental drivers (NED=8) and an novel environment copper sulphate (0.2 µM). The larger circles indicate how population’s selected in environments with either one environmental driver (solid green circle), or five environmental drivers (solid blue circle) cluster.
Figure 9.6 Plastic response of populations evolved in environments with one environmental driver (solid green points) and with five environmental driver (solid blue points) with respect to the first two principal components summarizing variation in their ability to grow in environments with either no environmental drivers (control), one environmental drivers (NED =1), five environmental drivers (NED =5), eight environmental drivers (NED=8) and an novel environment copper sulphate (0.2 µM). The circles indicate how population’s selected in environments with either one environmental driver (solid green circle), or five environmental drivers (solid blue circle) cluster.
Table 9-1 ANOVA of the effect selection NED (0, 1, and 5), Assay NED and similarity on the average rate of cell division ($d^{-1}$) in 17 different environments.

<table>
<thead>
<tr>
<th>Source</th>
<th>Mean Squares</th>
<th>df</th>
<th>Denominator df</th>
<th>$F$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selection NED</td>
<td>0.06</td>
<td>1</td>
<td>61.38</td>
<td>9.11</td>
<td>0.002</td>
</tr>
<tr>
<td>Assay NED</td>
<td>0.21</td>
<td>1</td>
<td>18.60</td>
<td>10.30</td>
<td>0.001</td>
</tr>
<tr>
<td>Similarity</td>
<td>0.18</td>
<td>1</td>
<td>228.48</td>
<td>0.003</td>
<td>0.064</td>
</tr>
<tr>
<td>Selection NED x Assay NED</td>
<td>0.51</td>
<td>1</td>
<td>209.64</td>
<td>12.57</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Table 9-2 Mixed effects model output showing the percentage variation of average rate of cell division ($d^{-1}$) of evolved populations explained by each source of the model.

<table>
<thead>
<tr>
<th>Source</th>
<th>Variance (%)</th>
<th>Mixed Effects Model Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Evolved Population(Selection Environment(Selection NED)) x Assay Environment(Selection NED)</td>
<td>30.89</td>
<td>Random</td>
</tr>
<tr>
<td>Assay Environment(Selection NED)</td>
<td>28.04</td>
<td>Random</td>
</tr>
<tr>
<td>Evolved Population(Selection Environment(Selection NED))</td>
<td>9.92</td>
<td>Random</td>
</tr>
<tr>
<td>Batch</td>
<td>1.94</td>
<td>Random</td>
</tr>
<tr>
<td>Selection Environment(Selection NED)x Assay Environment(Selection NED)</td>
<td>0.38</td>
<td>Random</td>
</tr>
<tr>
<td>Selection Environment(Selection NED)</td>
<td>0.00</td>
<td>Random</td>
</tr>
<tr>
<td>Assay NED</td>
<td>16.76 **</td>
<td>Fixed</td>
</tr>
<tr>
<td>Selection NED x Assay NED</td>
<td>2.46 **</td>
<td>Fixed</td>
</tr>
<tr>
<td>Selection NED</td>
<td>0.00 **</td>
<td>Fixed</td>
</tr>
<tr>
<td>Similarity</td>
<td>0.55</td>
<td>Fixed</td>
</tr>
<tr>
<td>Within Evolved populations</td>
<td>9.06</td>
<td></td>
</tr>
</tbody>
</table>

Number of observations: 1448, groups: Selection Environment x population Assay Environment, 715; Selection Environment x Assay Environment, 254; Selection Environment x population, 44; Assay Environment, 17; Selection Environment, 15; Batch, 4. Significance codes: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. 
Table 9-3 ANOVA of the effect selection NED (0, 1, and 5), Assay NED and similarity on the *correlated response* in 17 different environments.

<table>
<thead>
<tr>
<th>Source</th>
<th>Mean Squares</th>
<th>df</th>
<th>Denominator df</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selection NED</td>
<td>0.06</td>
<td>1</td>
<td>61</td>
<td>9.06</td>
<td>0.002</td>
</tr>
<tr>
<td>Assay NED</td>
<td>0.00</td>
<td>1</td>
<td>17</td>
<td>0.27</td>
<td>0.718</td>
</tr>
<tr>
<td>Similarity</td>
<td>0.18</td>
<td>1</td>
<td>226</td>
<td>0.00</td>
<td>0.075</td>
</tr>
<tr>
<td>Selection NED x Assay NED</td>
<td>0.53</td>
<td>1</td>
<td>209</td>
<td>12.86</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Table 9-4 Mixed effects model output showing the percentage variation of the *correlated response* of evolved populations explained by each source of the model.

<table>
<thead>
<tr>
<th>Source</th>
<th>Variance (%)</th>
<th>Mixed Effects Model Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Evolved Population(Selection Environment (Selection NED)) x Assay Environment(Assay NED)</td>
<td>47.08</td>
<td>Random</td>
</tr>
<tr>
<td>Evolved Population(Selection Environment (Selection NED))</td>
<td>15.29</td>
<td>Random</td>
</tr>
<tr>
<td>Assay Environment(Assay NED)</td>
<td>15.23</td>
<td>Random</td>
</tr>
<tr>
<td>Batch</td>
<td>3.16</td>
<td>Random</td>
</tr>
<tr>
<td>Selection Environment(Selection NED) x Assay Environment(Assay NED)</td>
<td>0.57</td>
<td>Random</td>
</tr>
<tr>
<td>Selection Environment(Selection NED)</td>
<td>0.00</td>
<td>Random</td>
</tr>
<tr>
<td>Selection NED x Assay NED</td>
<td>4.02 **</td>
<td>Fixed</td>
</tr>
<tr>
<td>Similarity</td>
<td>0.86</td>
<td>Fixed</td>
</tr>
<tr>
<td>Assay NED</td>
<td>0.00</td>
<td>Fixed</td>
</tr>
<tr>
<td>Selection NED</td>
<td>0.00 **</td>
<td>Fixed</td>
</tr>
<tr>
<td>Within evolved populations</td>
<td>13.80</td>
<td></td>
</tr>
</tbody>
</table>

Number of observations: 1448, groups: Selection Environment x population Assay Environment, 715; Selection Environment x Assay Environment, 254; Selection Environment x population, 44; Assay Environment, 17; Selection Environment, 15; Batch, 4. Significance codes: ***, P < 0.001; **, P < 0.01; *, P < 0.05.
Table 9-5 Mixed effects model output showing the percentage variation from each source of the model, of the average rate of cell divisions ($d^{-1}$) of evolved population in a novel environment, copper sulphate.

