Effects of temperature and phosphorus on growth, stoichiometry and size in three haptophytes

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Master thesis

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UNIVERSITY OF OSLO

01.06.2015

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http://www.duo.uio.no/

Printed: Reprosentralen, University of Oslo

Abstract

Temperature and elemental composition are key factors that affect life history traits in algae. Climate change is predicted to cause major temperature changes in the ocean that may affect algal populations, seasonal dynamic and stratification patterns with profound ecological impact. The determination of how species specific life history traits in algae may vary depending on bioavailable phosphorus (P) and temperature is therefore of vital importance. The growth rate and cell size is of fundamental importance for phytoplankton ecology and evolution. Theory predicts smaller cell size at increasing temperature, either directly related to temperature or indirectly through nutrient scarcity. To address these issues, a factorial experiment with *Emiliania huxleyi*, *Chrysochromulina rotalis* and *Prymnesium polylepis* was conducted. In order to effectively induce P limitation, the cultures with lower P were cultured as chemostats, while the cultures with elevated P were grown as turbidostats.

Responses to temperature and P were studied in terms of cellular RNA content, alkaline phosphatase activity, stoichiometry and genome size, combined with quantitative measurements of density and cell size. In general all parameters responded to P and temperature, yet with somewhat different responses for different algae. The results strongly indicate that temperature is the governing factor of plasticity in cell size as predicted from Temperature-Size Rules. The growth rate was primarily affected by P-treatment. The stoichiometric response show that N:P combined with RNA indicate a strong allocation to rRNA rich ribosomes with increased growth rate. The study of change in genome- to cell size was not conclusive, but the results indicate no significant correlation in the study.

These findings may indicate a selection for smaller cell size if the predicted climate change results in further increase in oceanic temperature. An increase in stratification and reduced mixing will also affect growth patterns and stoichiometric responses, and potentially seasonal dynamics with profound ecological impact.

Acknowledgment

This study was carried out at the Department of Bioscience, University of Oslo (UiO). Professor Dag O. Hessen has supervised on this thesis, along with co-supervisors Professor Bente Edvardsen and Professor Tom Andersen. All laboratory work was conducted at the Department of Bioscience, Section for Aquatic Biology and Toxicology (AQUA).

I want to extend my gratitude to my supervisor Dag O. Hessen who greeted me with an open door when I first came to talk to him about the project in the summer of 2013. And throughout this study that door was never closed. For all his knowledge, insight and wisdom, I am truly grateful. I also would like to thank my co-supervisors Tom Andersen and Bente Edvardsen. Tom taught me R to begin with during the bachelor study, and continued showing me a new world of statistics and ideas right up till the end. Bente taught me much on the different algae and was a continual source of inspiration and knowledge.

I would also like to thank Nita K. Shala and Dr. Marcin Wojewodzic. I consider myself a lucky person to have had the opportunity to learn so much from you during these years. I thank you for all your help, wisdom and fun times.

A special thanks to Jan Erik Thrane for his knowledge in stoichiometry, incredible R skills and positivity. I am very grateful for all your guidance and knowledge.

Furthermore I would like to thank Berit Kaasa, Sissel Brubak, Per Færøvig and Dr. Marwa Jalal for help with various technical tasks. Without you I would most likely be lost. And even though they were unfortunately not included, I would like to thank Wenche Eikrem for her help with electron microscopy preparations.

Lastly I want to thank my fellow students with special mention to Petra Mutinova, Anders Wold and Annie Evankow for your inspiring words, feedback and helping me through these years. Thank you all for helping me see through this to the end.

Oslo, June 2015

Lars F. Skau.

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1. Introduction

1.1 Effect of temperature on cell size and growth

Temperature is a key factor for all organisms. Many traits that are closely linked to fitness are either directly or indirectly affected by temperature (Angilletta 2009). The observed and predicted rise in temperature has sparked a recent interest for assessing how current and potential future conditions may affect biodiversity, species distribution and physiological responses (Thomas et al. 2004; Chevin et al. 2010). In 2014 the Intergovernmental Panel on Climate Change (IPCC) released their fifth assessment report. Through assessment of historical data they observed that the ocean on a global scale has had a temperature increase in the upper layers of 0,11^{o}C per decade from 1971 to 2010 (Stocker et al. 2014). Temperature not only affects organisms but also the physical properties of aquatic systems. Increased temperature in the upper layers of lakes and oceans may lead to an increased thermal stability. This may affect seasonal stratification patterns and hence also mixing and nutrient cycling in open waters (Sarmiento et al. 2004). This may have a profound impact on pelagic algae both in terms of metabolic rates and access to nutrients, thereby having indirect implications for all marine organisms on different trophic levels (Pauly & Christensen 1995; Scheffer et al. 2001).

Life history traits (LHT) are phenotypic characteristics that are closely linked to the survival and reproduction of an organism. However, during a change in the local environment, a considerable number of organisms can show remarkable resilience and flexibility in how they may acclimate, adapt and change to their surroundings. Organisms can adapt to their environment either through altering their behavioral or physiological responses. The degree of change is dependent of the plasticity of the traits (Angilletta et al. 2004). Phenotypic plasticity is the ability of a single genotype to produce different phenotypes under different environmental conditions (Westeberhard 1989). It can in the context of temperature be defined as the derived of a reaction norm (Angilletta 2009). A reaction norm is the distribution of phenotypes produced by a single genotype across a range of environmental conditions (Woltereck 1909). It can be traits such as size or a behavioral pattern, and while some responses may have a simple cause, others are more complex, making it difficult to predict exactly how and if different organisms will react (Reusch 2014; Schaum & Collins 2014).

Ectothermic organisms, as opposed to endothermic organisms, have negligible internal heat sources for regulating their body temperature (Davenport 1992). This implies that these organisms have to rely on their respective local environment to regulate their metabolic rate and other biochemical processes.

In 1847 Carl Bergmann attempted to explain the relationship between size and temperature, after observing a wide range of observations in endotherm organisms. Individuals within a species showed a tendency to be larger in low temperature habitats. This has become known as Bergmann's rule, and it was hypothesized that this was because a lower surface to volume ratio was more adaptive to colder environment to minimize heat loss, and has later been studied in both endotherms and ectotherms (Ray 1960).

The Bergmann's rule led to the formulation of a more general temperature-size rule (TSR) which predicts that ectothermic organisms will have smaller body size if reared at a higher temperature, and larger body size when raised in lower temperature (Atkinson 1995; Kingsolver & Huey 2008). The effects of temperature and size have been studied and explained in a wide range of ectothermic metazoans (Atkinson 1994; Angilletta et al. 2004; Aguilar-Alberola & Mesquita-Joanes 2014; Baudron et al. 2014), and also other organisms (Partridge et al. 1994; Arendt 2007). And while there are many exceptions to this rule (Shelomi 2012; Vinarski 2013), and hence disputable whether it actually is a "rule", the pattern is detectable on a global scale.

The potential growth pattern of an organism is of vital importance when studying thermal effect on size in different species. Multicellular organisms can grow by either increasing the number of cells or by changing the size of the cell (Hessen et al. 2013). Cell growth is however shown to be more sensitive to thermal constraint than cell division. This means that an organism with an approximately constant number of cells would be smaller due to reduction of the cells size at higher temperatures (Van Der Have & De Jong 1996). Increased temperature generally yields higher growth rate due to increased enzymatic and cellular activity (Dmitriew 2011).

1.2 Stoichiometry and growth

Ecological stoichiometry is the study on balancing multiple chemical substances in ecological interactions, and how it affects and is affected by organisms (Sterner & Elser 2002). A pioneer in this field was Alfred C. Redfield who hypothesized that the average chemical composition (element ratios) for algae was 106(C):16(N):1(P) (Redfield 1958). The 16:1 N:P ratio has served as a good indicator for the balance between N and P limitation in algae (Lenton & Watson 2000). However, later studies have shown that autotrophic growth is not as tightly bound to the Redfield ratio, such that it is better treated as a community average than a universal constant (Geider & La Roche 2002). The limiting nutrient might inhibit the uptake of other nutrients and thereby affect the elemental composition. This regulation is known as Liebig's law of the minimum (Sterner & Elser 2002). The optimal saturation point of the nutrients may vary between species, while the optimal ratio of elements may vary with environmental factors such as temperature (Sterner & Elser 2002; Allen & Gillooly 2009). The reason for variable elemental composition is that phytoplankton has a non-strict homeostatic regulation in response to environmental conditions such as light, temperature, nutrient supply and CO_2 levels (Hessen 1992; Sterner & Elser 2002). To understand the role of phytoplankton in the food-web and the biogeochemical cycles of the ocean, it is crucial to study how the C:N:P ratio may deviate from Redfield ratio (Falkowski 2000). By using culture experiments it is possible to study how these different environmental factors may influence the same traits in different species resulting in allocation of energy and change in elemental composition.

This study will focus on stoichiometry in the algae, with special interest on how P limitation affects growth rate, cell size and genome size. By looking into the RNA content and alkaline phosphatase activity in the cells it should also be possible to deduce to some extent where the cells allocate their phosphorus with regards to the growth rate hypothesis.

Growth rate is a key LHT that is highly plastic and closely related to fitness (Dmitriew 2011). The growth rate in algae responds rapidly to changes in nutrient level. Algae that do not experience nutrient limitations are able to support a faster growth than those that are lacking in resources. The faster growth is achieved since there is more energy to allocate to different cellular processes according to the principle of allocation (Brown et al. 2004; Dmitriew 2011). The N:P ratio is of particular interest since it not only relates to the limiting nutrient, but also

reflects the balance and demands for investing in N-rich photosynthetic proteins relative to P-rich ribosomes which are the sites of the protein synthesis (Klausmeier et al. 2004).

The growth rate hypothesis is based on the observed strong correlation between mass specific growth rate, phosphorus level and RNA. It predicts that in rapidly growing organisms there will be an increase in allocation to P-rich rRNA and hence a low N:P and C:P ratio (figure 1). This is because rapid protein synthesis provided by the ribosomes is necessary to support the fast growth rate (Hessen 1992; Elser et al. 2000).

Another hypothesis is that the N:P ratio can be positively correlated with growth rate. This is the same trend that has been observed in vascular plants (Reich & Oleksyn 2004). The reason for a higher N:P ratio is hypothesized to be due to an increase in ribosome efficiency at higher temperatures (Toseland et al. 2013). This implies that an increase in temperature would lead to a lower density of ribosomes in the cell. Sub-cellular processes that are positively affected by temperature, such as growth rate, is therefore expected to positively correlate with the N:P-ratio in algae (Yvon-Durocher et al. 2015).

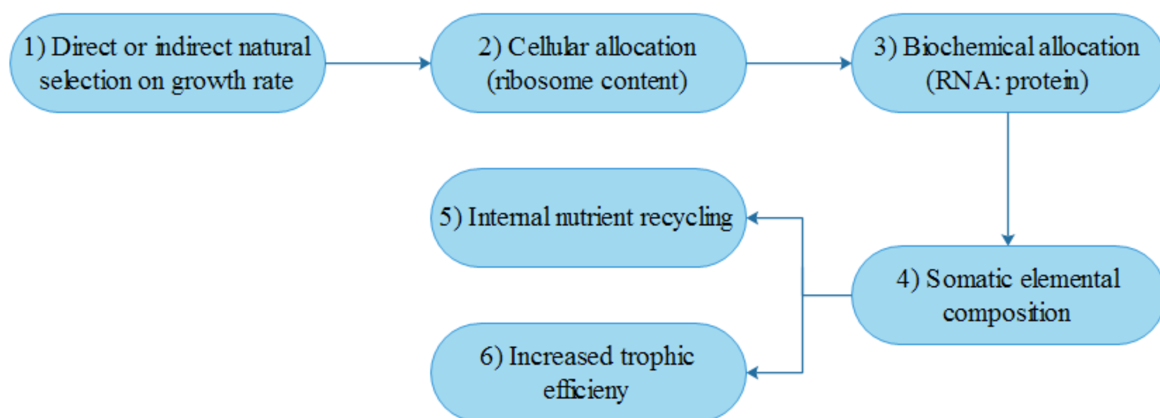


Figure 1. Conceptual diagram that shows how the mechanic for growth rate is connected with local environment and somatic elemental composition. (1) In certain habitats higher growth rate is favored by selection.(2) In order to grow more rapidly the organisms must raise their protein synthesis rate. This is done by allocation of P to production of P-rich ribosomes. (3) Since ribosomes are for the most part made from RNA, the consequence is that there is an increase in RNA:protein ratio in the cell. This change is largely due to rRNA.(4) Because RNA is a highly P-rich molecule, an increase in rRNA RNA implies that the organism needs a sufficient storage of P. (5) Because the high allocation of P to P-rich tissue, this leads to reduced internal nutrient recycling of P in rapid growing organisms. To sustain a high growth rate the P-rich organisms need to ingest more P leading to higher growth rate in organisms that are more efficient. This figure was obtained from Elser et al. (2000).

When algae have a limited supply of P, they may adapt by increasing the activity of the enzyme alkaline phosphatase. Alkaline phosphatase is a hydrolyzing enzyme that becomes active in times of stress due to P-limitation, making more P available in the cell (Litchman & Nguyen 2008). Hence alkaline phosphatase activity is a good indicator for P-limitation as demonstrated in several experiments (Dyhrman & Ruttenberg 2006; Wang & Liang 2014).

1.3 Genome size and cell size

The term C-value was first used to describe the amount of haploid DNA in a cell but has later been applied as a term for genome size (Swift 1950). The size of the genome and the total amount of DNA per cell varies greatly between different animals and plants, even between closely related species (Mirsky & Ris 1951). This variability in C-value irrespective of species complexity was coined "the C-value paradox". The paradox being that there is no apparent correlation between the genome size and the complexity of an organism (Thomas 1971).

The solution to the paradox is that the majority of the difference in C-value lies in amount of non-coding regions, but the role of these non-coding regions is to this day mostly unknown. Their ability to multiply and insert themselves as transposable elements (TE) has been instrumental to the selfish gene theory and the junk theory. The selfish-gene theory proposes that TE simple exist as "parasitic segments" (Doolittle & Sapienza 1980). The junk theory is the notion that dysfunctional genes will accumulate in the genome over evolutionary time (Ohno 1972). Regardless of their purpose, the TE has the possibility to regulate cell size to a certain extent, and a change in genome size has implications for the rest of the cell and ultimately the cell size itself.

Another process that may alter genome is polyploidization. This involves a partial or full duplication of the genome. It has been shown that this may be a strategy for organisms to combat stress (Scholes & Paige 2015). General studies have also shown that polyploidization generally leads to increased cell size (Otto 2007).

During the studies of the genome size, there have been several observations to support the notion that it is a strong positive correlation between cell size and genome size (Bennett 1987; Gregory 2001). The nucleoskeletal theory suggests a coevolutionary relationship between cell

size and genome size (Cavalier-Smith 1978). Generally genome size serves as a good proxy for cell size, and increased genome size should thus lead to increased cell size, and vice versa (Cavalier-Smith 1978; Gregory 2001; Hessen et al. 2013).

Another hypothesis is that genome size also could vary in size due to P limitation. The reason for this being that P is an essential element in growth and metabolic processes of the cell due to its important role in the synthesis of DNA, RNA and ATP (Sterner & Elser 2002). Under P-limiting conditions, studies suggest that P is reallocated from non-coding DNA regions to RNA (figure 2). This could result in a change in genome size under different selection pressure on growth rate (Hessen et al. 2010).

