MAST VISITING FELLOWSHIP
PROJECT SUMMARY

Building the first theoretical and experimental database for diatom short-term responses to changes in nutrient availability

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Introduction

Phytoplankton is a diverse group of photosynthetic microscopic organisms. Phytoplankton play a key role in global biogeochemical cycles, being responsible of approximately half of global primary production (Falkowski et al. 2004). In addition, phytoplankton fuels the rest of the trophic levels of the ecosystem affecting their production. In nature, these algae are subject to environmental conditions that change rapidly. Thus, unmasking the response of these organisms to changes in their environment is essential to understand phytoplankton community composition, the functioning of the marine ecosystems, and global biogeochemical cycles. To this aim, theoretical approaches are commonly employed.

Mathematical models represent in a simplified manner the growth and physiology of phytoplankton by including parameters related to growth and nutrient uptake. Most of those models assume that cells cannot acclimate to changes in nutrient availability. In addition, the few models that implement nutrient-uptake-related short term responses rely on few and contradictory data, most of them from experiments performed decades ago (Gotham and Rhee 1981, Caperon and Meyer 1972). Thus, we cannot use those data to estimate how nutrient availability affects the organismal traits which define the nutrient uptake. This information is required to develop meaningful mechanistic models incorporating acclimation to changes in nutrient availability (Bonachela et al. 2011).

This project aimed to fill that important gap in the experimental and modelling literature thorough quantifying short-term (i.e. acclimation) responses to changes in nutrient availability in the diatom *Phaeodactylum tricorunutm*. We focused on both steady (continuous cultures) and dynamic conditions (semicontinuous cultures), and measured important physiological traits such as maximum nutrient uptake rate, the half saturation constant or the intracellular nutrient concentration (quota) at different nutrient availabilities. To this end, we devised ourselves the necessary protocols. In addition, we conducted some additional experiments to check the effect of several factors like (antimycotics and photoperiod) on phytoplankton. The main output is a comprehensive compilation of trait values, which we will use to develop novel, mechanistic models capturing diatom acclimation strategies for both steady and dynamic environmental conditions.

Methods

Model species

The pennate diatom *Phaeodactylum tricornutum* (Fig. 1) is an especially interesting species because i) it is an emerging model diatom species in molecular and cellular biology studies ii) it has a fully sequenced and publicly available genome, which could increase

Fig. 1 Photography of *P. tricornutum*

the possibilities of our study, since it could enable the monitoring of genetic and transcriptional changes related to the changes in V_{max} and iii) *P. tricornutum* can storage lipids, being a potential microalgae energy source.

Continuous and semicontinuous cultures

We started and maintained two kind of cultures: continuous and semicontinuous cultures. In both techniques, a proportion of the culture is removed daily and replaced by the same amount of medium, composed by a mix of nutrients and vitamins dissolved in salt water, so that the volume of the culture remains constant. The rate at which parts of the culture and the medium are removed and supplied, respectively, is called the dilution rate. The difference between both methods is that the continuous culture is continuously diluted (by using a peristaltic pump), whereas the semicontinuous culture is diluted ("manually") only once per day. Continuous cultures were conducted in 360 mL glass chemostats. Air pumps connected to a timer insufflated an air bubble every 3 minutes to aerate and stir the culture. Semicontinuous cultures (200 mL) were carried out in glass flasks. They were homogenized by using small stirring bars rotating at 180 rpm. Both kind of cultures were kept at 20^{o} C under a 12-h light:dark photoperiod.

To detect the potential acclimation responses we were looking for, some specific conditions are required:

1. Nutrients, in our case phosphorous, must limit the growth rate of the cells.
2. Cultures must show time-prolonged (days-weeks) differences in nutrient (phosphorous) availability so that cells can potentially acclimate and show the differences in their traits.

To achieve the first requirement, i.e. nutrient limitation of the growth rate, we cultured our cells employing F/2 medium with a reduced concentration of phosphorous: 15 µM instead of 36 µM, the concentration commonly employed. We autoclaved the culture medium before adding vitamins to minimize the development of bacteria and fungi. On the other hand, to achieve the second requirement, we played with the system's dilution rates. The higher the dilution rate is, the higher the entrance of new medium with nutrients and, at least in semicontinuous cultures, the higher the phosphorous availability. In the case of the continuous cultures, we used tubes of different diameters to achieve different flows and therefore different dilution rates. In the case of the semicountinuos cultures we just daily removed and added a different amount of culture and medium, respectively.