<table>
<thead>
<tr>
<th>Source</th>
<th>Variance (%)</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Evolved Population(Selection Environment (Selection NED))</td>
<td>24.75</td>
<td>Random</td>
</tr>
<tr>
<td>Batch</td>
<td>10.12</td>
<td>Random</td>
</tr>
<tr>
<td>Selection Environment(Selection NED)</td>
<td>0</td>
<td>Random</td>
</tr>
<tr>
<td>Selection NED</td>
<td>54.88</td>
<td>Fixed</td>
</tr>
<tr>
<td>Within Evolved populations</td>
<td>9.25</td>
<td></td>
</tr>
</tbody>
</table>

Number of observations: 82, ID, 40; Selection Environment, 15; Batch, 4. Significance codes: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. 
Table 9-6: The observed distribution of rank order (out of 15) of the evolved NED = 1 and NED = 5 populations, assayed in 13 environments. Numbers inside bracket show the expected distribution calculated using Chi-squared goodness of fit test and the expected frequency that equal the frequency of rank order of the evolved control.

<table>
<thead>
<tr>
<th>Rank order</th>
<th>NED=1</th>
<th>NED = 5</th>
<th>Expected frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>9</td>
<td>2</td>
<td>5.5</td>
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<tr>
<td>4</td>
<td>7</td>
<td>5</td>
<td>6</td>
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<td>5</td>
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<td>4</td>
<td>5</td>
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<td>6</td>
<td>5</td>
<td>9</td>
<td>7</td>
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<td>7</td>
<td>6</td>
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<td>6</td>
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<td>8</td>
<td>6</td>
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<td>7</td>
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<td>9</td>
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<td>10</td>
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<td>11</td>
<td>5</td>
<td>9</td>
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<td>12</td>
<td>4</td>
<td>8</td>
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<td>13</td>
<td>4</td>
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<td>7</td>
</tr>
<tr>
<td>15</td>
<td>5</td>
<td>9</td>
<td>7</td>
</tr>
</tbody>
</table>
Appendix for Chapter 4

10.1.1 PE autofluorescence intensity per cell volume

A consistent feature cells growing MEDs was the presence of autofluorescence within the PE-a channel of the flow cytometer (Figure 10.4, pp 242; Ex-Max 488 nm/Em-Max 485/42 nm), which is commonly used to detect phycoerythrin autofluorescence found in red algae (Veldhuis & Kraay, 2000). *C. reinhardtii* does not contain phycoerythrin (Harris, 2001), and the source of PE autofluorescence has yet to be identified. I found that all chlorophyll negative populations are PE positive, and some chlorophyll positive cells are PE positive (cells positive for both chlorophyll and PE are referred to here as “dual expression”) (Figure 10.4). One possibility is that the PE autofluorescence channel is detecting chlorophyll breakdown products and may indicate cells that are dying or bleaching, i.e. losing their chlorophyll. Figure 10.5 demonstrates that little PE autofluorescence is detected in the control plastic response and the control evolved response. Before evolution there is a trend that PE increases with NED and this is significant (Figure 10.5b; *the effect of NED on the PE autofluorescence (µm³) before evolution, F₁,9₃ = 9.885, P = 0.002*). However, the relationship between NED and PE autofluorescence breaks down during evolution (Figure 10.5b; *the effect of NED on the PE autofluorescence (µm³) after evolution, F₁,5₉ = 1.304, P = 0.258*). In addition, the relationship between the proportion of PE autofluorescence per cell volume (µm³) and growth rate is stronger before evolution, however, following evolution PE autofluorescence is always absent in populations where growth rate is greater than 2.2 divisions (d⁻¹) (Figure 10.6).
10.1.2 Mass spectrometer analysis

The pigment responsible for PE autofluorescence in C. reinhardtii is yet to be characterised and so in order to better understand the metabolic differences between PE positive (chlorophyll negative) and chlorophyll positive (PE negative) populations, mass spectrometry analysis was performed in order to identify any significant differences between the proteins upregulated or downregulated by each of the populations.

Methods

Cell harvesting

Chlamydomonas reinhardtii evolved in NED = 5 (Temp/ pH/ LI/ UV/ ND), were grown for one transfer cycle and then sorted based on PE and chlorophyll autofluorescence properties, using FACs. See methods section 5.3.3 (page 144). Note that dual expression cells were excluded from this analysis (Figure 10.4).

Sample preparation

Cell pellets were reconstituted and lysed into 250 µl of 8M urea and a protein assay performed (Bradford Biorad). One mg of protein extract was digested, 25 µl of 1M ammonium bicarbonate and 25 µl of 200 mM dithiothreitol (DTT) were then added to enable denaturation and reduction of the samples. Samples were kept at room temperature for 30 minutes before cysteine alkylation with 25 µl of 500 mM iodoacetamide for 1h. 10 µg of trypsin was added and the digestions were performed overnight at room temperature. Peptide extracts were then cleaned on SPE reverse phase Bond Elut LMS cartridge, 25mg (Agilent). The samples were dried under low pressure (Speedvac from Thermo Jouan) and stored at -20 °C.
**HPLC-MS analysis**

The dried peptide samples were re-suspended in resuspension buffer (0.5% v/v trifluoroacetic acid in water) to give final concentration of 1 µg/µl. These samples were filtered using Millex filter before subjecting to HPLC-MS analysis. Nano-HPLC-MS/MS analysis was performed using an on-line system consisting of a nano-pump (Dionex Ultimate 3000, Thermo-Fisher, UK) coupled to a QExactive instrument (Thermo-Fisher, UK) with a pre-column of 300 µm x 5 mm (Acclaim Pepmap, 5 µm particle size) connected to a column of 75 µm x 50 cm (Acclaim Pepmap, 3 µm particle size). Samples were analysed on a 90 min gradient in data dependent analysis (1 survey scan at 70k resolution followed by the top 10 MS/MS).