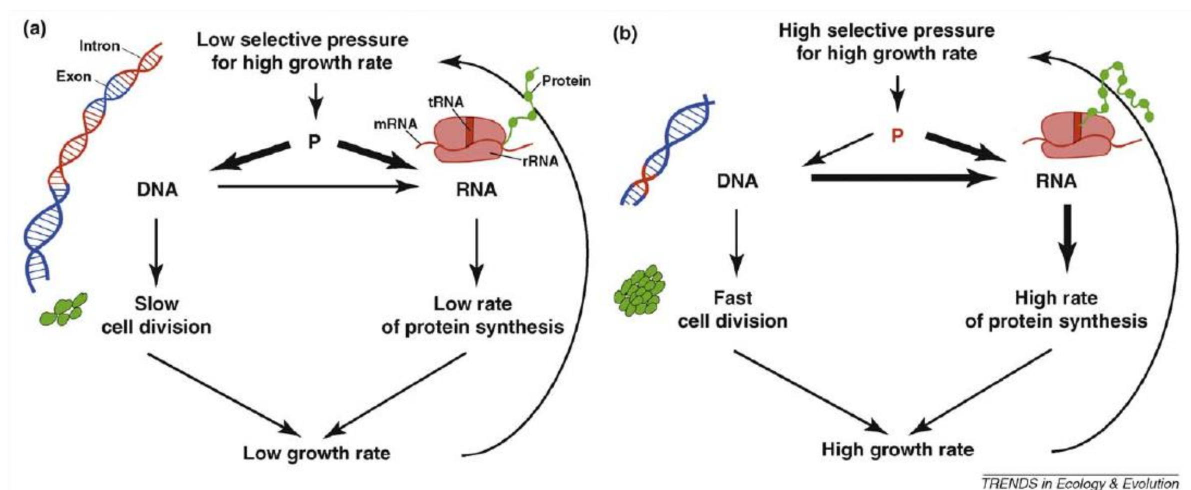


Figure 2. The two scenarios illustrate the ramification of different evolutionary consequence of allocating P to DNA or RNA. (a) Low selective pressure for high growth rate results in that a significant portion of P is allocated to DNA. This implies larger genome size, which cause slow growth rate and low rate of protein synthesis. (b) With Strong selection for high growth rate, there will be selective pressure for reallocating P from non-coding DNA to RNA. This is a necessity for promoting high growth rate. The thickness of arrows indicates relative importance of P allocation or causality. Scenario (a) indicates large genome, high intron:exon ratio, slow rate of protein synthesis and slow cell division as opposed to scenario (b). This figure was obtained from Hessen et al (2010).

1.4 Haptophyta and other algae in a changing climate

Microalgae are of great importance to the marine ecosystems and are responsible for the majority of carbon uptake in the oceans. The algae play a key role in the food web structure as primary producers. Through photosynthesis they utilize sunlight to convert inorganic carbon and water to organic carbon and O_2, and are responsible for approximately 45% of the global primary production (Field et al. 1998). They also serve important functions in other biogeochemical cycles. One algal group that is of particular significance in the ocean is division Haptophyta. Haptophytes has been given their name because of the *haptonema*, an appendage that may vary in length and superficially resembles a flagellum. It is mainly involved in food managing and attaching on to surfaces (Inouye & Kawachi 1994). Most haptophytes exist as planktonic forms in open and coastal waters, but some thrive in fresh water. They have chlorophyll *a* and *c*, and a cell covering that constitutes of organic or calcified scales (Edvardsen et al. 2000).

The haptophytes can occasionally form extensive algal blooms. These blooms can have profound ecological impact. An algal bloom is when the algae population is in a rapid increasing state, creating a high accumulation of biomass. One factor that causes algal blooms is high nutrient availability (Anderson et al. 2002). Two haptophyte groups of major ecological importance are orders Prymnesiales and Isochrysidales, both of which belong to class Prymnesiophyceae within division Haptophyta. In Prymnesiales there are several algae that can on occasion produce massive toxic blooms, also with large ecological impact (Edvardsen & Paasche 1998). Many coccolith-bearing haptophytes (coccolithophorides) also significantly contribute to the biological carbon pump by the vertical transportation of inorganic carbon (IOC) to the deep sea. The many functions of algae, both as a ground pillar in the food web and as a mediator in biogeochemical cycles, underlines the significance of mapping traits to changes in climate for a better understanding of climates impact on ecology and evolution.

1.5 Predictions and hypotheses.

One goal of this study is to test how specific growth rate in three different species of haptophytes is affected by a long-term exposure to phosphorus limitation at different temperatures. The underlying predictions are that growth rate is positively correlated with temperature and phosphorus treatment.

In order to support the fast growth rate there will be an allocation of P to P-rich rRNA molecules, according to the growth rate hypothesis. This implies that RNA is also expected to share a positive correlation with temperature and P-treatment. P-limited cultures are expected to show an increase in alkaline phosphatase activity (APA) as opposed to the P-rich cultures. This is simplified to two null hypotheses.

H_{0-1}: Temperature does not affect RNA content or APA.

H_{0-2}: Phosphorus availability does not affect RNA content or APA.

Another important aim of the study is to assess the long-term effects of temperature and P availability on cell size and genome size in the same 3 algal species. Cell size is predicted to be negatively correlated with temperature, and positively correlated with P-treatment. The genome size is expected to follow the same trends as the cell size. The null hypotheses are

H_{0-3}: Temperature does not affect the cell size.

H_{0-4}: Phosphorus availability does not affect the cell size.

H_{0-5}: Temperature does not affect the genome size.

H_{0-6}: Phosphorus availability does not affect the genome size.

These six hypotheses are an essential part of the puzzle to understand how fluctuations in the environment may impact algae.

2. Material and methods

2.1 Model organisms

In order to study the effect of phosphorus limitation and temperature, a long-term experiment with three different species of haptophytes was conducted. The species that were used in the experiment was *Emiliania huxleyi* (Lohmann) W.W.Hay & H.P.Mohler, *Chrysochromulina rotalis* Eikrem & Throndsen and *Prymnesium polylepis* (Manton & Parke) Edvardsen, Eikrem & Probert. These belong to the division Haptophyta and class Coccolithophyceae (=Prymnesiophyceae).

Emiliania huxleyi (figure 3a) was described in 1967 (Hay et al. 1967), and has become a popular model organism due to its widespread distribution, its major role in C-sequestration and marine production and its potential susceptibility to marine acidification. It typically has a cell diameter of 5-7 µm and is obligately autotroph (Graham et al. 2009). The laboratory stock of *E. huxleyi* used in this study (UIO212) was isolated in 2011 from outer Oslofjord (station OF2 near Missingene) by Shuhei Ota.

The second species, *Chrysochromulina rotalis* (figure 3b), was described in 1999 (Eikrem & Throndsen 1999). The genus is also known for its broad distribution, from polar to warmer waters, and a distinctively long haptonema. The species has a size range of 4-6 µm (Eikrem & Throndsen 1999). The C. *rotalis* strain used for this study (UIO044) was isolated at Torungen, Skagerrak in Southern Norway in Norway 1990 by Wenche Eikrem.

Prymnesium polylepis (figure 3c) was described in 1962 as *Chrysochromulina polylepis* (Manton & Parke 1962), but later moved to the genus *Prymnesium* (Edvardsen et al. 2011). It is highly euryhaline and euryterm, and is also known for producing toxic blooms like the event that caused fish death in 1988. The species has a wide size range from 4-30 µm. The *P. polylepis* strain used in this study (UIO037) was isolated from the Oslofjorden, near Torbjørnskjær, Skagerrak in Southern Norway in 1988, by Bente Edvardsen.

By using these three species from class Coccolithophyceae, it was possible to observe intra- and inter-species variability. This was done to assess whether the physiological responses to the change of temperature and phosphorus level were species specific, or more general for the whole algal group.

2.2 Medium

The algae where cultivated with 30 PSU IMR1/2 medium. The IMR1/2 is a medium that has half of the concentrations from IMR medium originally described by Eppley et al. (1967). Because this was for cultivating haptophytes, in addition to the chemicals from the original formulae, it was added 10 nM selenite to allow for better growth as shown in Appendix I (Edvardsen & Paasche 1992). The seawater used in the formulae was collected from Drøbak at approximately 40m depth. Prior to usage, the seawater was filtered through 1,2 µm mesh filter (Whatman GF/C). In order to get a salinity of 30 PSU, 900 ml l^{-1} of seawater was used in addition to 100 ml l^{-1} distillated water. After adding nitrate, phosphate and the rest of the solutions from the original formula, the medium was the post-filtered through a 0,22 µm mesh corning filter system (E&K Scientific), and pasteurized at 80°C for 15 min. The medium was then stored at 14°C.

2.3 Experimental design

The experiment was designed as a long-term, cross factorial setup in order to test the potential impact of temperature and phosphorus (P) on growth rate, cell size and genome size. The experiment involved three species of haptophyta, divided by two P-treatments and two temperatures. The cultures where cultivated in triplicates, resulting in 36 cultures.

Prior to the experiment the algae were cultivated with IMR1/2 medium for approximately 2 weeks at 16°C giving them a similar starting point. They were then moved to their respective temperatures for a time period of 7 days to allow them to acclimate before the experiment began. During the experiment these three species was cultivated at two temperatures, 13°C and 19°C and two levels of P in a factorial design. The low P-treatment (- P) was given a modified version of the IMR1/2 medium, with 2 µM phosphate. The high P-treatment (+P) received standard IMR1/2 medium with 25 µM phosphate. This results in a N:P ratio in the +P-treatment of 10 (mol:mol), and 124 (mol:mol) in the –P-treatment (table 1). The –P-treatment were treated as chemostats and semi continuously diluted with a constant volume (V). The dilution fraction equaled 1:2 (V:V) in *E. huxleyi* and *C. rotalis* and 2:5 (V:V) in *P. polylepis*. Chemostats are characterized by the constant supply of medium, without regards for density of the cultures. This allows for the cultures to grow to a higher density and stabilize at a stationary phase. The stationary phase is governed by the P limitation. In the stationary phase growth rate equals dilution rate.

Table 1. The relative N:P ratio in the IMR1/2 medium for different P-treatments (Edvardsen et al. 2013).

P-treatment	Amount of stock solution added (µl l^{-1})		Final concentration in media(µM)		Ratio
	KH_2PO_4	KNO_3	KH_2PO_4	KNO_3	N:P
+P-treatment	500	500	25	248	10
-P-treatment	40	500	2	248	124

The +P cultures were treated as turbidostats, meaning that after measurements of cell densities every 2-3 days the +P cultures was diluted accordingly to a constant number of cells. The cultures were diluted down to 50 000 cells ml^{-1} for *E. huxleyi* and *C. rotalis* and 100 000 cells ml^{-1} for *P.polylepis*.

By being diluted to a relatively low cell number and with a rich medium, the turbidostats were allowed to grow at their maximum growth rate without limitations. The reason for the difference between the dilution rates of particular algal species in turbidostats and chemostats was that *P. polylepis* had a much slower growth rate then *E. huxleyi* and *C. rotalis*. It therefore was treated accordingly. By combining turbidostats and chemostats it was possible assess the difference in how algae that experience P limitation responded, opposed to algae that was not limited. The stable dilution rate of the turbidostats, along with the first 100 days of *E. huxleyi* was cultivated and thought out by Nita K. Shala

All experiments were run in 40 ml nunclon filtercap flasks (Thermo Scientific). The cultures receives both cool white light and warm white light, 36W with a light intensity of 170 µmol photons $m^{-2}s^{-1}$. The light: dark-cycle was set to 14:10 hours. The experiment lasted approximately 280 days for *E. huxleyi*, and around 180 days for *C. rotalis* and *P. polylepis*.

During the study, different parameters were examined by various methods that are currently used in the assessment of culture and cell characteristics. First, the measurements incorporated the traditional estimations of number and size of cells, as well as culture growth rates. Second, stoichiometry, RNA and alkaline phosphatase analysis and flow cytometry was applied for estimating biochemistry of the cell and genome size. Using this approach allowed for the evaluation of the effect from P limitation and temperature on several parameters. This provided diverse, but comparable and highly relevant data. This could lead to the more general insight into the responses of coccolithophores to the changes of temperature and phosphorus levels.

2.4 Measurements of number and size of cells, and growth rate

A main goal of this study was to assess effect on size and growth rate of algae. These more traditional measurements provided the significant insight into the responses to the P-treatment and temperature on the levels of populations. The number and size of cells in the cultures were measured by the use of CASY electronic cell counter (Scharfe system Gmbh). Before analyzing the samples from the different cultures, they were diluted 1:10-1:100. They were diluted in order to be below the detection range of the electronic cell counter. The samples where diluted in a sterile room with IMR1/2 medium that was filtered through a 0.20 µm mesh filter.

The CASY electronic cell counter system utilizes a combination of a particle measuring technique that is referred to as the resistance measurement principle, with a more modern method called pulse area analysis. Such a technique is ideal for quality and quantitative measurements of cell culture.

The electronic particle counter's measuring is made up of an electrode on the outside and a glass pipe with another electrode on the inside. By applying a pulsed low voltage field to the measuring pore in between the two electrodes, it creates a defined electric resistance. When electrolytes (saline solution) and cell gets sucked into the pipe it has to pass the measuring pore at the bottom. The particle functions as an isolator, and creates a pulse increase in electric resistance when it passes the pore. The resistance is correlated with area of the cell. From this the machine assumes the particle to be spherical and calculates volume and diameter of the cell. Cells pass through the pore individually and the measuring signal is scanned at a high frequency. The amount of electric pulses registered gives the sample concentration. The electronic cell counter can count particles between ca 3 and 80 µm with the orifice of 150 µm. Below this size the cells are difficult to separate from background noise and debris. Cell size was measured with two different values mean cell size (MDI) and median cell size (PDI) at random throughout the experiment, after the cultures had stabilized.

During the experiment, after the chemostats reached (almost) stable cell growth rate, the BioTek synergy MX plate reader was incorporated as a means to more efficiently analyzing of quantitative data through. The plate reader was a necessity to incorporate due to the rather time-consuming use of the CASY electronic particle counter. The BioTek plate reader

measured in vivo fluorescence of chl *a*, and provided a decent estimate for culture density. The estimate correlated nicely with the data from the electronic particle counter. When cell size was not recorded with the electronic cell counter, it was supplemented with BioTek plate reader FL MX (BioTek, USA) that was calibrated and programmed according to preliminary CASY data with the assistance of Dr. Marcin Wojewodzic (UiO).

The mass specific growth rate (d^{-1}, MSGR) was determined as the relative change in cell abundance between two points in time. It was calculated with the formula:

$$MSGR(P-) = Log\ (N_1/(N_0/\ DF)\ /d$$

$$MSGR(P+) = Log\ (N_1/N_0)\ /d$$

In this case N_0 is the first measurement, and N_1 is relative abundance at the second measurement, d equals days in between. The dilution factor (DF) were equal to the total amount (40 ml) divided by the volume left after dilution. For *E. huxleyi* and *C. rotalis* DF=2, while for *P.polylepis* DF=1.6.

2.5 Stoichiometry

The ratio between carbon (C), nitrogen (N) and phosphorus (P) was estimated primarily in order to verify that the algae with –P-treatment did exhibit desired phosphorus limitation. Secondly, the content of C, N and P in the different cultures was mapped to observe the relative ratio between the elements.

Prior to the C-analysis, the algae in particular samples were counted by CASY and collected on a GF/C filter. A portion of particulate C in *E. huxleyi* would likely be allocated to the calcified structures. Hence in order estimate the amount of particulate organic carbon (POC) and particulate inorganic carbon (PIC), extra samples of *E. huxleyi* were treated with 2M hydrochloric acid (HCl) to remove PIC (Langer et al. 2009). The filters were then placed in a furnace at 530°C for a time period of 4 hours before being analyzed for C and N on a Thermo Finnegan EA 1112 series flash analyzer (Thermo Fisher scientific). The machine deduces the amount by burning small amounts of the samples in pure oxygen (Dumas method or flash combustion). The end product of combustion is then flushed through a catalyst where it is transformed to NO_2 and CO_2 at 1800°C. The product was then transported by a stream of helium gas into another chamber that is filled with copper. Copper reduces the nitrogen oxide

(NO_2) into nitrogen gas (N_2) These are again separated by an gas chromatic column and detected and estimated through their individual thermal conductivity (ThermoFisherScientific 2009).