We employed 5 (we lost one culture) different dilution rates (0.09, 0.22, 0.24, 0.36, 0.58 d^{-1}) with the continuous cultures and 10 different dilution rates with two replicates in the case of the semicontinuous cultures (0.09, 0.14, 0.20, 0.25, 0.36, 0.45, 0.50, 0.56, 0.60, 0.67 d^{-1}). We chose similar dilution rates for the continuous cultures and the semicontinuous cultures in order to compare both techniques. To maintain those differences in phosphorous availability over time, we

tried to reach and maintain our cultures in a quasi-steady state, which also allows us to make a meaningful comparison of the different treatments. After 45 days, the cultures reached a quasi-steady state, showing the abundances a low variability.

Maximum phosphorous uptake rate and half saturation constant

Most of the models describe the uptake of nutrients by phytoplankton as a function of the external nutrient concentration by using the Michaelis-Menten quinetic. Here, focused on phosphorous uptake:

$$V_p = V_{maxP} \times \frac{P}{K_P + P}$$

Where V_p is the uptake rate of phosphorous, V_{maxP} is the maximum phosphorous uptake rate, P is the phosphorous concentration in the environment and K_P is the half saturation constant for phosphorous, i.e. the environmental concentration at which $V_P = \frac{V_{maxP}}{2}$. In that equation, V_{maxP} and K_P are parameters, organismal traits, that determine the nutrient uptake ability of phytoplankton species. The V_{maxP} is related to the number of phosphorous uptake sites in the cell membrane and the capacity of the cell to take up nutrients at high phosphorous concentrations, whereas K_P is associated with the ability of the cell to take up nutrients at low phosphorous concentrations.

We set up a protocol to measure both V_{maxP} and K_P based on Lomas et al. (2014). Firstly, phytoplankton culture (10 mL) was added to the glass vials where the samples are incubated (Lomas et al. 2014). If the culture is very concentrated, it is recommended to dilute it. Then, increasing amounts of cold (non-radiactive) phosphorous, in our case from 0.1 to 10 µM (over the phosphorous concentration already present), were added to those vials in order to achieve different phosphorous concentrations in each vial (required to obtain a phosphorous uptake curve, see below). Those phosphorous concentrations must be chosen according to the phytoplankton species. Subsequently, 0.15 µCi of a work solution of $H_3{}^{33}PO_4$, obtained by diluting the stock solution (100 µCi, PerkinElmer), was added to the samples (Lomas et al. 2014). Additionally, we prepared two blanks, in which we added a very high amount of non-radiactive phosphorous (300 µM) so that the uptake of $H_3{}^{33}PO_4$ would be negligible. Samples were incubated for 30 min. Incubations were finished by the addition of a great amount of non-radiactive phosphorous (300 µM), so that the uptake of $H_3{}^{33}PO_4$ since then will be negligible.

After incubations, those 10 mL samples were gently filtered through glass fiber filters of 0.7 µm of pore diameter. Filters were washed with a solution of oxalic acid in order to remove the ^{33}P attached to the cells (Lomas et al. 2014). Then, filters were placed in scintillation vials containing scintillation liquid and kept in the dark for some hours. The activity in the vials was measured using a scintillation counter, which detects the light pulses produced by the incident radiation on the scintillation liquid. The rate of phosphorous uptake was finally estimated using the following equation:

$$V_p = \frac{\beta_{sort} \times e^{\lambda \times \Delta T} \times P}{n \times \beta_{TA} \times T_{inc}}$$

Where β_{sort} is the measured activity (after take into account, i.e. subtracting, the activity in the blanks), λ is the decay constant (0.0273 d^{-1} for ^{33}P), ΔT is the elapsed time from isotopic addition to counting, P is the phosphorous concentration in the incubation vial (phosphorous added + phosphorous in the culture), *n* is the cell concentration in the incubation vial, β_{TA} is the activity added and T_{inc} is the duration of the incubation. Cell concentration was measured just before the uptake experiment by using an inverted microscope and disposable count plates. Samples were diluted to 5 x 10^3 cells mL^{-1} when cell concentration was higher than 3 x 10^4 cells mL^{-1} to avoid the exhaustion of the $H_3{}^{33}PO_4$ during incubations and the consequent underestimation of phosphorous uptake rate.

By plotting the phosphorous uptake rate estimated in the different incubation vials against their corresponding phosphorous concentration, and by fitting the Michaelis-Menten function to those observations, we obtained a phosphorous uptake curve with an associated V_{maxP} and K_P. In doing that, we employed the software R. We repeated this procedure with the different cultures we had in order to estimate V_{maxP} and K_P in all of them.