**Data analysis**

Data from MS/MS spectra was searched using MASCOT Versions 2.4 (Matrix Science Ltd, UK) against the Chlamydomonas reinhardtii subset of the NCBI protein database with maximum missed-cut value set to 2. Following features were used in all searches: i) variable methionine oxidation, ii) fixed cysteine carbamidomethylation, iii) precursor mass tolerance of 10 ppm, iv) MS/MS tolerance of 0.05 amu, v) significance threshold (p) below 0.05 (MudPIT scoring) and vi) final peptide score of 20. Progenesis (version 4 Nonlinear Dynamics, UK) was used for LC-MS label-free quantitation. Only MS/MS peaks with a charge of 2+, 3+ or 4+ were taken into account for the total number of ‘Feature’ (signal at one particular retention time and m/z) and only the five most intense spectra per ‘Feature’ were included. Normalization was first performed based on the median of the ion intensities of these sets of multi-charged ions (2+, 3+, and 4+). The associated unique peptide ion intensities for a specific protein were then summed to generate an abundance value, from which was then transformed using an ArcSinH function (a log transform is not ideal considering the significant amount of near zero measurements generated by the current method of detection). Based on the abundance values, within group means were calculated and from there the fold changes (in comparison to control) were evaluated. One-way ANOVA was used to calculate the p-value.
based on the transformed values. Differentially expressed proteins were only considered significant in current study if the following conditions were fulfilled: i) p-values (pair-wise) less than 0.05, ii) number of peptides detected and used in quantification per protein was equal to or more than 2, and iii) absolute fold change was at least 1.5 (i.e. \( \geq 1.5 \) folds for up-regulated proteins while \( \leq 0.667 \) fold for down-regulated proteins).

**Results**

*Comparison of protein expression in chlorophyll positive and chlorophyll negative populations*

A total of 1,317 proteins were identified. Using a volcano plot I was able to plot the fold change between the two populations and the significance of the difference between them (Figure 10.7; Anova \( P \) value). Using thresholds for the fold change in proteins and significance (\( p < 0.05 \) and fold change; \( 0.667 \geq \text{ratio} \geq 1.5 \)), 719 proteins were selected for pathway analysis. Pathway analysis was used in order to understand the difference in proteins between the populations and any pathways that exist between them. Using the Uniprot database the EMBL accession number was converted into a Uniprot number. The Uniprot number could then be converted into a STRING number (using Uniprot database) (Table 10-1). STRING was used to visualise the pathways in downregulated and upregulated proteins (Figure 10.8), in the chlorophyll negative/ PE positive populations.

Table 10-1 list the number of Uniprot accession numbers for each group of peptides and condition of these peptides, showing the number of STRING numbers and the percentage of successful conversion.
<table>
<thead>
<tr>
<th>Group</th>
<th>Condition</th>
<th>Number of Uniprot numbers</th>
<th>Number of STRING conversion</th>
<th>Percentage conversion from Uniprot to STRING (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Proteins upregulated in PE populations (fold change &gt;1.5)</td>
<td>153</td>
<td>91</td>
<td>59</td>
</tr>
<tr>
<td>2</td>
<td>Proteins downregulated in PE populations (fold change &lt;0.667)</td>
<td>108</td>
<td>72</td>
<td>67</td>
</tr>
<tr>
<td>3</td>
<td>Proteins downregulated in PE populations (fold change &lt;0.667)</td>
<td>125</td>
<td>78</td>
<td>62</td>
</tr>
<tr>
<td>4</td>
<td>Proteins upregulated in PE populations (fold change &gt;1.5)</td>
<td>144</td>
<td>78</td>
<td>54</td>
</tr>
</tbody>
</table>

**Discussion**

Chlorophyll positive and chlorophyll negative subpopulations from the same evolved population were exposed to the same environmental conditions, yet their proteomes differ. The most notable difference between chlorophyll positive and chlorophyll negative subpopulations is the upregulation of ribosomal proteins in chlorophyll negative subpopulations (Figure 10.8). Chlorophyll negative/PE positive subpopulations lack a crucial element to carry out photosynthesis and since these cells are growing in a carbon free media without the capability to photosynthesise, differences in protein expression are expected. This is consistent with data by Klappenbach et al. (2000), who found a positive relationship between the number of rRNA operons inactivated and the time required to increase growth in response to added resources in *E. coli*, and this has been interpreted as evidence that more rRNA transcription allows *E. coli* to respond quickly to changing environmental conditions (Condon *et al.*, 1995). This may allow the chlorophyll negative subpopulations in my experiment to advantage of available resources such as leaked organic carbon.
Conversely in the yeast *S. cerevisiae*, Gasch (2002) found that rRNA and ribosomal protein genes are sharply reduced under stressful conditions and this is due energy costs of ribosome synthesis required. Using microarrays analysis the transcript levels of ribosomal proteins are downregulated (in some cases more than 80 fold) following environmental stresses (Gasch, 2002). A reduction in ribosomal proteins and histones in *E. huxleyi* is also seen under low pH conditions (Tomanek, 2014). The author suggests that these results are attributable to low cells division rates observed in populations growing in low pH.