The amount of total P was estimated by adding a solution of potassium peroxydisulfate ($K_2S_2O_8$) to the sample. In this case the samples were soaked in 10 ml of a 1% solution of potassium peroxydisulfate for 30 minutes at 120°C. After the incubation was complete the samples where processed by a BRAN+LUEBBE autoanalyser III (Bran Luebbe, Norderstedt Germany), with the method Nr. G-297-03 (multitest MT). This is called a colorimetric method, which means that it creates colored complexes that are detected in a spectrophotometer. The product is a combination of orthophosphate, molybdate and antimony. At pH< 1 the complex is reduced to ascorbic acid. This is a blue phosphorus-molybdate complex that can be measured at 880 nm wavelength.

The measurements were done 3 times throughout the experiment, at the beginning, middle and end. All measurements of C, N and P were performed by Berit Kaasa (UiO). The data was than normalized against the amount of cells for the respective samples to gain content per cell.

2.6 RNA.

Cellular concentrations of RNA was also included both as a proxy of growth rate and to judge the effect of P-limitation. The RNA_{total} also serves as a good indicator for the amount of rRNA (Flynn et al. 2010). The rRNA is one of the main components in the ribosomes and of high interest with regards to the growth rate hypothesis. In cases of P limitation, the growth rate hypothesis predicts a positive correlation between rapid growth and rRNA (Elser et al. 2000). A modified version of the RiboGreen fluorescence protocol (Turner BioSystems) was applied for measuring of the RNA content.

Prior to the isolation of RNA, the individual sample size was estimated by the use of the particle counter CASY. This was done in order to assess RNA content per cell. Secondly, 1-4 ml from the samples was extracted, filtered down and captured on a filter (nitrocellulose-membrane, 0.65 µm DAWP, Millipore). The samples was stored in nuclease free micro-centrifuge tube and swiftly snap frozen in liquid nitrogen before it was stored in a -80°C

freezer unit. At the day of the RNA analysis, each sample was placed on ice to minimize degeneration, and was added 120µl of the extraction buffer (1% sarcosyl, Sigma). The samples of *Prymnesium polylepis* were added 200 µl of the extraction buffer instead of the initial amount, because of the lesser amount of cells in the sample. The different amount of extraction buffer did however not change the extraction buffer: TE ratio as mentioned later. After the extraction buffer was added, the filters were drenched in the buffer by the use of sterile pipette tips to ensure that the entire filter was in contact with the solution. While still frozen, the samples were homogenized by ice-cold sonification process, executed with Branson Sonifier, S450A in a cuphorn (Brandson 101147048) for two minutes at duty cycle and output 80%. Once this process was done, the samples was again put on ice, and diluted 1:5 ratio with TE buffer (10 mM Tris-HCL,1 mM EDTA, pH 7.5). The duplicates with 75 µl from each sample were produced in individual slots of a 96-well plate (655076 Greiner Bio-one, USA). The duplicates were inserted pairwise into columns after the first column, which was reserved for standard. Into each one of the first duplicate columns a total of 20 µl RNase-free water (Gibco BRL1071) was added. The other duplicates was then added 20 µl of 0.1% RNase A (A7973, Promega). Immediately after the RNase mixture was added, the well plate was heat incubated at 37°C on a shaking table with the output 400RPM to ensure homogenous dispatch of the RNase, and digestion of RNA. After the incubation, 75 µl of 100 x diluted RiboGreen dye (R-11490, Molecular probes, USA) was added to each well by the use of an automatic 8-channel pipette. The plate was then inserted and analyzed by fluorescence using the BioTek plate reader FL MX (BioTek, USA) with excitation wavelength of 480 nm (bandwidth 20 nm) and an emission wavelength of 525/20nm (sensitivity 0.9).

The RNA analysis were conducted in cooperation with Dr. Marcin Wojewodzic (UiO) and normalized against cell numbers.

2.7 Alkaline phosphatase

Alkaline phosphatase is a hydrolase enzyme that is expressed when algae are experiencing a lack of phosphorus access. It serves well as a biomarker for phosphorus limitations. The enzymes physiological function of enzyme is to sever and remove bound phosphorus linked groups from molecules in the cytosol and thereby to make more phosphorus accessible to the cell. During the experiment the amount of alkaline phosphatase was estimated by the use of CDP-star chemo-luminescence method (Wojewodzic et al. 2011).

The samples were collected in a manner identical to that of the RNA samples and stored in a -80°C freezer unit. The day the analysis occurred, each sample was placed on ice to minimize degeneration and added 0,3 ml of Triton X-100 (T8787, Sigma) After the extraction buffer was added, the filters was pushed down and drenched in the buffer by the use sterile pipette tips to make sure that all of the filters were in contact with the buffer solution. While still in the frozen state, the samples were homogenized by ice-cold sonification process, executed with Branson Sonifier, S450A in a cuphorn (Brandson 101147048) in a similar matter to the RNA process.

The standards were then prepared by using AP type VII-S from bovine intestinal mucosa (P5521, Sigma). The standard curve ranged from 2-100 uU of the AP, diluted with 1% Triton. A unit is in this context the amount of enzyme that is required to hydrolyze 1 µM 4nitrophenyl phosphate/minute, at PH9.8 and a temperature of 37°C. After the preparation of the standards 20 µl of both the standards and the samples was transferred to a Pyrophosphate-free 96-well plate (Nunc, 236105), preserved on ice.

Afterwards 20 µl of 0,4 mM CDP-star was dispensed with and automatic 8-channel pipette to all the wells. The plate was put in the BioTek plate reader FL MX (BioTek, USA) for measurement. The hydrolase enzyme cuts phosphate rich groups on the CDP and thereby degrades it. Photons are released when the bounds are cut making it easily detectable for the plate reader. The machine was set to take measurement every 10 minutes for 1,5 hours. This resulted in a curve, which was integrating in order to make a calibration curve. The calibration curve was then used to find how much activity there was of AP in the samples.

The AP analysis were done in cooperation with Dr. Marcin Wojewodzic (UiO) and normalized against cell numbers.

2.8 Flow cytometry

Flow cytometry is a useful method to estimate genome size. It is a high-throughput analyzing tool that allows for detection of size in a high abundance of particles within a relatively short period of time. If there is a difference in ploidy level in a population, or other kinds of heterogeneity, this can also be identified in this test. This method has been applied in similar studies (Dolezel et al. 2007; Jalal et al. 2013), and is widely considered applicable to measuring genome size (Kron et al. 2007).

The Flow cytometry was used for estimating the cell size and DNA content (C-value). The FACS Calibur machine is composed of three components; flow system, optics and electronics. The flow system moves the particles in a single file past the center of detection, the focus point of the light. By moving it past this point the machine is able to detect objects through light scattering signals and fluorescence that is recorded by different sensors. By having the light scatter at two different angels the machine is able to detect several internal and external characteristics of each particle.

There are different categories of fluorescence detected, along with Forward scatter (FSC) and sider scatter (SSC). The FSC increase with an increased particle size, and SSC changes with difference in internal structure and granularity. Flow cytometry is useful since it allows for detection of size and fluorescence in a high abundance of particles within a relatively short period of time. If there is a difference in ploidy level in a population this can also be identified with this test.

In this work, flow cytometry was used for estimating the cell size and DNA content (C-value). The following paragraph is a description of the protocol that was used during this experiment, modified from Jalal et al. (Jalal et al. 2013). Prior to the flow cytometer analyzing the cultures was counted using the CASY, and 1 ml of the culture was collected as sample from each and placed in 2 ml nuclei free tubes. The living samples were centrifuged for 5 min at 1200 rpm/1230G at 10°C in Heraeus multifuge x3r, with Tx750 swinging bucket rotator (Thermo scientific). Afterwards excessive fluid was removed, and the algae was mixed with 400 µl grinding buffer (10 mM Tris-HCL, 10 mM CaCl2, 3 mM MgCl2, 0.5 % Nonidet P-40, pH 7.4) as in (Korpelainen et al. 1997). The grinding buffer breaks down the cells and intercellular material except for the nuclei. Afterwards in the dark and while the samples

where on ice 5 µl RNase was added to ensure that all RNA was fully denatured so that the iodine would only color the DNA. For the coloring process 50 µl of propidium iodide (PI) was added to each sample. Fresh (24-48h old, with added heparin) blood cells from chicken (*Gallus gallus domesticus,* chicken red blood cells, CRBC) were added to a mixture of grinding buffer, and were used as an internal and external standard (Galbraith et al. 1983). CRBC is an ideal standard because of the knowledge of its DNA content. The density of CRBC was 5.0x 10^5 cells ml^{-1} grinding buffer. This was followed by 1 hour of dark-incubation on ice to allow for the PI to bind. Samples were lightly mixed to ensure that the nucleii would not break, before they were transferred and filtered in to the test tube through a BD cell strainer filter cap with 35 µm mesh size (BD Falcon, ref 352235). In addition to the CRBC, 2.5 µm alignment beads (P-14831, Invitrogen Corporation, USA) was applied to ensure constant amplification and sample rate throughout the experiment. The analysis was done using FACS Calibur flow cytometer (Becton Dickinson, San Jose, USA) equipped with a 15 mW 488 nm air-cooled argon-ion laser and a standard filter set-up.

In order to quantify the data the software Cellquest Pro software package (Becton Dickinson, San Jose, USA) and FlowJo (Tree Star inc, USA) was then applied to analyze the data. To identify populations, data from all channels were used. The c-value (pg DNA $nucleus^{-1}$) was calculated according to the formula (Galbraith et al. 2001), for all calculations it was assumed that the governing ploidy level was diploid;

$$\text{Sample 2C DNA content (pg nucleus)} = \frac{\text{(Sample 2C peak mean)}}{\text{(Standard 2C peak mean)} * \text{ Standard DNA content (pg nucleus}^{-1})}$$

The flow cytometry analysis was performed by Dr. Marwa Jalal (UiO), and analysis of the data was done in collaboration with Per Færøvig (UiO).

2.9 Statistical method and modelling

All statistical calculations and modelling were executed with R. studio and R v3.1.1 (R Development Core Team, 2014). The method and modulation utilized from the software builds upon the assumption that the relationship can be analyzed with linear models. This assumption again builds upon that the data are independent and have normal distribution.

However, because the experimental design utilized semi continues cultures, there was a possibility that type 1 error could occur due to effects from pseudo replication. Pseudo replicates are either samples where experimental units are not independent or where treatments are not replicated (Hurlbert 1984). The samples were taken from the semi continues cultures between the time of 8-12 a.m., but since the same flasks were sampled each time it can be argued that these are not entirely independent. Equal light:dark periods can synchronize the cell cycle (Tamiya et al. 1953). However, variation could occur, due to different synchronization of the cell cycle caused by the different growth rate. This can affect size and density measures. Therefore, certain statistical steps were necessary to be done in order to minimize potential error. The data from approximately, 360 samples from CASY cell counter (MDI and PDI), and BIOTEK plate reader (MSGR). Through the function aggregate the samples was collected and involved in creating a new dataset with 12 mean values.

The nature of sampling regarding CNP, Alkaline phosphatase, RNA and Flow cytometry however differed from the CASY and BioTek analysis, and was therefore treated differently but for the same reason. Because fewer samples were taken in different stages of the experiment only the last measurement was taken into account. These samples is also the ones that best represent the physiological factors that coincide with the CASY and BioTek plate reader FL MX (BioTek, USA) aggregated data since it was in the same stage of the experiment.

3. Results

The first part of the results presents the analyses of data collected throughout the experiment. The results the mass specific growth rate (d^{-1}, MSGR) changes in response to different temperatures and concentrations of phosphorus (P) over time. To further confirm the P limited status of the algae, the specific contents of C, N and P, RNA and alkaline phosphatase activity (APA) was measured during the early phase of the time series and at termination and will be presented in context with the growth rate. The next section presents the cell size and genome size results. Nucleus size and DNA content (pg DNA $nucleus^{-1}$) was estimated several times throughout the experiment to see if there were changes in these parameters in response to temperature and P status over time.

3.1 Growth rate

The concentration of P was more important than temperature as a determinant of growth rate for all species, as shown by the analysis of variance (ANOVA). The temperature, P-treatment and interaction between temperature and P-treatments together explain approximately all of the observed variation (99.7%) in the species *Emiliania huxleyi* (table 2). The growth rate (d^{-1}) was significantly higher in the cultures with the +P-treatment at both temperatures (figure 3). The growth rate in the –P cultures was similar at 13 and 19°C, and was not clearly affected by temperature as the +P cultures was. Even though temperature, P-treatment and interactions of these appear significant, treatment alone explains close to 94.9% of the observed difference. The significant interaction pattern observed in table 2 indicates that the different P treated algae responds to temperature differently.

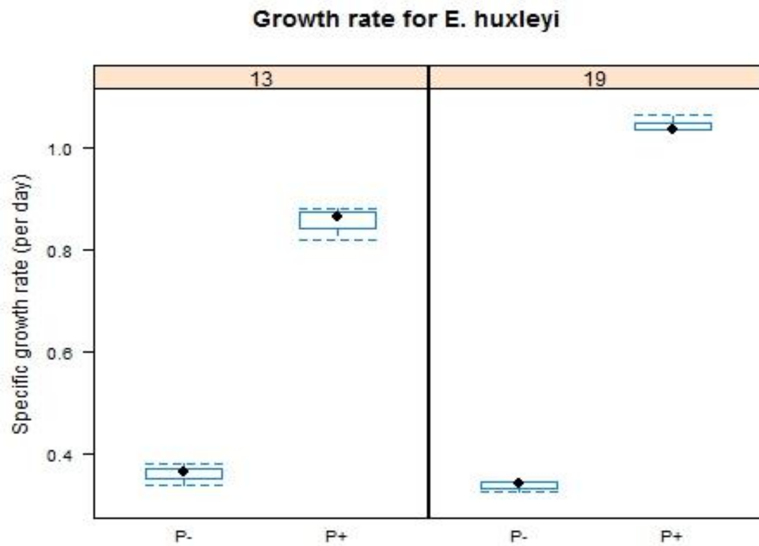
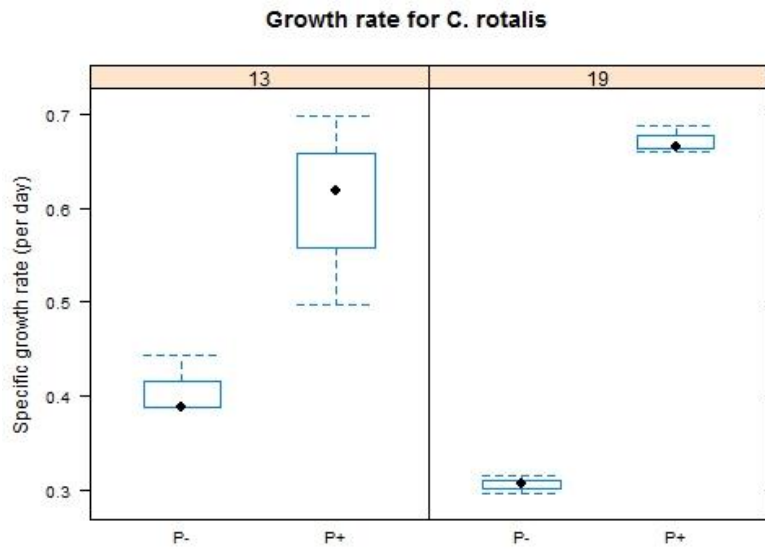


Figure 3. Variation in the MSGR (d^{-1}) to *Emiliania huxleyi* in response to two different P-treatments (-P=phosphorus limited, +P=phosphorus rich) and two different temperatures (13°C and 19°C).