The traits measured for the semicontinuous cultures showing a dilution rate higher than 0.50 d^{-1} are not provided due to the very low phytoplankton abundances, which hampered their accurate measurement. Additionally, we had some methodological problems with the continuous cultures related to the infection by fungi and the failure of the air pumps. For this reason, in the present report we exclude continuous cultures from most of the analyses, although we show their phosphorous uptake curves.

Phosphorous quotas and phosphorous concentration in the medium

We also set up a protocol to measure the intracellular phosphorous content (the phosphorous quota). Firstly, samples were filtered through 1.2 µm pore diameter glass fiber filters. The material on the filter was subsequently digested. To this end, most of the protocols immerse the filters in an acid solution and heat, although there are different ways to that. We tried several protocols with the guidance of Dr. Hugh Flowers at the School of Chemistry at the Glasgow University. We found that the best digestion was obtained by using concentrated nitric acid and heating (Fig. 2). In this way the material was completely digested, what was not achieved by employing softer acids like sulphuric acid. The solution was then neutralized by adding NaOH and the ortophosphate measured using a nutrient autoanalyzer. The same nutrient autoanalyzer was employed to estimate the ortophosphate concentration in the culture medium. Again, we could not accurately estimate the quotas in the semicontinuous cultures with a dilution rate ≥ 0.45 d^{-1}. The quotas reported for the continuous cultures could be biased.

Fig 2. Digestion with sulphuric acid (left) and with nitric acid (right).

Additional experiments

Effect of Antimycotics: Over the months we maintained the cultures so far we observed the growing of fungi in some of the tubes of the peristaltic pumps (Fig. 3).

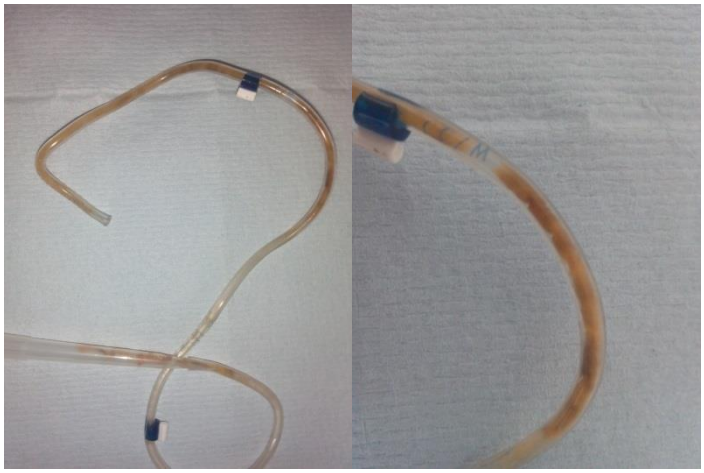

Fig. 3 Tube of peristaltic pump colonized by fungi.

The use of Amphotericin B as antimycotic is widely reported in the literature (e.g. Divan et al. 1982). Amphotericin B binds to ergosterol, a common lipid in the cell membrane of the fungi, forming pores in its membrane. These pores allow leakage of intracellular ions, leading to cell death. Before applying Amphotericin B to our cultures, we conducted a pilot experiment to check their potential effects on *P. tricornutum*. To this aim, we employed batch cultures with two replicated treatments: control (no Ampothericin B addition) and Amphotericin B addition (10 µg mL^{-1}).

Effect of photoperiod and autoclaving process: We conducted another pilot experiment using batch cultures to explore the effect of the autoclaving process and the photoperiod. The experimental design consisted in four treatments:

- 12-12 photoperiod (this is, cultures were grown under 12 h of light and 12 of darkness) with medium autoclaved.
- 12-12 photoperiod with medium not autoclaved
- Continuous photoperiod (24 h) with medium autoclaved.

- Continuous photoperiod with medium not autoclaved.

We did not replicate the treatments so the results are only exploratory.

Results

Maximum phosphorous uptake rate and half saturation constant

The employed methodology enabled us to obtain the phosphorous uptake curves for both continuous (Fig. 4) and semicontinuous (Fig. 5) cultures, together with their corresponding maximum phosphorous uptake rates and half saturation constants.