It is difficult to infer from the data in the present study if there is a benefit to upregulation of ribosomal proteins in chlorophyll negative populations, or the down regulation of the same proteins in the chlorophyll positive populations. Perhaps, one way to determine the role of ribosomal proteins is to compare the abilities of the subpopulations to grow in several new environments. Positive responses in the chlorophyll negative populations would confirm that ribosomal proteins are beneficial in changing environments, and suggest that the benefits outweigh the costs of ribosome synthesis in this case.
Figure 10.1 Standard curve of mean forward scatter (a.u.) and bead size (µm) measured using A FACS Canto.
Figure 10.2 Fluorescence image of three *C. reinhardtii* cells taken using a confocal microscope. Image shows fluorescence of rhodamine 123 dye that detects mitochondrial membrane potential (shown in red – top left panel), propidium iodide dye which passively diffuses across compromised cell walls (shown in blue - top right panel), chlorophyll autofluorescence (shown in green - bottom left panel), and the complete image with all fluorescence (bottom right panel).
Figure 10.3 Correlation between relative chlorophyll autofluorescence and cell size (µm) and before (a) and after evolution (b). Colour of points is scaled from red (low NED) to blue (high NED).
Figure 10.4 Using flow cytometry subpopulations can be identified using autofluorescence (labelled on plot). Each point indicates the autofluorescence of a cell in channels PE-A and PerCP-Cy5-5-A.
Figure 10.5 PE autofluorescence ($\mu$m$^3$) in populations of *C. reinhardtii* under 0 to 8 environmental drivers. Black data points and bars represent means and standard deviation between regimes for each NED. Coloured points indicate the average growth rates among replicates populations within each regime ($\pm$ SD) (96 regimes total, see Figure 7.11 for identity of regimes). Panel labels indicate the plastic response and evolved response to MEDs. Dashed line shows the PE autofluorescence ($\mu$m$^3$) of the control populations in the control environment. Upper and lower panels show the same data however, the lower panels have different y axis so that the differences growth rate as NED increase are clear.
Figure 10.6 Correlation between proportion of PE positive cells and the rate of cell division before (a) and after evolution (b). Colour of points is scaled from red (low NED) to blue (high NED). Horizontal dotted line indicates the growth rate where no or very little PE autofluorescence is detected (>2.2 divisions per day⁻¹).
Figure 10.7 Differences in proteins in *C. reinhardtii* that were grown under MEDs (Temp/ pH/ LI/ UV/ ND) and sorted using FACs into two populations: chlorophyll positive, PE negative and chlorophyll negative, PE positive populations. Red circles indicate proteins which are significantly different between the two conditions (*p* < 0.05), with a fold change in proteins between the two conditions (*0.667 ≥ ratio ≥ 1.5*).
Figure 10.8 Pathways between proteins upregulated in PE populations, generated by STRING. Protein ID’s that begin with “RP” are ribosomal proteins, shown here in a highly connected cluster.
Figure 10.9 A comparison of the number of divisions of *C. reinhardtii* growing in regimes of the case study, before (a), and after evolution (b).
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12 Appendix papers

Brennan and Collins (2015) – attached
Growth responses of a green alga to multiple environmental drivers

Georgina Brennan and Sinéad Collins*

One feature of global change is that biota must respond not to single, but to multiple environmental drivers. By growing a model photosynthetic microbe in environments containing between one and eight different drivers, including changes in CO$_2$, temperature, and pH, in different combinations, we show that the number as well as the identities of drivers explain shifts in population growth rates. This is because the biotic response to multiple environmental drivers depends on the response to the single dominant driver, and the chance of a driver of large effect being present increases with the number of drivers. Interactions between drivers slightly counteract the expected drop in growth. Our results demonstrate that population growth declines in a predictable way with the number of environmental drivers, and provide an empirically supported model for scaling up from studies on organismal responses to single drivers to predict responses to large numbers of environmental drivers.
Some aspect of organismal function, such as growth, is plotted as a function of the environment experienced by the organism, with the value of ‘Environment’ being determined by multiple environmental drivers. Initially, organismal function is high (solid black circle). When single drivers change, organismal function changes (patterned filled circles). Although the effect of each driver may be unknown, as an increasing number of drivers occur, the likelihood of at least one driver or driver interaction having a large detrimental effect on organismal function increases. This thought experiment does not require that the population be in its optimal environment, just that, among the environments sampled, the control environment be one where organismal function is high. Figure 2a shows that this is the case here, as the control environment is among the ‘best’ environments available in this experiment. Note that this cartoon is meant to illustrate our thought process, and not to indicate the quantitative effects of the specific environments used in this experiment. Please refer to Fig. 2 for quantitative data.

Our key finding is that the number of environmental drivers can explain population growth. Population growth rate declines as the number of drivers in test environments increases (Fig. 2). We see that the number of drivers is the strongest predictor of population growth, explaining approximately 37% of the decrease in growth rate independently of the particular combination of environmental drivers involved, which is in line with our hypothesis that knowing the number of environmental drivers alone is informative ($F_{1,22} = 11.1766, P = 0.001$, Fig. 2a; see Supplementary Methods). Regime (the particular drivers in any unique test environment) explains some (32%) of the decrease in population growth rate in test environments, and the overlap in the environmental drivers between regimes also explains some (about 10%) of the variation in growth ($F_{1,22} = 3.8777, P = 0.052$, Fig. 2a). As expected, extinction is more likely in test environments with a greater number of drivers ($F_{1,22} = 3.310, P = 0.072$, Supplementary Fig. 1).

Because regime explains some of the variation in population growth, we further investigate whether this is due to interactions between drivers, or to the actions of single drivers within regimes. We find that the drop in population growth rate can be explained by the single driver in a regime that has the largest effect on growth when it is experienced alone ($r^2 = 0.43; P < 0.0001$, Fig. 2b). This is consistent with population growth rates being largely determined by one overriding driver, rather than by interactions between them, at least with the drivers investigated here. The relationship is thus best described by a simple comparative model (Supplementary Table 4). Antagonistic interactions occur, where the effect of the most detrimental driver is often mitigated if other drivers are present. Because of this, the realized average population growth rate is higher than predicted by the comparative model. Here, high CO$_2$ (Fig. 3), which increases population growth in C. reinhardtii and many other chlorophytes,$^{27,43–46}$ counteracts the growth effects of detrimental drivers and gives rise to antagonistic interactions. When CO$_2$-enriched test environments are removed from our data set, populations in the remaining test environments have lower average growth rates, and fit the predictions of the comparative model without antagonistic interactions ($r^2 = 0.58; P < 0.0001$, Supplementary Fig. 2).