For *Chrysochromulina rotalis* there was also a significant response in growth rate to the P-treatments, and 84% out of the 91.7% explained where due to P-treatment alone (table 2). There was no significant effect of temperature alone, but a weak interaction effect, and a similar pattern regarding highest and lowest average growth rate as observed in the *E. huxleyi* (figure 4A).

The MSGR (d^{-1}) of *Prymnesium polylepis* had a corresponding response as in *E. huxleyi*, with major impact of P-treatment and a minor, additive impact of temperature. The linear model explained 96.4% of the observed difference, of which 85.4% was related to P-treatment (table 2). Somewhat surprisingly, the highest growth rate was found in the 13°C cultures, however. Also the interaction pattern is reversed if compared with *E. huxleyi*. In *E huxleyi* the gap in growth rate became larger as the temperature increased, while for *P. polylepis* it is the opposite (figure 4B).

A



B

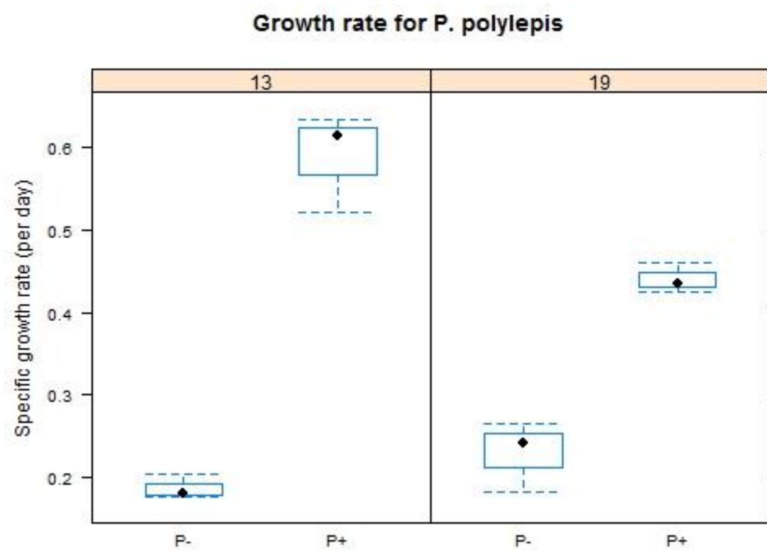


Figure 4. Variation in MSGR (d^{-1}) to (A) *Chrysochromulina rotalis* and (B) *Prymnesium polylepis* in response to two different P-treatments (-P=phosphorus limited, +P=phosphorus rich) and two different temperatures (13°C and 19°C).

Table 2. ANOVA table of MSGR (d^{-1}) that shows to what extent the different variables effect the growth rate in all species. The PVE column added to the ANOVA shows percentage variation explained by the individual factors (total PVE=R^2).

Species	Source of variation	DF	SS	MS	PVE (%)	F-stat.	P-value
E.hux.	P-treatment	1	1.08616	1.08616	0.949	2359.444	3.570e-11 ***
	Temperature	1	0.02077	0.02077	0.018	45.123	0.00015 ***
	P-Treat. X temp.	1	0.03397	0.03397	0.030	73.790	2.607e-05 ***
	Total	3	1.14458	1.14136	0.997		
	Residual	8	0.00368	0.00046			
C.rot.	P-treatment	1	0.238208	0.238208	0.840	81.0605	1.848e-05 ***
	Temperature	1	0.000856	0.000856	0.003	0.2911	0.60418
	P-Treat. X temp.	1	0.021035	0.021035	0.074	7.1581	0.02812 *
	Total	3	0.283608	0.263038	0.917		
	Residual	8	0.023509	0.002939			
P.pol.	P-treatment	1	0.283501	0.283501	0.854	187.4334	7.807e-07 ***
	Temperature	1	0.008543	0.008543	0.026	5.6481	0.044785 *
	P-treat. X temp.	1	0.027968	0.027968	0.084	18.4909	0.002615 **
	Total	3	0.332112	0.321525	0.964		
	Residual	8	0.012100	0.001513			

3.2 Stoichiometry

The specific and relative concentrations of C, N and P also responded strongly to nutrient treatments, and to a variable degree also on temperature.

In *E. huxleyi* the –P treated cultures had a significantly higher C:P ratio compared to the +P-treatments (figure 5). The linear model shows that 92.1% of the variation observed is accounted for in the C:P measurements, as shown in the ANOVA (table 3). Somewhat strangely there was no significant observed effect in particulate carbon from the HCL treated filters, which means there was no significant difference in particulate organic carbon (POC) and particular inorganic carbon (PIC) according to these data.

The data from *C. rotalis* also showed a trend that the cultures with –P-treatment had a significantly higher C:P ratio (figure 6A), the same trend observed in *E. huxleyi*. The model used explains 94.8%, out of this 73.1% is explained by difference in phosphorus treatment (table 3). There was no significant temperature or interaction effect observed in the *P. polylepis*. With regards to *P. polylepis*, the same trend observed in the other species was also observed in the +P treated cultures (figure 6B). The model presented explains 80.5% (table 3).

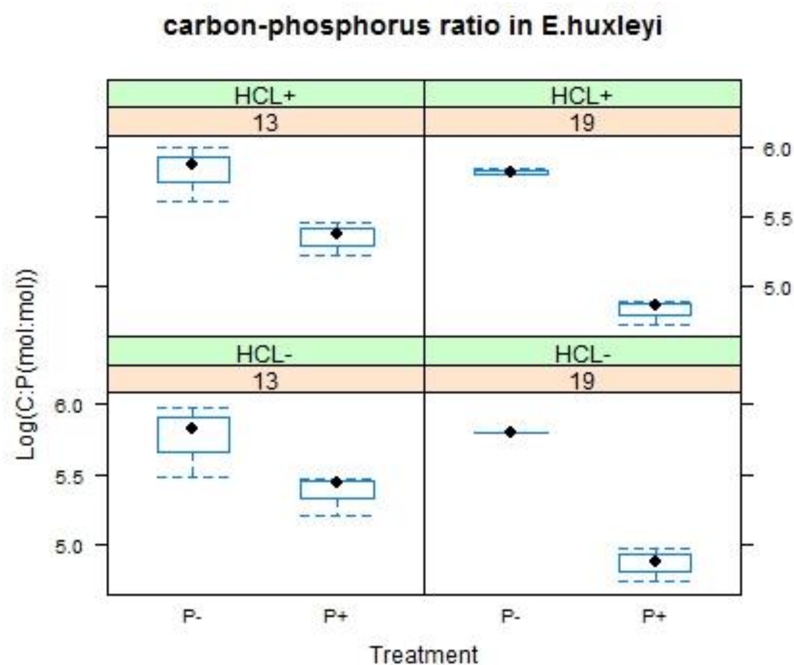
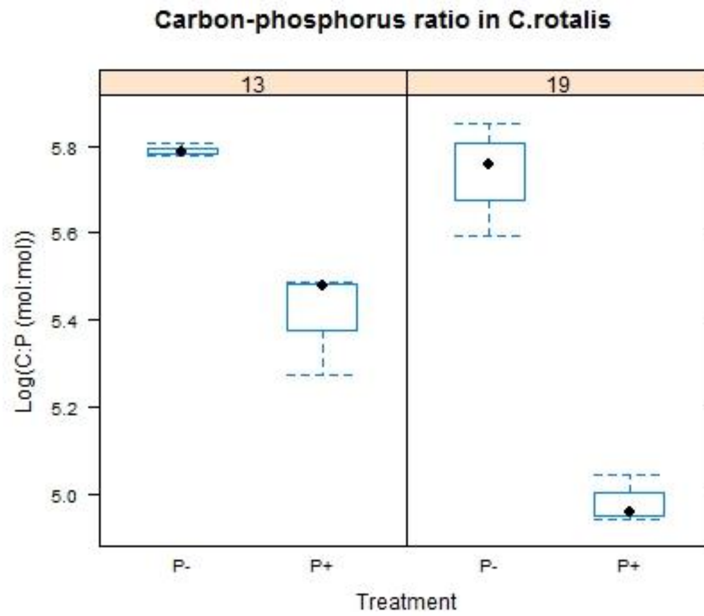


Figure 5. The ratio of carbon and phosphorus (mol:mol) in *Emiliania huxleyi* as response to two different treatments and two different temperatures within samples treated with hydrochloric acid (top), and samples not treated with hydrochloric acid (bottom).

A



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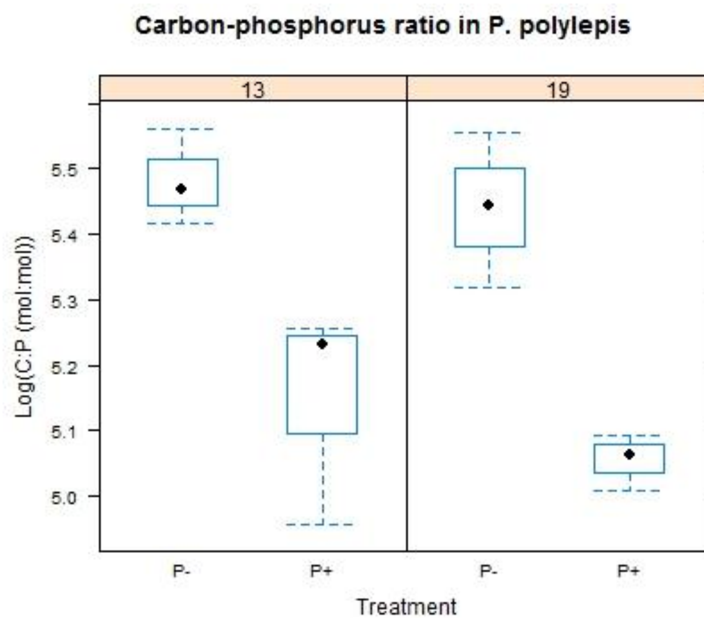


Figure 6. The ratio of carbon and phosphorus (mol:mol) in (A) *Chrysochromulina rotalis* and (B) *Prymnesium polylepis* as response to two different treatments and two different temperatures (13°C and 19°C).

Table 3. ANOVA table shows how the explanatory variables affect the carbon: phosphorus ratio in all species. The PVE column added to the ANOVA shows percentage variation explained by the individual factors (total PVE=R^2).

Species	Source of variation	DF	SS	MS	PVE	F-stat.	P-value
E.hux.	P-treatment	1	2.92206	2.92206	0.722	145.5654	1.902e-09 ***
	Temperature	1	0.37552	0.37552	0.093	18.7067	0.0005226 ***
	HCL	1	0.00022	0.00022	0.000	0.0107	0.9188229
	P-Treat. X temp.	1	0.42026	0.42026	0.104	20.9356	0.0003110 ***
	P-treat x HCL	1	0.00791	0.00791	0.002	0.3939	0.5390893
	Temp x HCL	1	0.00102	0.00102	0.000	0.0507	0.8246630
	P-treat x temp x HCL	1	0.00022	0.00022	0.000	0.0112	0.9170156
	Total	7	4.04839	3.74728	0.921		
	Residual	16	0.32118	0.02007			
C.rot.	P-treatment	1	0.95795	0.95795	0.731	111.739	5.6e-06 ***
	Temperature	1	0.17751	0.17751	0.135	20.706	0.001874 **
	P-Treat. X temp.	1	0.10608	0.10608	0.081	12.373	0.007874 **
	Total	3	1.31012	1.25011	0.948		
	Residual	8	0.06858	0.00857			
P.pol.	P-treatment	1	0.38713	0.38713	0.773	31.7603	0.0004895 ***
	Temperature	1	0.01420	0.01420	0.028	1.1647	0.3119617
	P-treat. X temp.	1	0.00189	0.00189	0.004	0.1553	0.7038432
	Total	3	0.50073	0.41541	0.805		
	Residual	8	0.09751	0.01219			

Regarding the ratios between other elements ratio, the analyses were done and their results are presented similarly to the analyses of C:P ratio. The C:N ratio in *E. huxleyi* had a significant higher ratio in the +P-treatment (figure 7). Otherwise, the C:N ratio showed no significant differences in response to the effects of other parameters on *E. huxleyi*. Also there was no significant difference in in response of *C. rotalis* and *P. polylepis* to any parameter (figure 8). However, *C. rotalis* shows a non-significant response in 13°C that are similar in trend as the E. huxleyi. It has to be stressed again that there was no differences in this ratio between the samples of *E. huxleyi* that were treated with HCL and those treated without HCL (table 4).

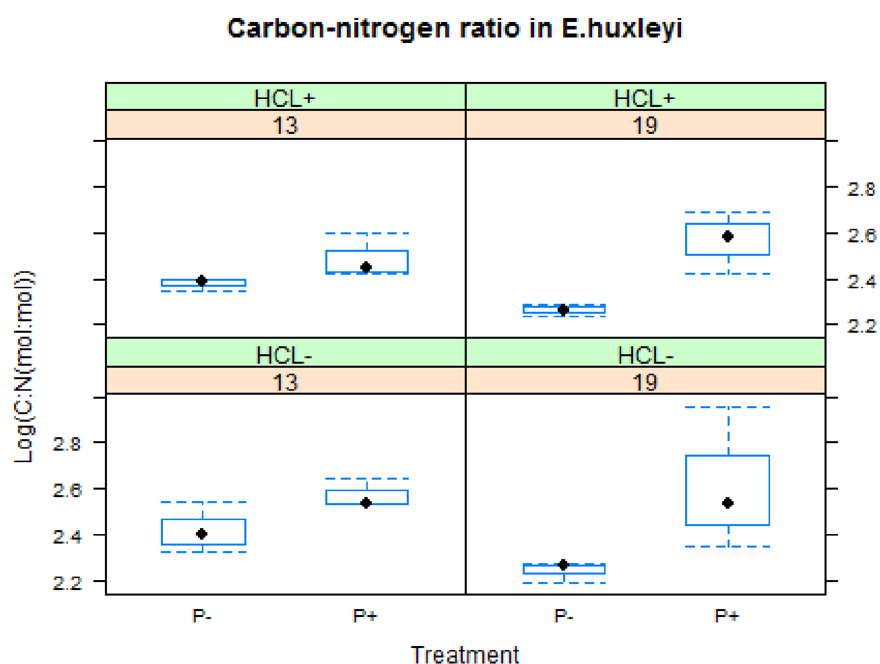
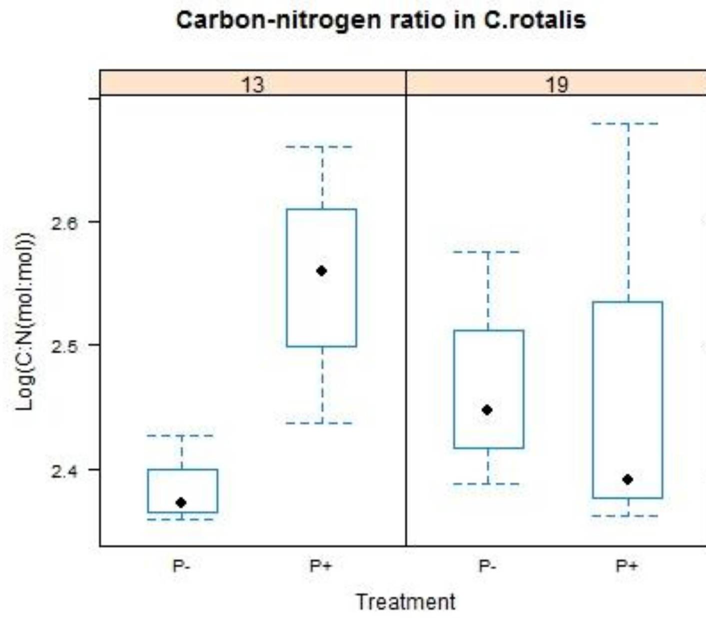


Figure 7. The ratio of carbon and nitrogen (mol:mol) in *Emiliania huxleyi* as response to two different treatments and two different temperatures (13 °C and 19 °C).