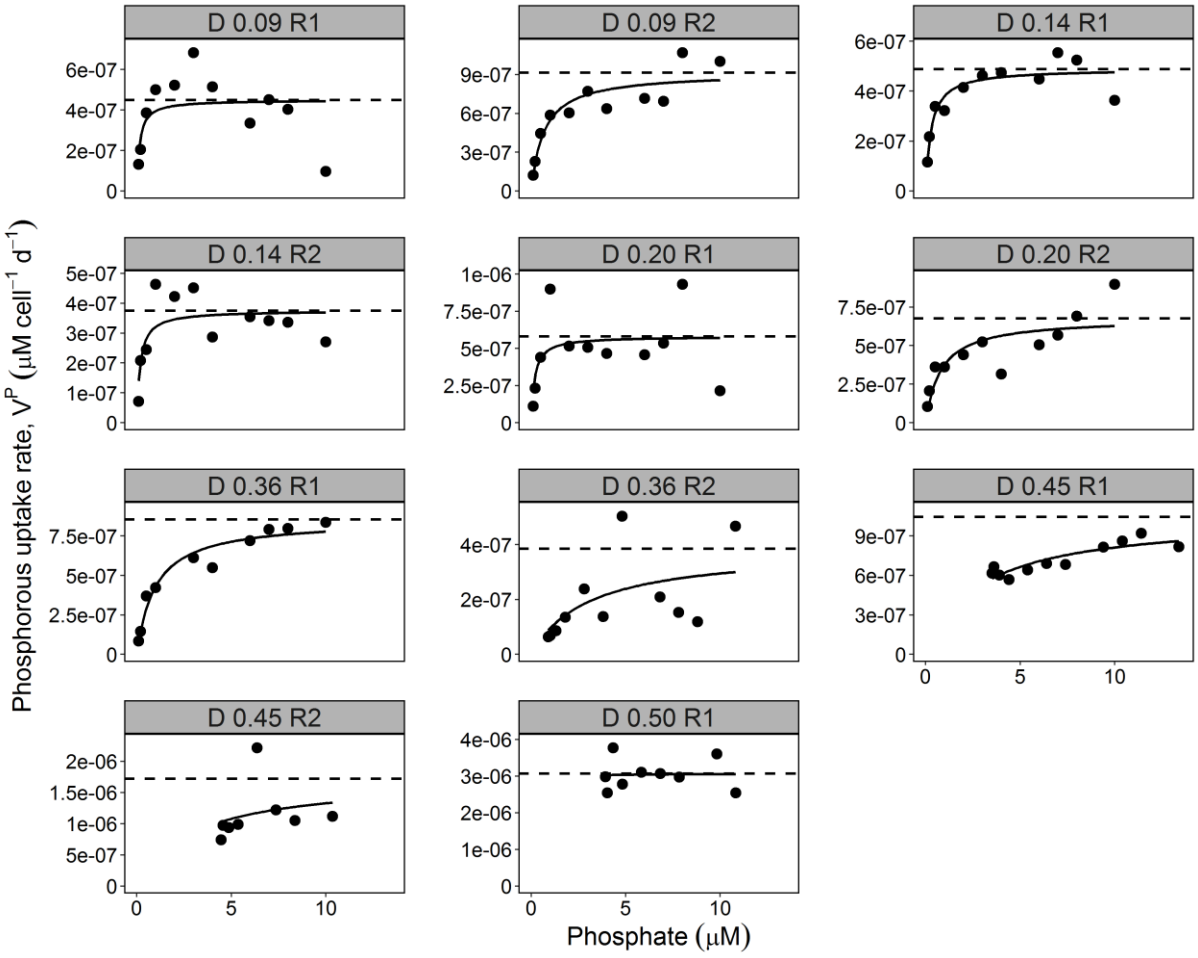

Fig. 4 Phosphorous uptake curves obtained for the different semicontinuous cultures. The label at the top of each panel indicates the dilution rate and the replicate. Solid line represents the fitting of the Michaelis-Menten model. Dashed line points out the maximum phosphorous uptake rate (V_{maxP}) deduced from that curve fit.