Our key finding is that the number of environmental drivers can be used to predict growth in the test environments even without knowing which drivers make up each test environment. We propose a hypothesis is based on how reactions to changes in the environment affect organismal growth, drivers are environmental values that differ from the usual laboratory environment of the particular population used to start this experiment. The control environment is thus not arbitrary, even though it may differ from the optimal environment for other strains of C. reinhardtii that have been maintained under different conditions. Whenever possible, control and test environments reflect anticipated changes in the natural world. For example, the control environment uses 430 ppm CO$_2$, whereas the test environments containing high CO$_2$ use 2,000 ppm CO$_2$, in line with IPCC predictions.$^{39}$ In other cases, the usual laboratory environment for this strain required that we choose the test environment value using pilot studies. This experiment requires that the test environments be different from the environment usually experienced by this particular strain at the beginning of the study, not that the control environment be the average or optimal one for this species over many studies. See online methods for a detailed discussion of each test environment. In each test environment, we measured population growth, a trait commonly used to predict how populations will fare under environmental change,$^{47}$ including whether they are likely to persist.$^{27}$ See Methods and Supplementary Information for test environments and experimental design.
that this is because test environments with a greater number of drivers are more likely to contain at least one severely detrimental driver or driver interaction and that, once a severely detrimental driver is present, the addition of other drivers is unlikely to depress growth much more, barring extinction. This can be seen in Fig. 2a, where the lowest fitness at NED = 2 is about 0.16 divisions/day (regimes for the two lowest points are pH + phosphate starvation and phosphate starvation + low light, both have the same average growth rate), but at higher NED this minimum does not decrease, indicating that once a very stressful driver or driver interaction is present, further drivers or driver interactions do not, on average, depress growth more. However, interactions do matter for low NED: the populations with the lowest growth rates at NED 2 (lowest average growth rate for a regime is 0.16 divisions/day) do far worse than those with the lowest growth rates at NED 1 (lowest average growth rate for a regime is 0.30 divisions/day). Interestingly, this shows that if the goal of empirical studies is to predict population responses to MEDs when many drivers are present, the most useful course of action when only a limited number of populations can be observed is to determine which single drivers affect growth most, or even determining how many drivers are likely to co-occur. In contrast, focusing on interactions between a few specific drivers may produce results dominated by interactions that sum nearly to zero when more realistic scenarios of environmental change are considered.

The goal of this study was to disentangle the role of the number of environmental drivers from the specific drivers present in test environments. Each driver is used only at a single intensity in our study (see Methods for explanations of the choice of intensities of particular drivers), and the rank order of the driver effects are probably due to both intensity and identity. In our data set, the most detrimental drivers are herbicide presence and phosphate starvation. Presumably, neither would be as detrimental if we had used lower concentrations of herbicide and higher concentrations of phosphate, respectively. However, it is reasonable to suppose that in most natural environments, drivers will vary in intensity as well as identity. Our interpretation of our data hinges on the growth effects of drivers, not their identities per se, and we expect that if this experiment were repeated with different drivers, or a different organism, the qualitative results would be the same—the drivers with the largest effects on growth would determine responses even when populations experienced them together with numerous other drivers. Although the size of our study precluded multiplying it to measure the relative contributions of identity and intensity of drivers to organismal responses, this indicates a direction for future experiments.

Figure 2 | Population growth rate of C. reinhardtii under zero to eight environmental drivers. a. Black data points and bars represent means and standard deviation between regimes for each NED. See Supplementary Table 3 for regimes. Different shapes within each NED indicate individual regimes. Dashed line in a indicates growth in the control environment. b–d. Population growth rates (mean and standard deviation) predicted by a model (white triangles) alongside measured values (black circles), followed by goodness-of-fit, for three models: comparative model ($r^2 = 0.43, P < 0.0001$) (b), multiplicative model ($r^2 = 0.33, P < 0.0001$) (c) and additive model ($r^2 = 0.25, P < 0.0001$) (d); extinction (indicated by dashed line in panels b–d) is predicted in environments with >5 changes.
Environmental similarity

In our experiment, test environments become more similar as the number of drivers increases, although this similarity explains less than 11% of the variation in growth. If increases in environmental similarity were driving our results, we would expect that variation among regimes drop as the number of drivers within regimes increases, but this is not the case (correlation between the number of drivers and variance among regimes with the same number of drivers: post hoc fit $r^2 = 0.06$, $P = 0.53$). Increasing environmental similarity with an increasing number of drivers per test environment is a limit of performing an experiment with a finite number of drivers. To understand how increasing environmental similarity affects our data, we simulated the same experiment using infinite environments with the same distribution of effects on growth for single environmental changes as in our experiment. We found that using a finite number of possible environmental changes in our experiment slightly underestimates growth rates in regimes with many drivers, but the effect is small (Supplementary Fig. 3), confirming that the increase in similarity between regimes with an increasing number of drivers does not explain the overall pattern of our data.

Case study involving temperature, CO₂ and pH

To understand how interactions between focal drivers change when additional drivers are present, we measured the effects on population growth of increased CO₂, increased temperature and decreased pH—either alone, in pairs, all together, or all together in the presence of other drivers (Fig. 3). When these focal drivers occur singly, populations grow fastest under CO₂ enrichment, slower under low pH, and slowest under high temperature. In pairs, the effect of CO₂ enrichment counteracts that of high temperature so that these populations have higher growth rates than those under high temperature alone, whereas the combined effects of CO₂ enrichment and low pH reduces growth. Populations grown in low pH and high temperature grow faster than those subjected to either driver alone, and populations subjected to all three drivers together grow faster than any of the paired or single cases. In these test environments, containing between one and three drivers, specific interactions between responses to drivers determine growth effects, and the most informative way to explain changes in growth is by investigating the physiological mechanisms involved.