A



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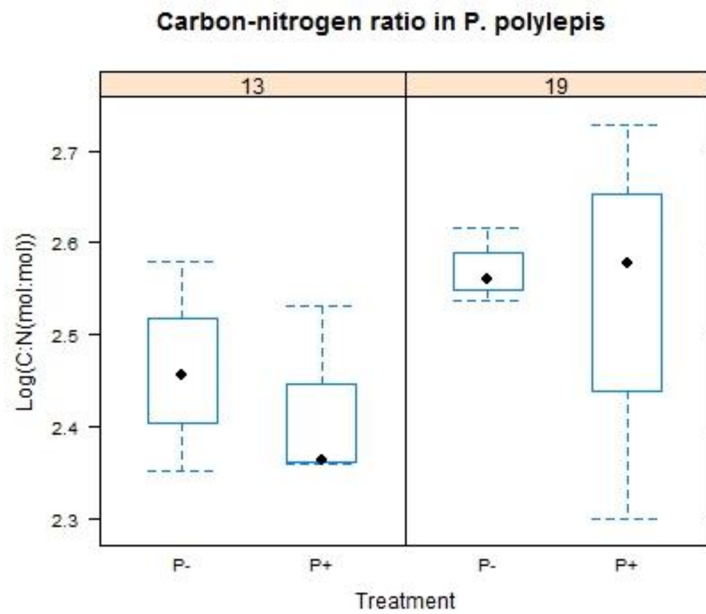


Figure 8. The ratio of carbon and nitrogen (mol:mol) in (A) *Chrysochromulina rotalis* and (B) *Prymnesium polylepis* as response to two different treatments and two different temperatures (13 °C and 19 °C).

Table 4. ANOVA table shows how the explanatory variables effect the carbon: nitrogen ratio in all species. The PVE column added to the ANOVA shows percentage variation explained by the individual factors (total PVE=R^2).

Species	Source of variation	DF	SS	MS	PVE	F-stat.	P-value
E.hux.	P-treatment	1	0.32502	0.32502	0.460	18.0097	0.0006192 ***
	Temperature	1	0.01142	0.01142	0.016	0.6328	0.4379852
	HCL	1	0.01015	0.01015	0.014	0.5622	0.4642774
	P-Treat. X temp.	1	0.06320	0.06320	0.089	3.5018	0.0797015 .
	P-treat x HCL	1	0.00428	0.00428	0.006	0.2373	0.6327412
	Temp x HCL	1	0.00376	0.00376	0.005	0.2081	0.6543827
	P-treat x temp x HCL	1	0.00016	0.00016	0.000	0.0087	0.9270459
	Total	7	0.70674	0.43604	0.591		
	Residual	16	0.28875	0.01805			
C.rot.	P-treatment	1	0.022570	0.022570	0.152	1.6869	0.2302
	Temperature	1	0.000074	0.000074	0.000	0.0055	0.9425
	P-Treat. X temp.	1	0.018772	0.018772	0.126	1.4030	0.2702
	Total	3	0.148454	0.054796	0.279		
	Residual	8	0.107038	0.013380			
P.pol.	P-treatment	1	0.004966	0.004966	0.027	0.2779	0.6124
	Temperature	1	0.038552	0.038552	0.207	2.1574	0.1801
	P-treat. X temp.	1	0.000062	0.000062	0.000	0.0035	0.9546
	Total	3	0.186536	0.061450	0.234		
	Residual	8	0.142956	0.017870			

In case of the N:P ratio, the analyses revealed approximately similar trends as for the C:P ratio. As evident from table 5, the P-treatment was again the most important factor determining the N:P ratio within all algal species. Identically to the results of C:P ratio, there were detected lower N:P ratio in the +P-treatments, reflecting elevated cellular P-content in the +P-treatments (see figure 7 for *E. huxleyi* and figure 8 for *C. rotalis* and *P. polylepis)*. In both *E. huxleyi* there was again a significant interaction effect, as previously shown in the C:P ANOVA. However there is no temperature effect with regards to the N: P ratio in *E. huxleyi*. For *C. rotalis* however there is a temperature effect but no interaction effect.

Therefore, it can be claimed that the cultures of all three species undergoing the P- treatment were most probably P limited. Such a stage within the experiments was desirable in order to investigate the effect of P on algal growth rate.

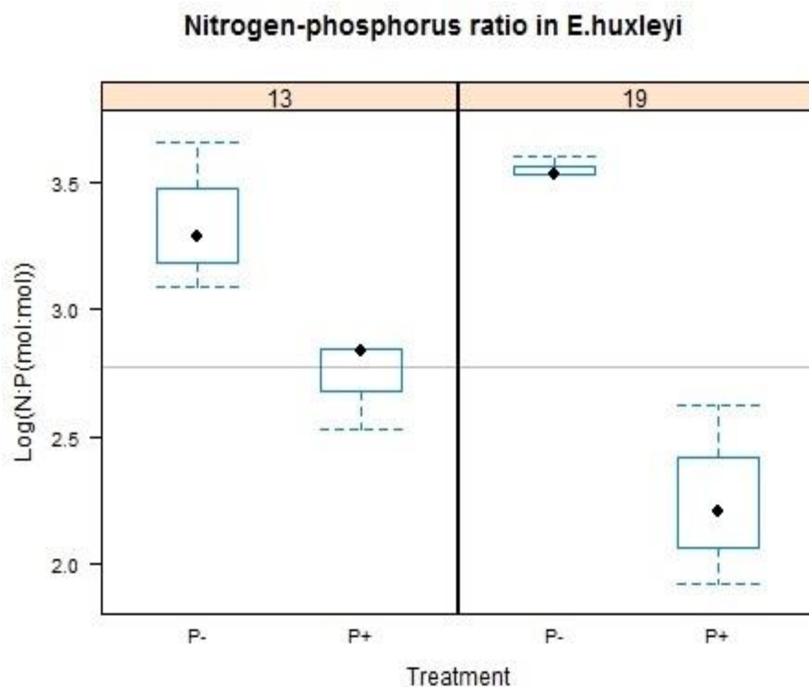
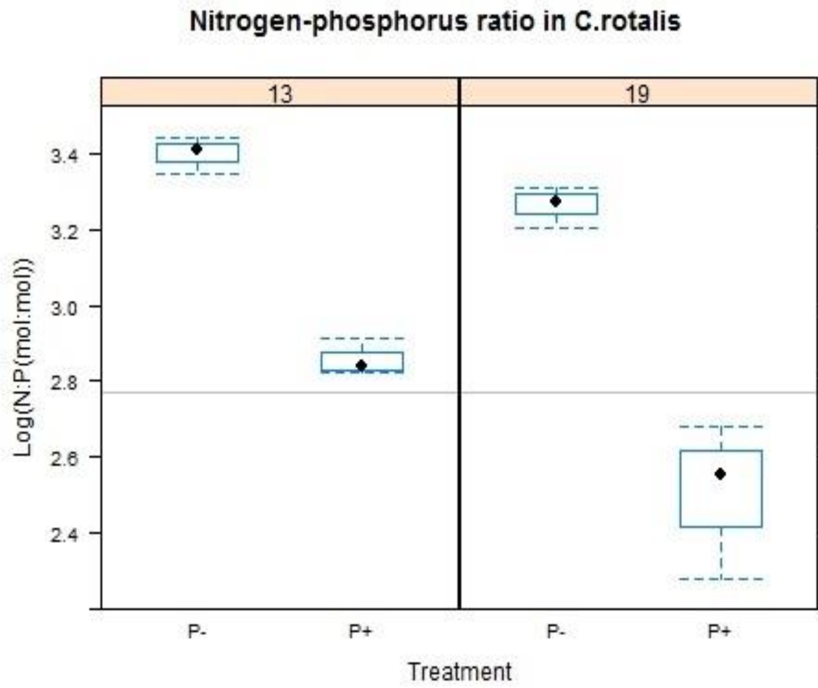


Figure 9. The ratio of nitrogen and phosphorus (mol:mol) in *Emiliania huxleyi* as response to two different treatments and two different temperatures(13 °C and 19 °C). The gray line illustrates Redfields' ratio.

A



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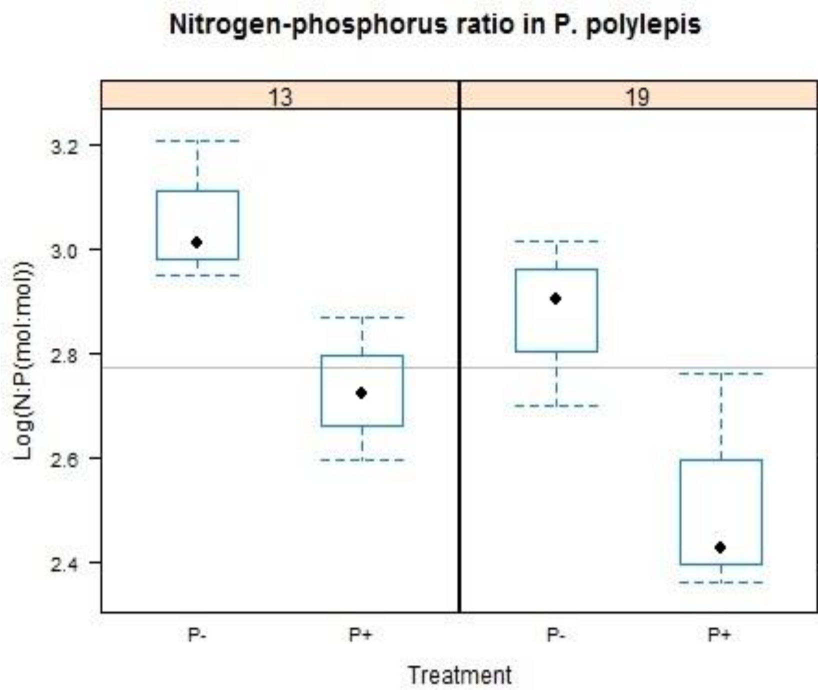

Figure 10. The ratio of nitrogen and phosphorus (mol:mol) in (A) *Chrysochromulina rotalis* and (B) *Prymnesium polylepis* as response to two different treatments and two different temperatures (13 °C and 19 °C). The gray line illustrates Redfields' ratio.

Table 5. ANOVA table shows how the explanatory variables affect the nitrogen: phosphorus ratio in all species. The PVE column added to the ANOVA shows percentage variation explained by the individual factors (total PVE=R^2).

Species	Source of variation	DF	SS	MS	PVE	F-stat.	P-value
E.hux.	P-treatment	1	2.72250	2.72250	0.751	44.7437	0.0001544 ***
	Temperature	1	0.05622	0.05622	0.016	0.9239	0.3645894
	P-Treat. X temp.	1	0.36103	0.36103	0.100	5.9334	0.0408275 *
	Total	3	3.62652	3.2006	0.866		
	Residual	8	0.48677	0.06085			
C.rot.	P-treatment	1	1.27808	1.27808	0.801	104.0627	7.314e-06 ***
	Temperature	1	0.18351	0.18351	0.115	14.9419	0.004772 **
	P-Treat. X temp.	1	0.03502	0.03502	0.022	2.8518	0.129749
	Total	3	1.59486	1.50889	0.938		
	Residual	8	0.09825	0.01228			
P.pol.	P-treatment	1	0.34850	0.34850	0.511	12.9323	0.007021 **
	Temperature	1	0.11671	0.11671	0.171	4.3310	0.070994 .
	P-treat. X temp.	1	0.00064	0.00064	0.001	0.0236	0.881657
	Total	3	0.68143	0.49280	0.684		
	Residual	8	0.21558	0.02695			

3.3 RNA

The variation in the specific content of RNA (µg cell^{-1}) responded strongly on the P-treatment for all species, yet there also were interspecific differences. In general algae in the +P-treatment displayed a higher RNA content (µg cell^{-1}), than those at -P-treatment.

There was a significantly higher RNA content in *E. huxleyi* cultures that was treated with +P-treatment. The model however explains 93.1% of the observed difference (table 6). The P-treatment alone explains 83.4%. The same positive trend appears visible at both temperatures (figure 11). There is also a significant effect of temperature, and a trend showing that RNA content is negatively correlated with temperature.

In the same way, the cultures of *C. rotalis* treated with +P-treatment showed significantly higher values of RNA (cell^{-1}), than the cultures treated with –P-treatment (figure 112). There was no significant effect of temperature. However, there was a significant interaction effect between temperature and P-treatment which states that the RNA content responded differently to the P-treatment at different temperatures. The model presented explains 98.7% of the variation. From this 91.2% is explained by P-treatment alone.

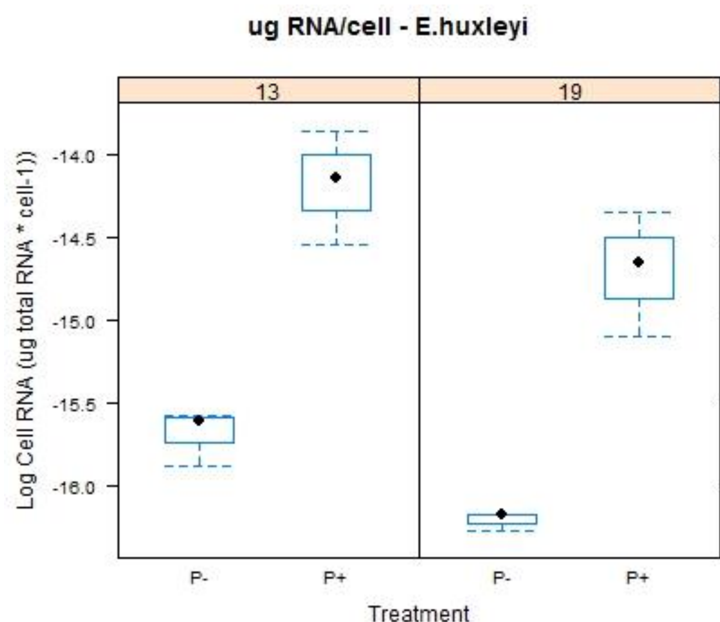

Figure 11. Concentration of µgRNA (cell^{-1}) as a function of phosphorus level and temperature in the species *Emiliania huxleyi.*

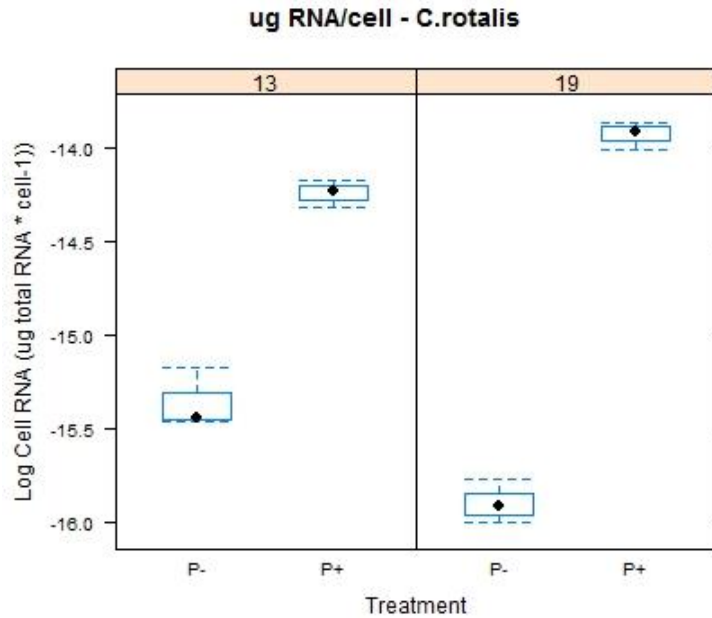

Figure 12. Concentration of µgRNA (cell^{-1}) as a function of phosphorus level and temperature in the species *Chrysochromulina rotalis.*

Unlike the other two algae, *P.polylepis* shows a pattern where RNA content is positively correlated with temperature. However, there are both significant differences found in the different P-treatments and at different temperature (table 6). The data of *P. polylepis* were more variable than in case of other algal species, the model explained 66.9% of the observed. However, similarly to *C. rotalis*, the *P. polylepis* also had higher RNA content (µg cell^{-1}) in the +P-treatment (figure 13).