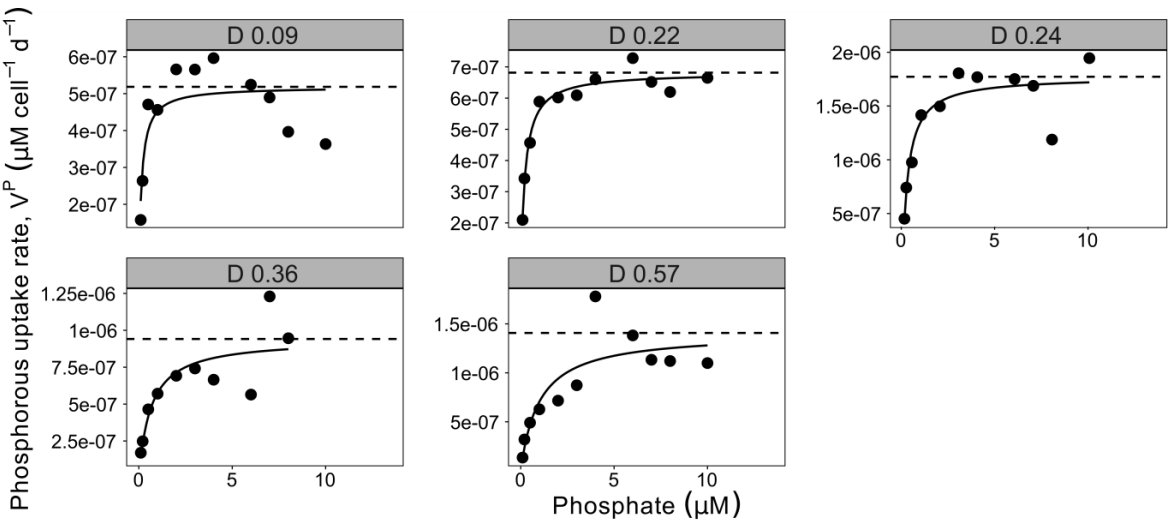

Fig 5. Phosphorous uptake curves obtained for the different continuous cultures. The label at the top of each panel indicates the dilution rate. Solid line represents the fitting of the Michaelis-Menten model. Dashed line points out the maximum phosphorous uptake rate (V_{maxP}) deduced from that curve fit.

In the case of the semicontinuous cultures we found a positive relationship between V_{maxP} and the phosphorous measured when phosphorous uptake experiments were conducted, almost 24 h since the last dilution, i.e. the last addition of culture medium (Fig. 6). Because of the low number of observations, we can not exclude the existence of nonlinear relationships. That positive trend could mean that cells acclimate and take advantage of the higher nutrient concentrations by increasing their nutrient uptake capacity. It is opposite to the trend observed in other studies conducted in the field (Lomas et al. 2014) and in some laboratory studies using continuous cultures, in which maximum uptake rate reduces as nutrient availability, that is, cells increase their maximum uptake rate in response to nutrient scarcity. Thus, the kind or the frequency of nutrient inputs could influence the sign of the acclimation responses.

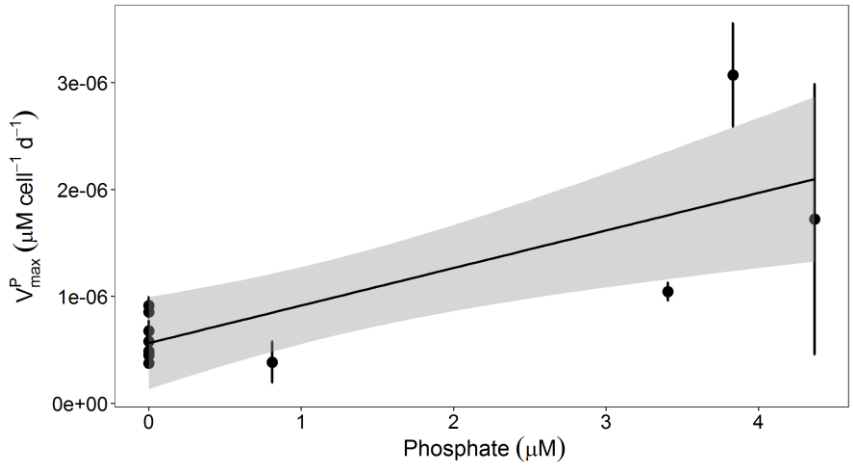

Fig. 6 Relationship between maximum phosphorous uptake rates (V_{maxP}) and phosphate concentration in semicontinuous cultures. Vertical bars represent the standard error. Solid

line shows the trend obtained by fitting a linear regression. The grey shaded area shows the 95 % confidence interval of the regression line.

The relationship between V_{maxP} and the pulse phosphorous daily added seems to be also positive (Fig. 7). This implies that cells could take advantage of nutrient pulses, which might be associated with luxurious uptake responses. In this case, nonlinear relationships seem even more plausible.