In contrast, when elevated CO₂, low pH, or high temperature co-occur with other drivers, changes in population growth are predictable from the effects of single drivers. This prediction is more robust when a greater number of drivers are present in the test environments. For example, if CO₂, pH and temperature change, decreasing light intensity does not affect growth further, as expected from the small effect of light intensity on growth alone relative to the effect of other drivers already present in the regime. In contrast, the presence of herbicide, which has a drastic effect alone, reduces growth when it is added to a test environment that already contains several other drivers. The addition of nutrient depletion has very little effect on growth and is masked by the dominant effects of herbicide.

These interactions are all expected under the simple comparative model. Interestingly, at high NED, phosphate limitation has an antagonistic interaction when herbicide is present. This is surprising, as both herbicide and phosphate are dominant environmental drivers. The herbicide used here is atrazine, which directly blocks the photosynthetic electron transport chain, reducing photosynthetic efficiency. Phosphate is a limiting factor in many natural environments, yet it is a necessary
macronutrient that photosynthetic organisms such as *C. reinhardtii* require in large amounts\(^5\). Previous work\(^3\) suggests protection mechanisms such as nonphotochemical quenching of excess light energy and adjustment of the photosystem stoichiometry to explain the antagonistic interaction observed between atrazine and very high light in *C. reinhardtii* and arrested growth with no loss in viability in low light conditions. Similar protection mechanisms may be in place here to protect populations from the lethal effects of atrazine under limited resources at high NED. Antagonistic interactions between phosphate depletion and other environmental changes have also been found in a nitrogen-fixing species (*Trichodesmium*\(^4\)), where phosphate-limited populations are also CO\(_2\)-limited, so that high CO\(_2\) can increase population growth rate when phosphate is limiting. Our case study broadly supports the observation that elevated CO\(_2\) can partly mitigate the drop in growth in test environments with MEDs, including phosphate-limited environments. However, we also find some exceptions where growth is not increased by high CO\(_2\), such as when nutrients and phosphate are co-limiting.

Although interactions between drivers increase variation in the reduced data set that excludes high-CO\(_2\) test environments (Supplementary Fig. 2) relative to the full data set shown in Fig. 2, the overall relationship between population growth and the number of drivers is the same. When many drivers co-occur, the effects of individual drivers, in particular of the driver with the single largest effect alone, are reasonable predictors of population growth. Our data also show that even if the individual effects of drivers on growth are unknown, the number of drivers offers a good estimate of the expected growth rate when large numbers of drivers co-occur. As with the full data set, this is due to test environments with a greater number of drivers having a higher chance of containing at least one severely detrimental driver so that, generally, growth decreases as the number of drivers increases.

Conclusions

Global change involves many environmental drivers, but biotic responses are often studied using few environmental drivers, so it is vital that we explore if and how studies using few environmental changes inform predictions of biotic responses to higher numbers of drivers. Mechanistically understanding all interactions between the relevant drivers in aquatic systems\(^6\) cannot be tackled experimentally—with current methods, full factorial experiments are simply too large to carry out. Therefore, we can make a tradeoff between a mechanistic understanding of interactions between specific drivers and predicting overall biotic reactions to MEDs. One well-established way to do this is by using scenarios\(^7\) where suites of environmental variables are changed in concert and organismal responses measured. Here, we propose a complementary method suitable for situations where a larger number of drivers is considered, based on data showing that average changes in population growth in a model microalga are largely predictable from either the number of environmental drivers, or the effect of the single most detrimental driver, in cases where a large number of environmental drivers occur together. As with scenarios, our approach trades mechanistic understanding for predictive power. Although the ideal solution to understanding organismal responses to MEDs may be to replace ‘black box’ approaches such as ours with a mechanism-based understanding that allows prediction, this may not be realistic given current knowledge. Our approach is appropriate when constructing scenarios of environmental change carries significant uncertainty, because of uncertainty in predicting the intensities of individual drivers, of correlations between changes in drivers, or even in the identity of the particular drivers involved at the relevant geographical and temporal scales for focal organisms. It is also useful when data on responses to drivers or scenarios cannot be gathered for all organisms where it is needed. Another use of our method is in making between-species or between-genotype comparisons by uncovering differences in sensitivities to particular drivers. If the effect of many individual drivers is measured on different species or genotypes, then studies can be used to both understand differences in responses between species or genotypes, and to predict the likely range of responses to MEDs within communities containing many species or genotypes.

We show that specific interactions between drivers determine growth responses when only a few drivers change, but these interactions do not need to be taken into account to predict average growth responses when many drivers change. This provides hopeful evidence that continuing to build our understanding of how single drivers affect population growth is indeed informative for understanding population-level responses to MEDs.

Methods

Methods and any associated references are available in the online version of the paper.

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References


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Author contributions

G.B. and S.C. designed the experiment, G.B. performed experiments and S.C. supervised laboratory work. Both authors contributed to statistical analysis and writing the article.

Additional information

Supplementary information is available in the online version of the paper. Reprints and permissions information is available online at www.nature.com/reprints. Correspondence and requests for materials should be addressed to S.C.

Competing financial interests

The authors declare no competing financial interests.
Methods

Experimental design. All populations were founded from a single cell of *C. reinhardtii* (CC-2931, mt−; Chlamydomonas Resource Center, University of Minnesota). Suokas high concentrated medium (HSMT, ref. 39), under continuous rotation (50 r.p.m.) at 25 °C and constant light at 32 µmol m−2 s−1 photon flux density (Fisher Scientific Traceable Dual-range Light Meter), at 420 ppm CO2 (Supplementary Tables 1 and 2). These variables were controlled using incubators (Infor AG CH-4103). This strain of *C. reinhardtii* is from a culture collection, and has been grown in our lab for over seven years—this medium, temperature and light levels represent the usual benign growth conditions for this strain.