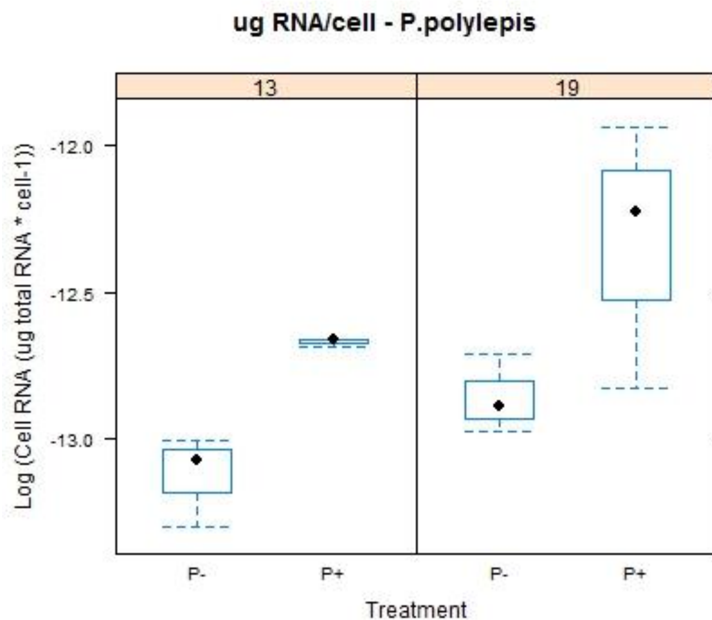

Figure 13. Concentration of µgRNA (cell^{-1}) as a function of phosphorus level and temperature in the species *Prymnesium polylepis.*

Table 6. ANOVA table shows how the explanatory variables affect the RNA content (µg $cell^{-1}$) in all species. The PVE column added to the ANOVA shows percentage variation explained by the individual factors (total PVE=R^2).

Species	Source of variation	DF	SS	MS	PVE	F-stat.	P-value
E.hux	P-treatment	1	6.7763	6.7763	0.834	93.837	1.076e-05 ***
	Temperature	1	0.8065	0.8065	0.097	11.168	0.0102 *
	P-Treat. X temp.	1	0.0000	0.0000	0.000	0.000	0.9988
	Total	3	8.3436	7.8381	0.931		
	Residual	8	0.5777	0.0722			
C.rot	P-treatment	1	7.1471	7.1471	0.913	564.8950	1.046e-08 ***
	Temperature	1	0.0380	0.0380	0.005	3.0041	0.1212824
	P-Treat. X temp.	1	0.5435	0.5435	0.069	42.9542	0.0001777 ***
	Total	3	7.8298	7.7413	0.987		
	Residual	8	0.1012	0.0127			
P.pol	P-treatment	1	0.72425	0.72425	0.483	11.6568	0.009166 **
	Temperature	1	0.27391	0.27391	0.183	4.4086	0.068972.
	P-treat. X temp.	1	0.00419	0.00419	0.003	0.0675	0.801592
	Total	3	1.49940	1.06448	0.669		
	Residual	8	0.49705	0.06213			

3.4 Alkaline phosphatase

The alkaline phosphatase activity (APA) trend in the -P treated algae displayed higher activity of the hydrolase enzyme. This is a good indicator for P limitation. The results of *E. huxleyi*, revealed a strong effect of P-treatments on APA ($cell^{-1}$). The cultures that were grown at a low phosphorus treatment produce a higher amount of the AP enzyme then the algae grown under +P-treatment (figure 14). The linear model presented explains close to 99% of the observed variation, as shown in the ANOVA (table 7). 98.4% of this is due to difference from P limitation.

In *C. rotalis* the estimation of the enzyme activity shows no significant difference between either temperature or treatment (table 7). However, visually the same trend is present in the mean values of the data (figure 15). The model explains only 42.9% of the observed variation.

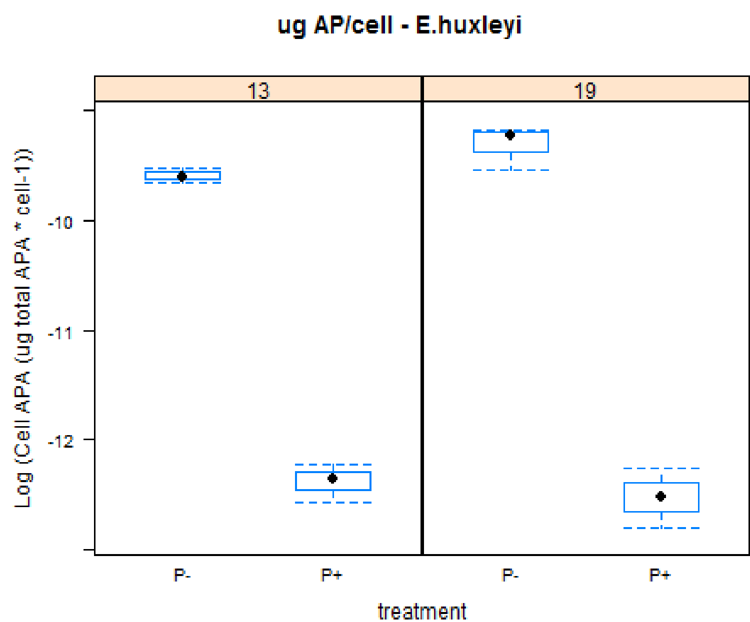

Figure 14. Activity of the alkaline phosphatase enzyme ($cell^{-1}$) as a function of phosphorus level and temperature in the *Emiliania huxleyi*

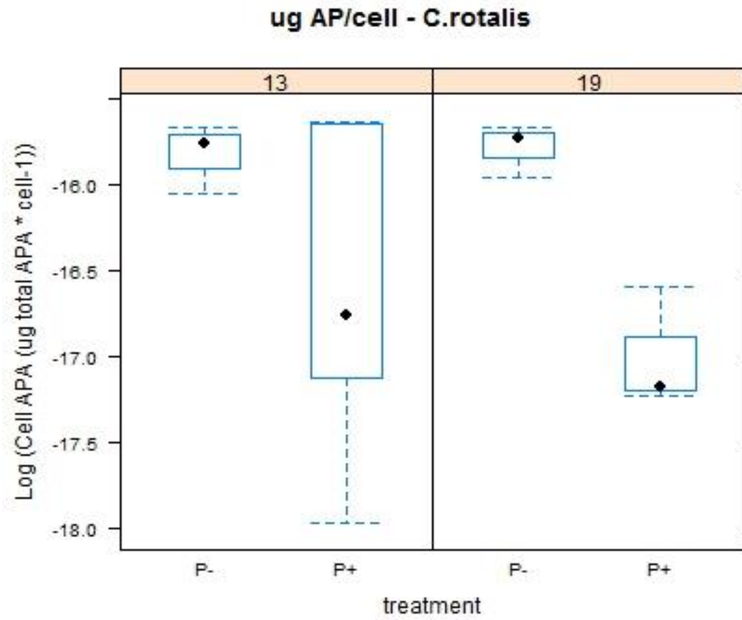

Figure 15. Activity of the alkaline phosphatase enzyme (cell^{-1}) as a function of phosphorus level and temperature in the *Chrysochromulina rotalis.*

In *P polylepis* there was a clear significant effect from temperature, P-treatment and also an interaction effect between the two which shows that the reacted differently to the P-treatment depending on the temperature they were cultivated at (figure 16). The linear model explains 95.6% of the variation (table 7), 85.5% of this is due to P limitation.

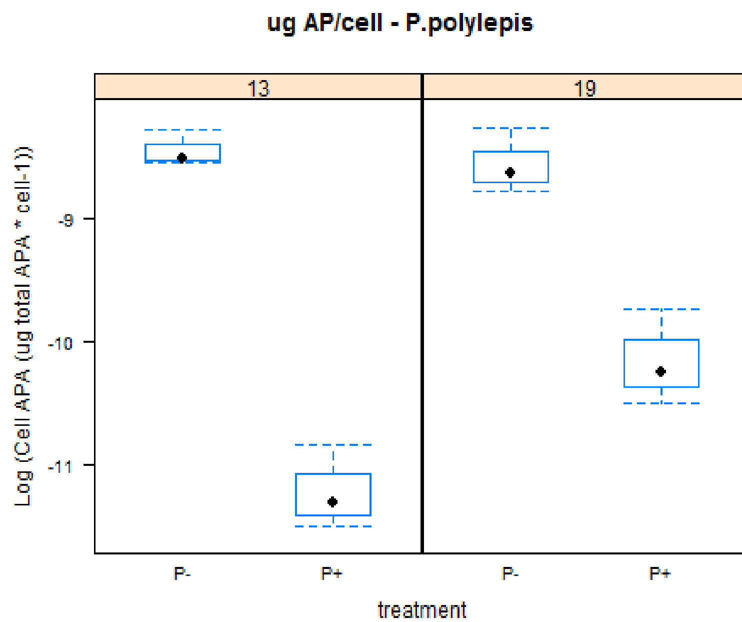

Figure 16. Activity of the alkaline phosphatase enzyme (cell-1) as a function of phosphorus level and temperature in the *Prymnesium polylepis.*

Table 7. ANOVA shows to what extent activity of the alkaline phosphatase enzyme ($cell^{-1}$) is affected by phosphorus level and temperature in all species. The PVE column added to the ANOVA shows percentage variation explained by the individual factors (total PVE=R^2).

Species	Source of variation	DF	SS	MS	PVE	F-stat.	P-value
E.hux	P-treatment	1	26.9594	26.9594	0.984	749.1109	3.423e-09 ***
	Temperature	1	0.01390	0.01390	0.001	0.3853	0.55206
	P-Treat. X temp.	1	0.13790	0.13790	0.005	3.8310	0.08602.
	Total	3	27.3991	27.1472	0.989		
	Residual	8	0.28790	0.03600			
C.rot	P-treatment	1	1.68697	1.68697	0.313	4.3865	0.06954.
	Temperature	1	0.21646	0.21646	0.040	0.5629	0.47460
	P-Treat. X temp.	1	0.40542	0.40542	0.075	1.0542	0.33459
	Total	3	5.38552	2.69343	0.429		
	Residual	8	3.07668	0.38458			
P.pol	P-treatment	1	14.4131	14.4131	0.855	156.6123	1.556e-06 ***
	Temperature	1	0.66500	0.6650	0.039	7.2263	0.02757 *
	P-treat. X temp.	1	1.03470	1.0347	0.061	11.2426	0.01004 *
	Total	3	16.8460	16.2048	0.956		
	Residual	8	0.73620	0.09200			

3.5 Cell size

The results of the cell size analysis were assessed by mean cell size (MDI) and peak cell size (median, PDI). For both *E. huxleyi* and *C. rotalis* the MDI was higher than the PDI, which implied that these curves were right skewed. For *P. polylepis* the curve were left skewed. In the results there is a clear negative correlation with cell size and temperature within all species, with exception to *C. rotalis* –P-treatment, which showed an opposing trend.

The estimation of cell size (µm) revealed that temperature was the dominating factor for MDI (table 8) and PDI (table 9) in both *P. polylepis* and *E. huxleyi*. For *C. rotalis* there was no significant difference in temperature or P-treatment with regards to MDI but there were present a strong interaction between the two factors. The results show that the linear model presented accounted for 98.5% of the observed variation. 90.5% is due to temperature. P-treatment and interaction effect is also affecting *E. huxleyi* but to a very little extent. For the median cell size in *E. huxleyi*, the linear model explains 95.2% of the observed variation. From this 75.2% is due to temperature alone. In *C. rotalis,* the linear model explained 82% of the observed variation as shown in the ANOVA (table 8). Approximately 70% of the variance was due to the interaction effect, and temperature. Furthermore in the PDI there is only a minor interaction effect and a significant strong effect from P-treatment in the *C. rotalis*. For *P. polylepis* the linear models for MDI (table 8) and PDI (table 9) showed that 90.4% and 96% respectively of the observed variance was accounted for. Temperature alone explained over 60% of the variance in both.

The MDI of *P. polylepis* shows the largest cell size at 13°C –P-treatment (figure 17E), while both *C. rotalis* and *E. huxleyi* have largest average cell size at 13°C +P-treatment (figure 17 A and C). The median cell size of *E. huxleyi* shows a slight different trend, the largest cells is still in the 13°C treatment but at –P-treatment (figure 17 B). *C. rotalis* has the largest cell size at 19°C –P-treatment (figure 17 D), and *P. polylepis* continues to exhibit the largest cell size at 13°C – P-treatment (figure 17 F).

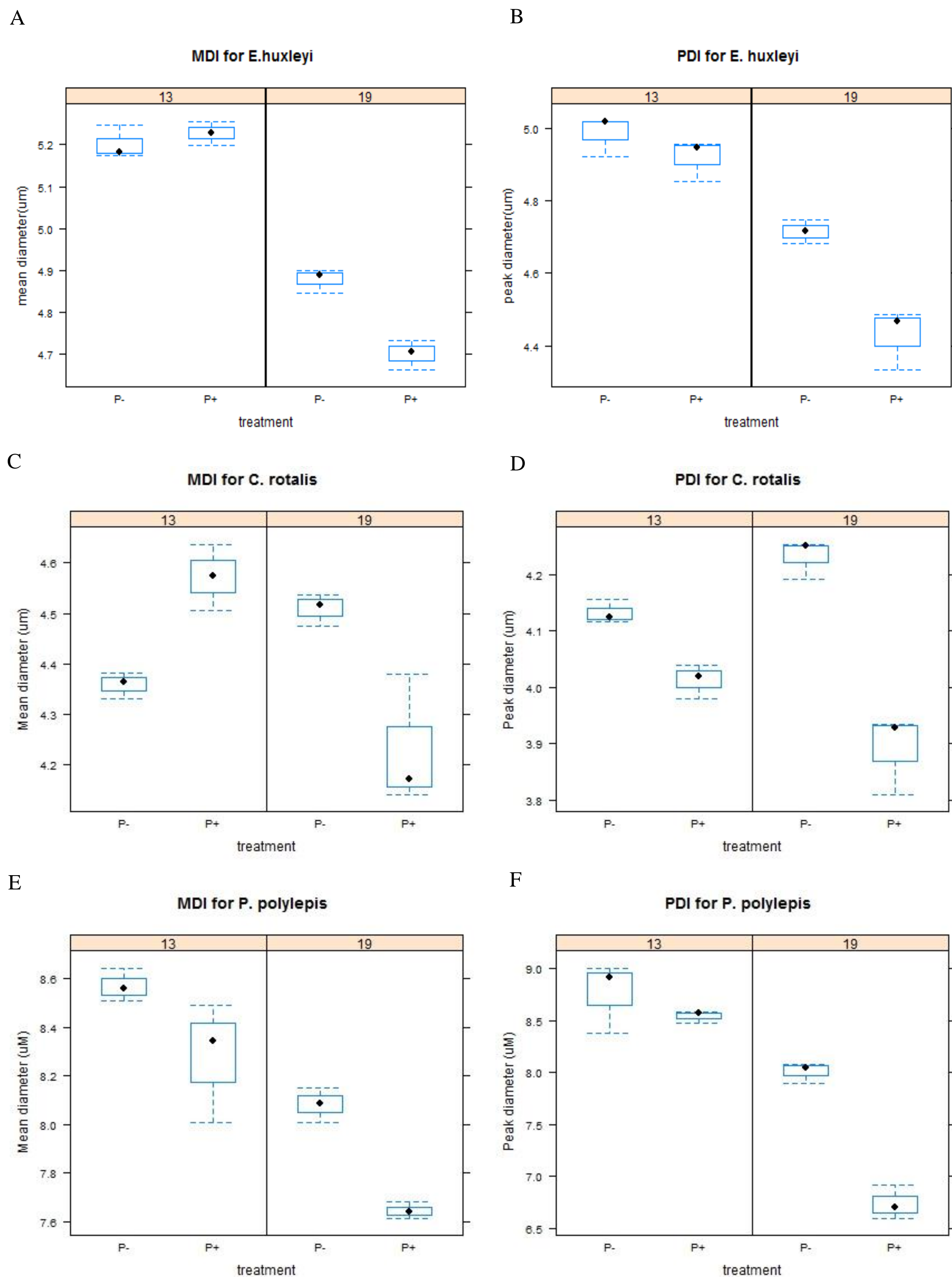

Figure 17. Cell size for (A) *Emiliania huxleyi* (MDI), (B) *Emiliania huxleyi* (PDI), (C) *Chrysochromulina rotalis* (MDI), (D) *Chrysochromulina rotalis* (PDI), I *Prymnesium polylepis* (MDI) and (F) *Prymnesium polylepis* (PDI) as a response to temperature and phosphorus level.