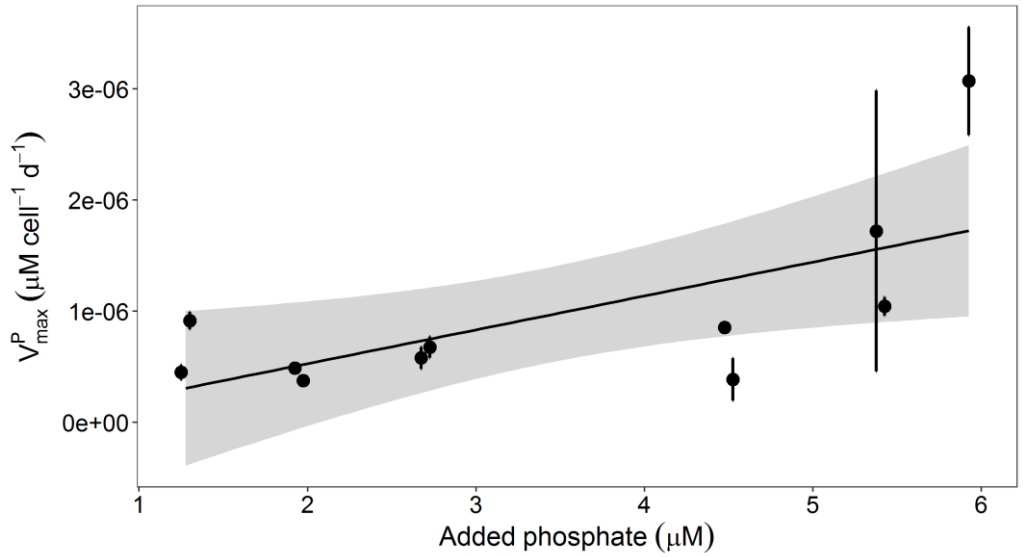

Fig. 7 Relationship between maximum phosphorous uptake rates (V_{maxP}) and phosphate daily added in semicontinuous cultures. Solid line shows the trend obtained by fitting a linear regression. Vertical bars represent the standard error. Dots are slightly moved in the x-axis to avoid the overlapping of the error bars. The grey shaded area shows the 95 % confidence interval of the regression line.

The K_P showed a positive relationship with V_{maxP} (Fig. 8). It implies the existence of a trade-off between the traits that mainly determine the uptake of nutrients at high (V_{maxP}) and low nutrient concentrations (K_P). Other studies have reported this trade-off before (e.g. Litchman et al. 2007), but they rely on data collected for several species, showing an across taxa relationship. To our knowledge, this is one of the few investigations reporting the trade-off between V_{max} and K within taxon.

We did not show here the results obtained for the continuous cultures due to the mentioned problems associated with the infection by fungi and the failure of the air pumps, which hampered the reaching of a quasi-steady state, the acclimation of the cultures to specific conditions, and the reliable estimation of V_{maxP} and K_P.

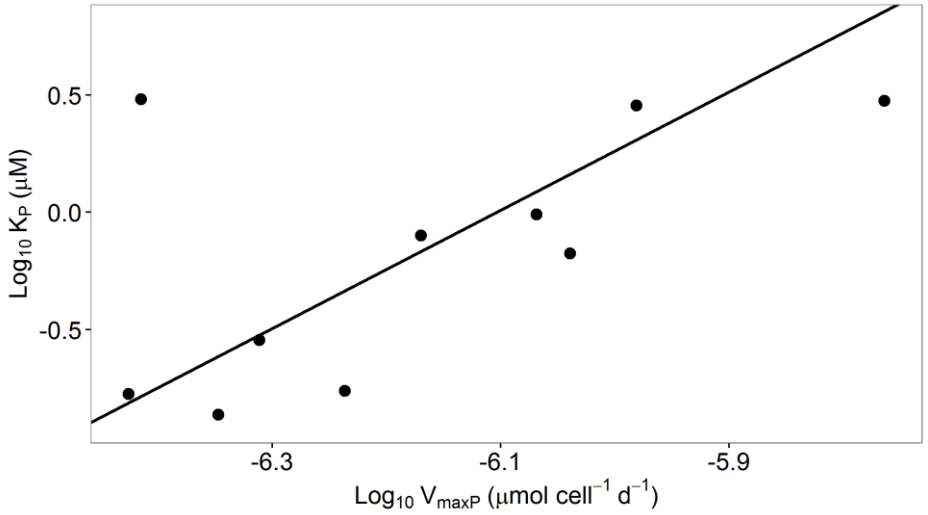

Fig. 8 Log-log relationship between maximum phosphorous uptake rate (V_{maxP}) and half saturation constant (K_P) estimated from semicontinuous cultures. Solid line shows the trend obtained by fitting a reduced major axis regression. Note that semicontinuous culture with a dilution rate equal to 0.50 d^{-1} was excluded from the analysis due to we could not measure uptake rates at values close to the K_P.