Experimental environments. Experimental populations were grown for approximately three generations in replicate test environments that differed from the benign control environment (430 ppm CO2, pH 7.2, temperature 25 °C, full light and nutrients, no herbicide and no ultraviolet), by between one to eight of the following parameters: increased CO2 to 2.000 ppm, temperature to 26 °C, decreased pH to 6.5, light levels to 18 µmol m−2 s−1, reduced phosphate to 1.69 mM, general nutrient depletion by 75%, and added 0.5 µM of the herbicide atrazine. In addition, test environments with ultraviolet were exposed to a dose 8.1 kJ m−2 ultraviolet radiation once a week as part of the batch culture protocol (Supplementary Tables 1 and 2). There are 96 test environments in total in this study and 288 populations (3 independent replicate populations per test environment ×96 test environments, Supplementary Table 1). The large size of this experimental design using *C. reinhardtii* as the model alga, as it grows easily in small volumes in media that it is already adapted to that have sufficient buffering capacity to control pH when CO2 is varied, has a wealth of information available on responses to the individual drivers used in our study, and is a common model system in algal physiology and evolution. Cultures were grown in 48-well plates containing 1.6 ml of culture media. Each population was acclimated to its test environment for seven days (three generations), and then transferred to fresh test environment medium for each regime.

Details of how individual drivers were manipulated and our reasoning behind specific manipulations are below. In general, driver intensities were kept in line with future climate change scenarios where possible27,29, but modified to accommodate logistics, the starting point of the benign lab environment, the need that each driver affect growth, and avoiding rapid extinction in environments that contained only one driver. Although extinction is one possible outcome of populations being exposed to changes in environments, the goal of our study was to learn how responses to one environmental driver predicted responses to multiple environmental drivers; this requires meaningful measures of growth in the single-driver environments. With the exception of CO2/pH, we did not attempt to control chemical interactions between drivers; these interactions may contribute to organismal responses and to subsequent patterns of how response scales with the number of drivers. Because this study aimed to understand average biotic responses with increasing numbers of drivers, we had more power to detect a pattern by including a greater number of drivers rather than focusing on specific chemical interactions among drivers.

Temperature. A conductive heat mat (Exo Terra Heat Wave substrate heat mat) was placed under experimental plates to increase the temperature of the culture media to 26 °C. This did not affect the control temperature set within the incubator and was controlled using a thermostat (Rootit Heat Mat Thermostat). Our reasoning is that a 1 °C rise in temperature could be produced without affecting the overall temperature of the incubator or causing condensation on the culture vessel lid, falls within the range of predicted temperature rises for aquatic ecosystems29 and produces a change in growth rate in *C. reinhardtii*—and can thus act as a driver—but does not cause mortality (we wanted to avoid large numbers of extinctions during the experiment).

CO2. Sterile breathable films (AeraSeal breathable sealing film) were used instead of the of the 48-well plate lids that came with the plates. This allows increased CO2 diffusion by the media, although we did not quantify the precise level of CO2 in the media, growth in the high-CO2 conditions was stimulated, indicating that it was acting as a driver, which is all that was needed for the purpose of this study. CO2 levels in the test environments were chosen based on projected CO2 levels, and are in line with other experiments investigating microalgal responses to CO2 enrichment.

pH. The pH of the culture media was altered by adding 2% HCl. This required one to two drops per litre of HSMT, so the concentration of nutrients was not altered by changes in volume. The pH was measured with a pH meter (Thermo Orion Star A121 pH Portable Meter) and buffered by adding Tris-HCl. Even though this drop in pH (0.7 units) is large relative to changes expected in marine ecosystems31 it is well within those experienced in freshwater systems32. On the basis of pilot work, this drop reliably affects growth in the *C. reinhardtii* in our laboratory cultures.

Ultraviolet. A ultraviolet lamp (UVM-57) was used to provide a dose of ultraviolet radiation at acclimation and at T0 (Supplementary Fig. 5). The breathable films were removed from the culture plates under sterile conditions during although the lamp was housed 5.1 cm from the surface of the culture plates, providing an irradiative exposure of 33.75 W cm−2. Populations were irradiated for 4 min, which corresponds to a ultraviolet dose of 8.1 kJ m−2.

Light intensity. Overall light intensity was reduced by approximately 40% using a neutral density light filter (0.15 optical density), designed to reduce the light intensity across all wavelengths equally and attenuate light by absorption with minimal reflection. The filter was secured to the top of the experimental plates, allowing sufficient room for CO2 to circulate. Our rationale for decreasing light was pragmatic; it is possible to put a filter on some of the culture vessels, but difficult to selectively increase light levels reliably for only a few populations during an experiment of this size. Furthermore, increasing light levels for *C. reinhardtii* often lead to bleaching and mortality54,55. We found that with this strain the light intensity used was high enough for growth, but limited the amount of bleaching in populations.

The strain we used (CC-2391) has been used by other experiments where light levels were 60 µmol m−2 s−1, equivalent to 60 µmol m−2 s−1 (ref. 53), and 50 µmol m−2 s−1 (ref. 54). These light intensities are lower than the ‘low light’ intensity reported in ref. 13, although a different strain of *C. reinhardtii* (CC-125) was used. However, previous experiments used cultures that differed from ours in terms of volume, as well as other details, and so should not be directly compared. We have been growing this strain in the laboratory for several years under the light levels used in this experiment (32 µmol m−2 s−1), and as the experiment depends on using environmental change (that is, change relative to a control environment that the organism usually experiences), deviation from the light levels usually experienced is needed. We verified that the light levels in the control environment allow faster growth than the light levels in the test environments (see Supplementary Fig. 9). Neutral density filters were used to decrease light levels, and we show that the filter used in our test environments (0.15 optical intensity) significantly reduced growth relative to the control light levels, and that the control light levels are not low down on the growth curve.

Herbicide. Atrazine was used at a concentration of 0.5 µM in HSMT. Atrazine was then added to the culture media used for this treatment freshly whenever populations were transferred into fresh media. On the basis of pilot work, this concentration of atrazine reliably affects growth in the *C. reinhardtii* genotype used.