Table 8. ANOVA shows how mean cell size (MDI) is effected by phosphorus treatment and temperature in all species. The PVE column added to the ANOVA shows percentage variation explained by the individual factors (total PVE=R^2).

Species	Source of variation	DF	SS	MS	PVE	F-stat.	P-value
E.hux.	P-treatment	1	0.01703	0.01703	0.028	15.552	0.0042740 **
	Temperature	1	0.54255	0.54255	0.905	495.621	1.752e-08 ***
	P-Treat. X temp.	1	0.03104	0.03104	0.052	28.358	0.0007066 ***
	Total	3	0.59938	0.59171	0.985		
	Residual	8	0.00876	0.00109			
C.rot.	P-treatment	1	0.003088	0.003088	0.012	0.5441	0.4818279
	Temperature	1	0.027313	0.027313	0.106	4.8121	0.0595811 .
	P-Treat. X temp.	1	0.181302	0.181302	0.705	31.9424	0.0004804 ***
	Total	3	0.257110	0.217379	0.823		
	Residual	8	0.045407	0.005676			
P.pol.	P-treatment	1	0.39805	0.39805	0.264	22.0734	0.001545 **
	Temperature	1	0.94831	0.94831	0.629	52.5880	8.79e-05 ***
	P-treat. X temp.	1	0.01599	0.01599	0.011	0.8867	0.373937
	Total	3	1.50661	1.38038	0.904		
	Residual	8	0.14426	0.01803			

Table 9. ANOVA shows how median cell size (PDI) is effected by phosphorus treatment and temperature in all species. The PVE column added to the ANOVA shows percentage variation explained by the individual factors (total PVE=R^2).

Species	Source of variation	DF	SS	MS	PVE	F-stat.	P-value
E.hux.	P-treatment	1	0.09373	0.09373	0.159	26.316	0.0008961 ***
	Temperature	1	0.43259	0.43259	0.732	121.462	4.09e-06 ***
	P-Treat. X temp.	1	0.03612	0.03612	0.061	10.143	0.0129051 *
	Total	3	0.59093		0.952		
	Residual	8	0.02849	0.00356			
C.rot.	P-treatment	1	0.159852	0.159852	0.754	85.4443	1.522e-05 ***
	Temperature	1	0.000352	0.000352	0.002	0.1882	0.675884
	P-Treat. X temp.	1	0.036852	0.036852	0.174	19.6982	0.002173 **
	Total	3	0.212023	0.198927	0.929		
	Residual	8	0.014967	0.001871			
P.pol.	P-treatment	1	1.6582	1.6582	0.215	43.115	0.0001755 ***
	Temperature	1	4.9390	4.9390	0.640	128.423	3.312e-06 ***
	P-treat. X temp.	1	0.8164	0.8164	0.106	21.228	0.0017386 **
	Total	3	7.2130	7.45210	0.960		
	Residual	8	0.3077	0.0385			

3.6 Genome size

The results of relative genome size (FSC) and DNA content (pg cell^{-1}) through the experiment showed no drastic change in trend during the experiment. But unfortunately due to technical difficulties in converting the data to statistically measurable units, there was no possibility to interpret the intraspecific variance in nucleus size. This makes it difficult to state anything substantial about the effect of temperature or phosphorus on genome size throughout this experiment. But the overall trend shows no large differences within species (figure18 A-F).

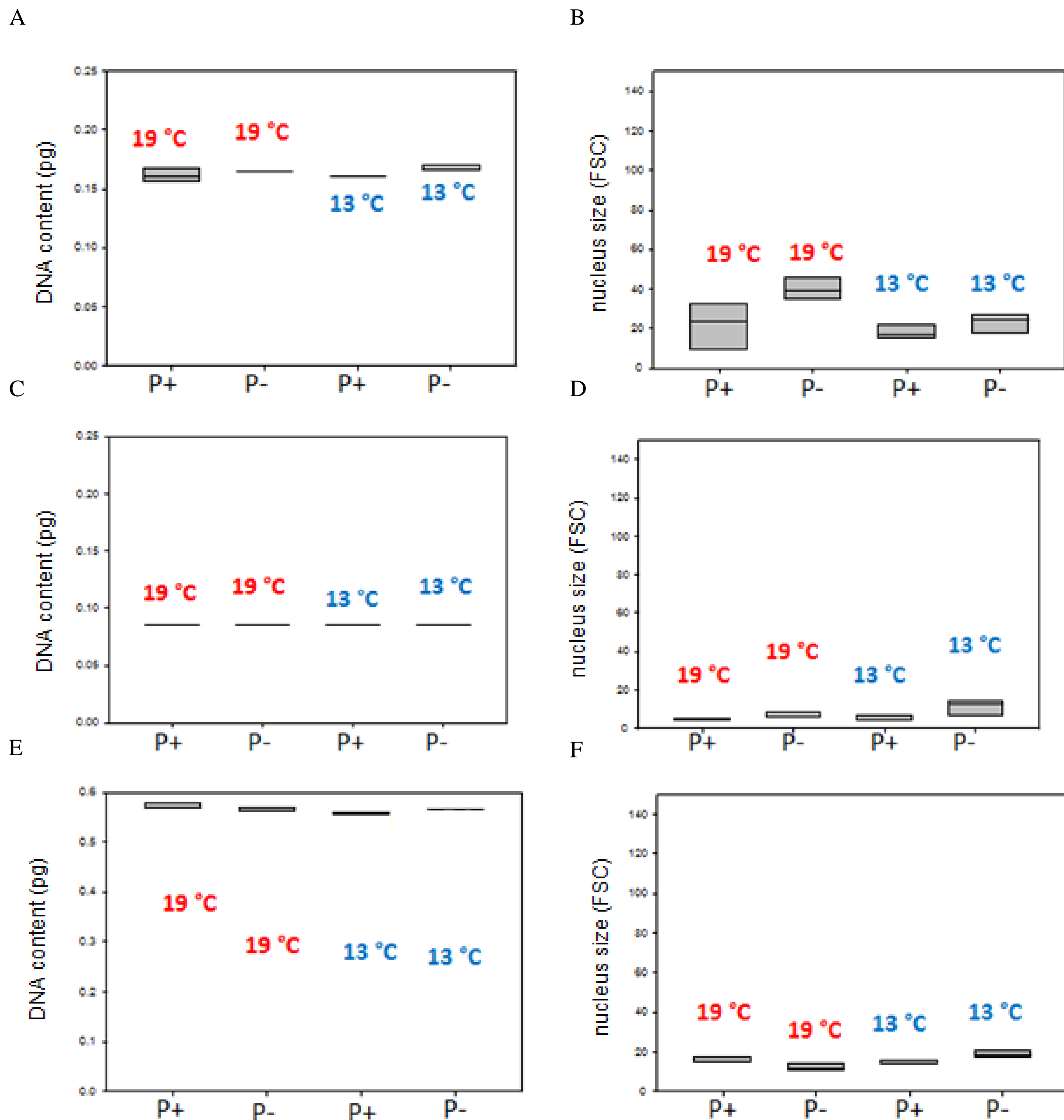

Figure 18. The DNA content (pg cell^{-1}) for (A) *Emiliania. huxleyi*, (C) *Chrysochromulina rotalis*, and (E) *Prymnesium polylepis*. And also the relative nucleus size based on analysis of forward scatter in (B) *Emiliania huxleyi*, (D) *Chrysocromulina rotalis* and (F) *Prymnesium polylepis*.

4. Discussion

In this study, the long-term effect of phosphorus (P) limitation and temperature on growth rate, size, stoichiometry and indicators of nutrient deficiency in haptophytes was studied. The main objective was to predict how these environmental variables could influence algal populations. The findings indicate that phosphorus is more important for regulating growth rate, while temperature governs plasticity range of cell size. The genome size has little observed difference between temperature and treatment.

4.1 Experimental design and P-limitation

One essential premise for this study was to ensure two stable culture lines of treatment that significantly differed in P concentration for the duration of the experiment. This was essential to further map how P limitation can affect growth rate, cell size and genome size in the haptophytes. In order to induce P limitation, the +P-treatment of algae was cultivated as semi-continually turbidostats and the –P-treatment was cultivated as chemostat. The chemostats gets a constant supply of medium, without regards for density of the cultures. This allows for the cultures grow and stabilize at a stationary phase that is governed by the P limitation. In the stationary phase growth rate equals dilution rate. The turbidostats was diluted every other day to relatively small density. With a rich medium the turbidostats are allowed to grow at their maximum growth rate. By combining turbidostats and chemostats it is possible to observe the contrast in how algae that experience P limitation acts on contrary to algae that are not limited. Previous studies have shown that this type and similar kinds of experimental design work well to successively induce nutrient limitation in cultures (Andersen 1981; Hutchins et al. 2003; Borchard et al. 2011). The –P cultures were cultivated with IMR1/2 medium that had a N:P ratio ≈ 124, and the +P cultures was cultivated with IMR1/2 medium with standard N:P ratio ≈ 10 (table 1). To ensure that P-limitation was successfully induced, results from several parameters were considered alkaline phosphatase activity, RNA content and measurements of the algal stoichiometry.

Alkaline phosphatase activity (APA) is a hydrolase enzyme that increases in activity during times of P-limitation. The results from APA revealed that the activity of the enzyme was significantly higher in the –P cultures. As observed in earlier studies with P limitation (Litchman & Nguyen 2008). RNA is P rich molecules that were expected to significantly deviate in content between P-treatments. Earlier studies have shown that a decrease in RNA

content is common in P starved cultures (Berdalet et al. 1994). The trend shows a significant positive correlation between RNA-content and P, thereby indicating P limitation. Finally the N:P and C:P ratio has served as indicators in terms of to what extent the values deviate from proximal values of Redfield ratio (Lenton & Watson 2000). The C:P values may however be affected by the haptophytes' ecology. Haptophytes can produce and store chrysolaminarin as storage product, which may result in elevated C content in the algae. Regardless the results show all values deviate to some extent from Redfield. The ones that deviate the most with regards to both N:P and C:P is the –P-treatment. The combination of these different parameters measured during the experiment, all strongly indicates successful P limitation was induced in the –P cultures.

When studying impact of nutrient limitation it is necessary to ensure that the limited culture is not influenced by other external and internal factors. Such factors may include cell density and CO_2 availability (Andersen 2005). Some experimental design included bubbling to ensure a stable supply of CO_2. Bubbles were not used during the experiment. However +P-treatment was semicontinuously diluted to a relative low amount of cells:volume ratio. Hence it is highly unlikely that the +P-treatment was limited by C. The –P-treatment however had a higher density of cells, making C limitation possible. When the pH increase beyond 9, it is a good indication that inorganic C is a limiting factor in the culture (Andersen 2005). However, since pH was not measured during the experiment it is difficult to state that to what extent the algae suffered from C limitation.

Some algae are mixotrophic. This means they can utilize bacteria as a source for P through phagotrophy (Graham et al. 2009). The *P. polylepis* is a mixotrophic species, which makes alternate P sources of high relevance. Previous studies with nutrient limitation have shown an effect of the presence from bacteria (Paasche 1973; Vadstein et al. 1988). The strains of algae that were used were not bacteria free. However the medium added was pasteurized and filtered to remove further contaminants and bacteria. This implies that if the bacteria were present in a culture, the bacteria would utilize the same source of P as the algae, and not give any new input in the system. Also since this was a long-term experiment, the bacteria would also be affected by P-limited medium, making them poor in P. Hence in this experiment the effect from bacteria as an alternate source of P is assumed negligible.

To sum up, the data from the RNA content and APA analyses revealed some variation between species. Only the *P.polylepis* exhibited an effect from temperature with regards to APA. However, analysis of RNA content revealed an effect of temperature in *E. huxleyi* and *C. rotalis*, but not *P. polylepis*. The P-treatment showed a significant effect on APA in all species except for *C. rotalis*. Since temperature and P-treatment significantly affected RNA content or APA in all species, both H_{0-1} and H_{0-2} are discarded. However, the observed variation underlines the significance in using multiple parameters when confirming nutrient limitations.

It is not possible to conclusively state that C was not limiting in the –P cultures. However, phosphorus has been shown through several measured parameters to be a limiting factor. Hence the goal of inducing P limitation in the –P-treatment with respect to the +P cultures are therefore considered successful.

4.2 Stoichiometric responses

Elemental composition can be used to look at adaptation and allocation strategies in extreme environment. In order to study the effect of P limitation and temperature on cell size, growth rate and genome size C:N:P was of major interest to see how the elemental balance changed according to the difference in traits. The results showed with regards to C:P and N:P a significant negative correlation with temperature towards Redfields ratio and below. The –P-treatment did exhibit an N:P level of significant lower value than that of the medium. This implies either that there is luxury uptake of P present or that N uptake is limited by P. The latter would be an example of Liebig's law of minimum. The +P-treatment had a N:P ratio between the medium concentration and Redfield (N:P ≈ 16) at higher temperature but at lower temperature the N:P ratio was above Redfield's ratio (N:P > 16).

Previous studies have shown that temperature affects subcellular processes that regulate the nutrient uptake, such as growth rate (Agren 2004; Hessen et al. 2013) and cell size (Furnas 1978). Because of the reduction in cell size with increased temperature, and P changed little with temperature, it is implied that excessive uptake of P is not useful for the increased cell size. Hence, it is more likely that the elevated P uptake is connected to the growth rate, which also shows significant effect of P-treatment. The correlation between N:P and growth is covered in depth in the next section.

Another interesting aspect with stoichiometry in response to P limitation and temperature is the C:N ratio. The results showed that the C:N ratio did significantly differ in *E. huxleyi* between P-treatments. If one looks at the ratio in this case as not a result of low levels of N but instead higher levels of C. Since *E. huxleyi* belong to the *coccolitophorides* it is possible for the species to stack up on high levels of carbon in its organic shells, this difference in carbon assimilation may explain the relative difference observed in the C:N ratio in this species (Tsuji et al. 2009). However, there were no observed difference between the HCl treated samples and the untreated samples as would be expected if the reason for elevated C lied in the calcification process (Gerecht et al. 2014).

The C:N ratio could be explained by a lower protein synthesis. But if we assume a static ratio of RNA:protein in eukaryotic organisms to be approximate 1:2 (Sterner & Elser 2002), the variation would then be best described due to intraspecific differences. Former studies have shown that the C:N ratio also may correlate with cell size and nutrient limitation (Mei et al. 2011).