Phosphorous quotas

The growth rate (dilution rate at steady state) showed a positive relationship with the phosphorous quota (Fig. 9), in agreement with the Droop model (Droop 1973). In semicontinuous cultures, quota ranged from 8.17 x 10^{-9} to 2.04 x 10^{-5} µmol P $cell^{-1}$. This range is one order of magnitude higher than the reported in the literature (Reynolds 2006). Nevertheless, the low abundance and cells counted at dilution rates ≥ 0.45 d^{-1} entails a high uncertainty associated with the highest quotas. Thus, results must be taken with caution.

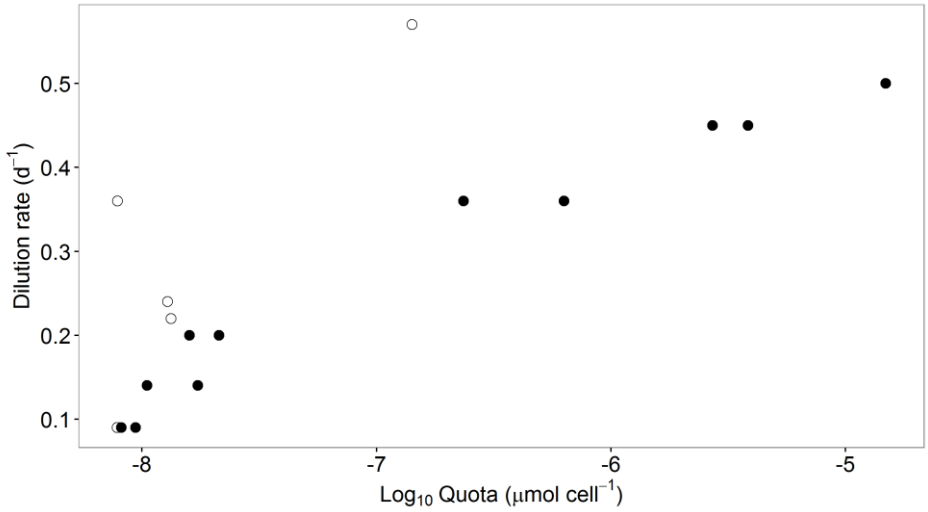

Fig. 9 Relationship between dilution rate and Log_{10} phosphorous quota in semicontinuous (black dots) and continuous cultures (white dots).

Additional experiments

Effect of Antymicotics: We observed negative effects of amphotericin B on phytoplankton during the first week (Fig. 10); the abundance of *P. tricornutum* was lower in the both cultures in which amphotericin B was added, indicating a reduction in its growth rate. However, that effect vanished during the second week, this is, from the day seven (Fig. 11). There could be several explanations for the lack of effect at a longer time term. For example, the starvation of nutrients in the control treatment during the first week as a consequence of phytoplankton uptake and growth could reduce the growth rate of phytoplankton in the second week. On the contrary, in the antimycotic treatment there still would be nutrients during the second week. These results warn against the use of amphotericin B in phytoplankton cultures at steady state, since its effect on the phytoplankton growth rate could alter that steady state.