Nutrients. All nutrients within Hutner’s trace elements (HTE) were reduced equivalently to a concentration factor of 0.25 relative to the control concentration (see Supplementary Table 2 for concentration of each nutrient within HTE). As laboratory strains are used to growing in rich media such as HSMT, increasing trace nutrients has no measurable effect on growth. The reduction in nutrients needed to act as a driver in this experiment was determined empirically during pilot studies.

Phosphate. Phosphate was reduced to a concentration factor of 0.125, relative to the control concentration43. Salts lost by the removal of dipotassium phosphate (K2HPO4) and monopotassium phosphate (KH2PO4) were replaced with potassium chloride (KCl). The level of phosphate needed to act as a driver was based on pilot work and previous studies44.

Population growth. Cells were counted by flow cytometry every 24 h for a total of 120 h using a BD FACSCanto II (BD Biosciences) flow cytometer calibrated with Cytometer Setup and Tracking (CS&T) beads. The data were acquired with the BD FACSDiva v6 software. Each culture was counted twice. The cell counts were transformed into cells per millimetre and the number of divisions per day per starting cell was calculated using equation (1):

\[
\text{Rate of division (day}^{-1}) = \frac{\log(N_f/N_i)}{(t_f - t_i)}
\]

where \(N_i\) is the cell density (cells ml−1) at time \(t_i\) (hours) and \(N_f\) is the cell density at time \(t_f\) (hours). This calculation was used because different environments produced different shaped growth curves (Supplementary Fig. 6), and the usual metric of maximum growth rate was not useful, whereas this measure averages the average number of divisions per day per founder cell in a transfer cycle, and allows comparison of populations with different growth strategies50. In particular, this measure gives the average number of divisions per day that have taken place per founder cell in the population, where cells divide by binary fission, as is the case here. It is also a metric that is not affected by \(N_0\), which is required because the population size reached during the acclimation period differs between environments—this is to be expected given that the environments were chosen to have a range of effects on growth. Here, even though many of the curves seen in Supplementary Fig. 6 do not appear exponential, an exponential process (binary fission) underlies them, and thus justifies the use of equation (1). There are several
reasons why an exponential process may fail to produce a full exponential growth curve, such as the rate of cell division not being constant over the entire time period measured, or a low number of division events occurring within the time window of interest. In different environments, maximum cell division rates, the tempo of cell division events over the transfer cycle, the presence and length of lag phases, and carrying capacities, in any combination, may differ. The metric of the average number of reproductive events per unit time over the time window of interest is a general one, although a different equation would have to be used in the case where the organism being studied did not reproduce by binary fission. In our experiment, populations in the control environment were never nutrient-limited (cultures never reached carrying capacity). For comparison, we also used a more conventional measure of population growth (see Supplementary Methods) that simply measures the slope of the growth curve, which is the average number of cell divisions per day in the entire culture, rather than per cell in the starting population. In this case, it is possible to have a larger number of cell divisions simply by having a higher population density at the end of the acclimation period, leading to a larger value of N, so the measure of slope is sensitive to small differences in the initial population size. The two methods reach the same conclusions, probably because starting population sizes were similar over different populations in our experiment.

Statistical analysis. The effect of the identity and NED on growth was analysed using a mixed model in R (ref. 56), using the packages lme4 and lmerTest. Number of environmental drivers (0–8; referred to as NED) is a fixed factor, as is overlap between regimes within each level of NED (measured as the average number of shared drivers between different test environments for a given NED—see below). Regime and replicates within each regime are random factors. To directly compare the contributions of fixed and random factors to variance, the percentage contribution of fixed factors (Supplementary Table 5) was estimated by using equation (2).

\[
\text{Percentage of fixed effect variance} = \left( \frac{\sigma_f^2 \times (b^2 - se^2)}{\sigma_s^2} \right) \times 100
\]

(2)

where \(\sigma_f^2\) is the variance of the fixed effect, \(b\) is the slope of the fixed effect estimated by the mixed effects model, se is the standard error of the fixed effect as estimated by the mixed effects model and \(\sigma_s^2\) is the variance of the response variable.

Post hoc analysis. A post hoc mixed model was used to detect effects of particular drivers (for example, of CO\(_2\) or pH) where the identities of each driver were nested within NED were added to the random part of the model in place of regime and overlap, as described above.

Overlap of NED between regimes. Each regime is unique—however, because regimes become more similar as the number of environmental drivers increases, overlap between regimes for a given NED was calculated as an average pairwise difference between regimes, where each environmental driver is coded as a binary variable (present or absent). Average overlap for each NED is calculated as \(1 - (\text{average pairwise distance})\). The overlap for test environments with 0, 8 and 1 environmental drivers is zero as there is only one control regime, one regime with all eight environmental drivers and in test environments with 1 driver, all eight changes were assayed alone. The analysis was performed the same way for each subset of the presented data (including the case study and full data set less CO\(_2\)). The effect of sampling from a finite number of possible environmental drivers was explored using a simulation written in R (Supplementary Information).

Models. Expected numbers of division (\(N_{\text{exp}}\)) for each regime were calculated for each of three models (simple comparative, multiplicative and additive), using the observed number of divisions (\(N_{\text{obs}}\)) measured for NED = 1, where each driver is experienced alone. For the simple comparative model, \(N_{\text{exp}}\) is equal to the most dominant individual environmental driver relative to the control (1 – \(N_{\text{obs}}\)). For example, if herbicide is found to elicit the largest change in population growth, any other driver present within that regime would have no additional effect. For the additive model, \(N_{\text{exp}}\) is calculated as the sum effects of all individual drivers present in the regime when experienced alone (at NED = 1). For the multiplicative model, \(N_{\text{exp}}\) is the product of \(N_{\text{obs}}\) for each of the drivers present in the regime when they are experienced alone (at NED = 1). Model fits were compared using the \(r^2\) values. The expected number of divisions for each model (simple comparative, multiplicative and additive) was fitted against the observed fitness using a linear model. This was completed in R using the lm function within the R basic stats package.

Data. All data and R scripts are available from Datadryad (http://dx.doi.org/10.5061/dryad.jt1fb).

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