Regardless, during the experiment, *E. huxleyi* went through physiological transformation in the +P cultures where it lost its coccoliths. Unfortunately this change was not registered or looked further into in this study and can thereby not be looked as proof of conditions in the culture. But judging from former studies done on nutrient limitation and *E. huxleyi* it is plausible that the cultures went through a transformation from C-cells to N-cells. A transition that might occur when *E. huxleyi* is cultivated with high access to nutrients (Andersen 1981).

Former studies have shown that C: N: P ratio will vary as a function to growth rate (Goldman et al. 1979), cell size (Peter & Sommer 2015) and intraspecific variation (Sterner & Elser 2002). When stoichiometry is affecting multiple traits, and the traits affect the nutrient supply, it creates a complex pattern of interactions with interspecific variance. This is why it is difficult to create rules for how C: N: P will change in a changing climate.

4.3 Growth rate as a response to P limitation and temperature.

To assess the growth rate in the division Haptophyta, data from the mass specific growth rate (MSGR), N:P ratio and RNA were observed. The results from MSGR in this study indicate that growth rate is governed by nutritional availability more than temperature as seen in the ANOVA (table 2). While there was some interspecific variance with regards to the growth rate trend and reaction to temperature, the growth rate generally showed positive correlation with increasing temperature in the +P-treatment. With increasing temperature it was also shown that the relative P uptake increased, along with the amount of RNA content. This strongly supports that faster growth rate is reliant on allocation to ribosome machinery to uphold the growth rate.

The growth rate trend with regards to temperature exhibited interspecific variation. The growth rate in both +P-treatment of *E. huxleyi* and *C. rotalis* showed a positive correlation between growth rate and temperature (figure 3 and 4A). This correlates with the previously described trend that is that with increased temperature leads to increased cellular processes (Atkinson 1994; Dmitriew 2011). However *Prymnesium polylepis* did not respond in similar manner as the other algae (figure 4B).

One potential reason for this is that *P.polylepis* had difficulties acclimating to the 19°C. *Prymnesium polylepis* did exhibit slower growth rate during the start of the experiment which was the reason to why it was treated differently from the other species. The reaction norm of alkaline phosphatase differed in *P. polylepis* compared to the other two species. Hence it is plausible that the low growth rate is a symptom of a slight P-limitation that the P+ treatment, for whatever reason, did experience. This may have been caused by variation in interspecific need that was not accounted for during this experiment. A former study that involved the *P. polylepis* strain, UIO037, found it grew worse at temperatures close to 20°C, compared to lower temperature (Edvardsen & Paasche 1992).

Despite the positive trend observed in *E. huxl*eyi and *C. rotalis* with regards to temperature, the temperature and interaction does not account for much of the total variation explained in the algae. This can implicate that nutritional availability is a governing factor for regulation of plasticity of growth rate in algae. This is in support of previous studies (Peter & Sommer 2013).

However The N:P ratio (figure 9 and 10) shows the opposite response to growth rate (figure 3 and 4) in the +P-treatment with regards to temperature. Hence, it implies that more rapid growing algae have a higher uptake of phosphorus.

In the recent years, several explanations have been proposed to predict the effect from P limitation and temperature can have on growth rate in algae. Two of them are the temperature-dependent physiological hypothesis and the growth rate hypothesis. The first predicts N:P ratio will increase with growth rate, showing a positive correlation. This prediction is built on the assumption that ribosomes become more efficient with temperature, so that less P-rich ribosomes are necessary to support the fast growth (Toseland et al. 2013). This prediction is supported by the observations from meta-analysis conducted by Yvon-Durocher et al (2015), which shows the same positive correlation trend observed in vascular plants on land. The other prediction with regards to P and MSGR is built upon the growth rate hypothesis (GRH). The growth rate hypothesis predicts a negative correlation between N:P and MSGR. This is because an allocation to rRNA rich ribosomes is necessary to support rapid growth (Elser et al. 2000). The hypothesis has been applied mostly to heterotrophic organisms, but is the GRH applicable to algae? Some studies find that there is no correlation between the expected pattern from GRH and the trend that can be viewed *in vitro* algae cultures (Flynn et al. 2010). In the recent years, more data accumulated by various experiments shows a tendency to support the first hypothesis. Stating that N:P and growth rate is positively correlated. However the trend is highly variable, and even in the metadata analysis that Yvon-Durocher utilizes some studies support negative correlation between N:P and growth rate. The combination of stoichiometric and

The results from this experiment have shown that growth rate is negatively correlated with phosphorus content per cell. This trend, along with an increase in RNA content with growth rate is pattern that one would expect to observe according to GRH. The trend supports the concept that that chemical composition of algae is a highly dynamic relationship that is strongly influenced by their surrounding water mass (Goldman et al. 1979; Agren 2004; Hessen et al. 2013). But if the difference in phosphorus is due to luxury uptake of P or other limiting factors is difficult to state with a certainty. However these results contradict prediction that algae grown at higher temperature need less P to allocate to ribosomes due to an increase in efficiency that is with increased temperature.

Growth rate changes quickly with environmental change as is assumed to be under strong selection due to its effect on fitness (Dmitriew 2011). Previous studies have shown growth rate is N:P is correlated. The temperature trend in N:P with regards to growth is an opposite of what is predicted by the temperature-dependent physiological hypothesis. The variation inn results from several studies (Agren 2004; Daines et al. 2014), including this one, underline the need for more data on specie specific adaption of growth both to N: P and temperature.

4.4 Cell size, mean and median as a response to temperature and P limitation

An issue recognized as a possible source of error is that different stages of the cell cycle implements difference in size. The light: dark time synchronizes the cell cycle in algae (Tamiya et al. 1953). However different growth rate can affect the cell cycle which leads to the potential source of error. However, by applying statistical tools, the effect of difference that is due to the cell cycle is minimized. This should also minimize methodical errors if variance occurred due to which sample that was measured first.

The results showed a difference in mean and median values of cell size. The median is proven to be less sensitive to extreme observation and outliers. The mean value however reflect the variation with regards to all values. This makes it more likely to be affected by extreme values, meaning that the more rear extreme phenotypes is also taken into account. The mean cell diameter (MDI) and median cell diameter (PDI) of *E.huxley* and *P. polylepis* showed significant effect from temperature. The clear negative trend highly indicates that cell size is negatively correlated with temperature. However, the *C. rotalis* shows a different trend with regards to the –P-treatment in both MDI and PDI. While the +P treated samples of *C. rotalis* shows a negative correlation between cell size and temperature, the –P treated cultures shows a positive trend. This creates a significant effect from interaction pattern to P-treatment and temperature means that the different P treated cultures respond different to temperature, or that the P-treatment forces the cultures to respond differently to temperature.

Previous studies of plasticity in size as a variable to temperature predicts a negative trend in cell size of ectotherms as a response to temperature, this is known as the temperature-size rule (TSR) (Atkinson 1995; Kingsolver & Huey 2008). This trend have been observed in a wide range of organisms (Arendt 2007; Angilletta 2009; Hessen et al. 2013), however not all

studies find the predicted trend expected from TSR. Because of this it has been suggested that nutrient availability is more important for mediating cell size (Peter & Sommer 2013; Maranon 2015).

The TSR predicts a negative correlation between size and temperature. This prediction correlates with observations in both *E. huxleyi* and *P. polylepis*. However, the *C. rotalis* trend shows that the different P-treatments respond differently with regards to temperature. This can be explained by several scenarios. For whatever reason, not all ectotherm has been found to follow the temperature-size rule. However, this does not explain the subcellular respond leading to the opposing of TSR. If altering in size is not only a response to temperature but also a mean to acclimate to stress, combined with the more plausible explanation is that plasticity in size as a response to temperature is strongly mediated by nutrient availability which has been proven in former studies (Peter & Sommer 2013). Previous studies have shown that temperature may counteract the effect of phosphorus limitation (Shatwell et al. 2014). This implies that size is potentially bottom-up and top-down regulated. Variation in terms of stoichiometry may be induced due to interspecific variation in optimal C:N:P ratio to size (Sterner & Elser 2002).

Thus, the results show overall the same trend as shown in previous work with regards to the TSR. The results indicate that while temperature is the main factor for determining plasticity range in cell size, nutrient availability also is significant factor. This implies that both $H_{0\text{-}3}$ and $H_{0\text{-}4}$ is discarded. The degree of possible change in size is dependent on intraspecific variation.

4.5 Genome size

During the study, the amount of DNA (pg cell^{-1}) was estimated using the FACS Calibur system, and attempted analyzed with a combination of the software as described in the method section. Due to the initial output from the FACS Calibur machine there was unfortunately not possible to transform the data to a statistically manageable format. This made difficult to interpret the smaller differences with regard to temperature and treatment on intraspecific variation. However, larger changes would still be detectable.

The result from the experiment indicated that there was little or no intraspecific difference between temperature and P-treatment with regards to genome size or amount of DNA. But larger events of change like polyploidization can be ruled out with a degree of certainty. The fact that there is no implication that genome size changes with cell size is an important results, because it implicates that there is potentially other factors that affect the evolutionary drive of changes in size in the algae.

Because of the lack of statistical data it is difficult to say to what extent P-limitation and temperature affect the genome size. Whiteout usage of statistical analyzing tools the null hypothesis $H_{0\text{-}5}$ and $H_{0\text{-}6}$ cannot be confirmed or discarded. However the correlation of cell and genome size appears not to present in the results. The lack of a clear response with regards to P makes also the streamline forming of DNA to allocate P to other functions when P is limiting in the environment.

Earlier studies show indication of increase in nucleus size in organisms reared a lower temperature has enlarged genome size and body size (Gregory 2001; Smith & Gregory 2009; Jalal et al. 2013). An observation with such a pattern would support the notion that temperature affects body size according to the temperature-size rule (TSR) and that genome and cell size could be correlated. However this study show no immediate larger trend in effect of temperature or P-treatment, as would be expected if streamlining of DNA occurred during P limitation (Hessen et al. 2010). However, due to lack of statistical analysis it is difficult to state anything conclusively on these findings.

4.6 Implications and effect of changing climate

All the different parameters measured were necessary to assess to what extent P limitation and temperature could influence traits of vital importance for population dynamic and structure of marine algae. This study showed that both nutrient availability and temperature has the potential to affect important life history traits.

The size tended to be more controlled by temperature than P limitation. This correlates well with finding from other studies and TSR (Angilletta et al. 2004). That temperature is a mediator of size corresponds with field observations from in the North Atlantic Ocean. This was mostly due to picophytoplancton with elevated temperatures by Moran et al. (2010).

However, such field survey is difficult to interpret due to the possible interacting effect (Yvon-Durocher et al. 2011). While these data implicate that TSR is applicable to algae, a central issue then becomes to predict how this will shape the algae in the open ocean. A potential in selection for smaller individuals in the open oceans could have a profound ecological and evolutionary impact in stoichiometric composition and biogeochemical cycles. This study did not find any conclusive correlation between genome size and cell size. However, several other studies have clearly shown a viewable trend with regards to this (Gregory 2001; Smith & Gregory 2009; Jalal et al. 2013), making it an important piece of the puzzle in mapping the sub cellular mechanisms that governs cell size.

This study showed that nutrient availability had a significant effect on growth rate. This coincides with other studies (Maranon 2015). A combination of these findings and the prediction of increased stratification and reduced mixing in open waters (Sarmiento et al. 2004) could have a strong effect on the algal community. The effect on algae with regards to nutrient limitation implicate that if climate change results in reduced mixing, this could significantly alter seasonal dynamic and growth of populations in algae (DeLong & Hanson 2011). As a ground pillar in the food web a potential shift in seasonal cycle could have a strong adverse effect on life cycle of organisms leading to a regime shift in the ocean and possible migration toward coastal regions, where mixing is induced through upwelling. However, such regions may suffer from other limiting factors during times of climate change (Pauly & Christensen 1995; Scheffer et al. 2001; Di Lorenzo 2015). But how these factors will affect taxonomic diversity, evolution and ecology with climate change is uncertain.

Regardless, temperature dependent traits and mechanisms combined with interspecific variance are of high importance for algal stoichiometry. This predicts possibly large effects on population and seasonal dynamic of climate change. Considering that close to approximately half of the total primary production is produced by the phytoplankton, this should serve as a strong motivator for future studies to map the interspecific pattern of reactions to climate related factors.

5. Future work and prospect

This study provides an insight in studies of how temperature and P limitation potentially may affect growth rate, cell size, genome size and stoichiometry of algae. There are many studies in different systems that show the relationship between nutrient availability and temperature. However, several of these make predictions about long-term change from extrapolation of short term data set. In order to predict how the algae will respond to future climate changes it is necessary with more long-term experiments. Future studies should also focus on assessing the correlation between growth and ribosome efficiency with regards to temperature. To further map how allocation of P and other potential limiting nutrient affects different groups of algae

In order to further predict change in genome as a response to P limitation and temperature. A combination of flow cytometry and qPCR could be useful to assess changes in regions of the DNA that is connected to P limitation. This could also give valuable insight into speciation and evolution of the algae genome and size regulation.

More research is necessary to more accurately assess how the cell size, genome size, growth rate and elemental composition in different algae is affected by changing environmental conditions.

6. Conclusion

The experimental setup successfully induced Phosphorus (P) limitation in the –P treated algae. This made it possible to observed effect of different temperature and P-treatment. Stoichiometric responses with regards to C:P and N:P showed P availability as the most dominant factor. The trends showed a negative correlation with temperature in the +P-treatment. This implies that P uptake increase with temperature, if P is present. The N:P declined towards Redfield. The elevated C:P is likely to be due to an increase in chrysolaminaran. Phosphorus treatment was more important than temperature with regards to growth rate. The N: P combined with the growth rate trend differs from what would have been expected to observe if the temperature-dependent physiological hypothesis was correct. The pattern presented resembles more the trend one would expect to observe with regards to the growth rate hypothesis. Phosphorus limitation showed strong effect on RNA content and alkaline phosphatase activity. Temperature had little or no effect on these parameters.

The study shows a strong effect of temperature on cell size as predicted by temperature-size rule. But there is also a significant effect of P-treatment found in the mean values. Ihis implicate that size regulation is also adjusted by nutrient availability or underlying mechanisms that rely on similar resources, like growth rate. The genome size showed no indication of any large difference. However these findings were inconclusive with regards to any smaller differences. But with regards to the large differences genome size does not change with cell size.

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Appendix I

Supplementary Table 1. Recipe for IMR 1/2 modified after (Eppley et al. 1967). Seawater was collected in Drøbak from 40m depth. It was prefiltered with 1.2 µm mesh, added the solutions, postfiltered with 0.22 µm, and pasteurized at 80 ^{0}C for 20 minutes.

Compound	**Amount**
Seawater (34 PSU)	900 mL
Distillate water	100ml
Nitrate	0.5 mL
Phosphate	0.5 mL
Trace metal solution	0.5 mL
Vitamin solution	0.5 mL
Selenite solution	1 mL

Supplementary Table *1*. Stock solutions for IMR 1/2. All stock solutions used in this experiment was created by Sissel Brubak

Compound	**Formula**	**Total of 1000 mL (added to MilliQ)**
Nitrate	KNO_3	50 g
Phosphate	KH_2PO_4	6.8 g
Trace metal solution	Na_2EDTA	6 g
	NaFeEDTA	1360 mg
	$MnSO_4$ x 1 H_2O	620 mg
	$ZnSO_4$ x 7 H_2O	250 mg
	Na_2MoO_4 x 2 H_2O	130 mg
	$CoCl_2$ + $CuSO_4$ [4 mg/L]	1 mL
Vitamin solution	Tiamin B_1	100 mg
	Cyanokobalamin B_{12}	1 mg
	Biotin	1 mg
Selenite (dilute 1:100)	Na_2O_3Se x 5 H_2O	263 mg