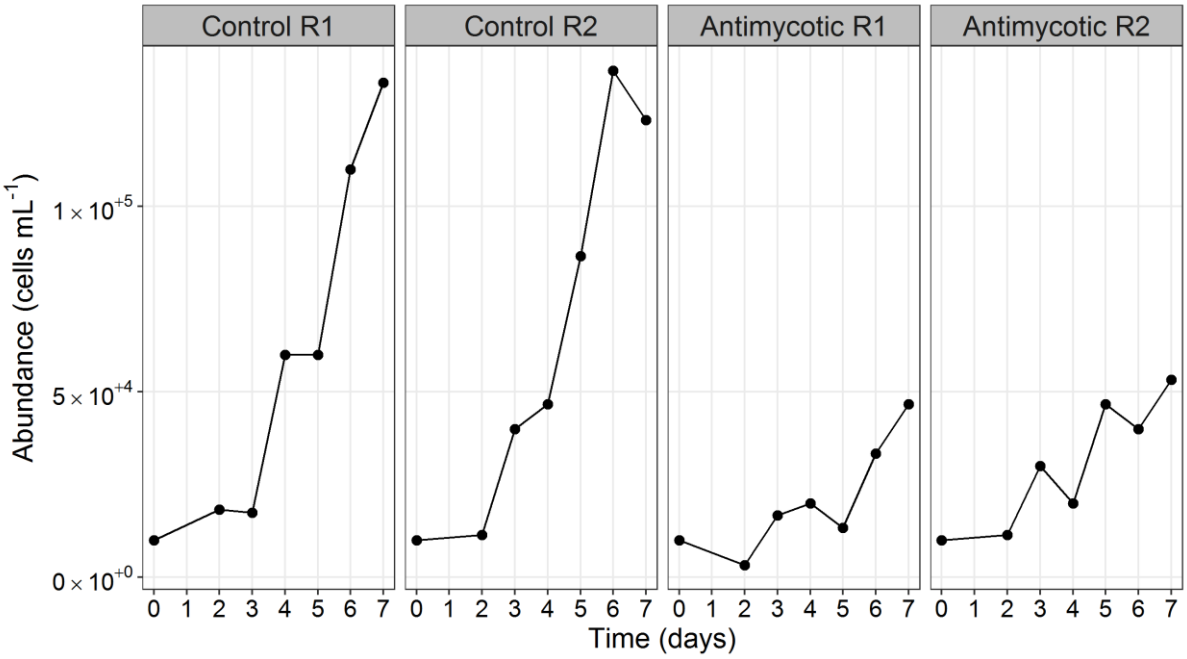

Fig. 10 Abundance of *P. tricornutum* during the first week of the experiment carried out to check the effects of Amphotericin B. Each panel represent a different culture. Treatment and replicate are indicated at the top of each panel.

Effect of photoperiod and autoclaving process:

The abundances of *P. tricornutum* were higher in the case of the continuous photoperiod, indicating a positive effect on the growth rate (Fig. 12). The autoclaving of the medium had a negligible effect on the abundance of phytoplankton growing at both continuous and 12-12 photoperiod, which means that it does not greatly affect phytoplankton growth.

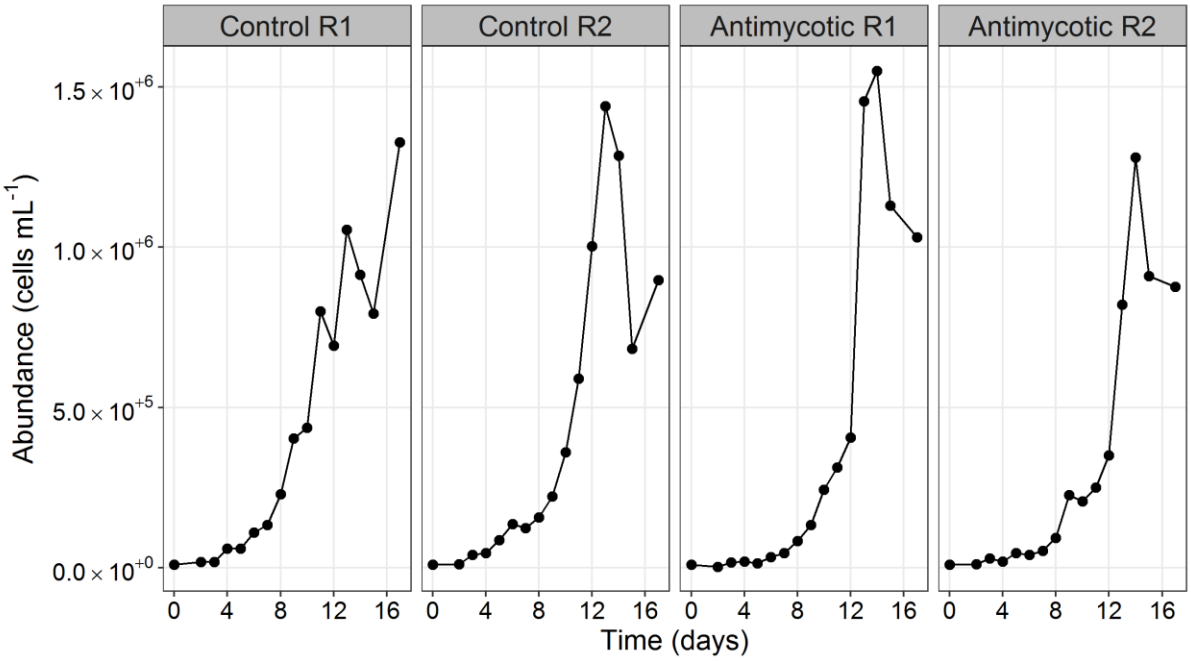

Fig. 11 Abundance of *P. tricornutum* along the experiment carried out to check the effects of Amphotericin B. Each panel represent a different culture. Treatment and replicate are indicated at the top of each panel.

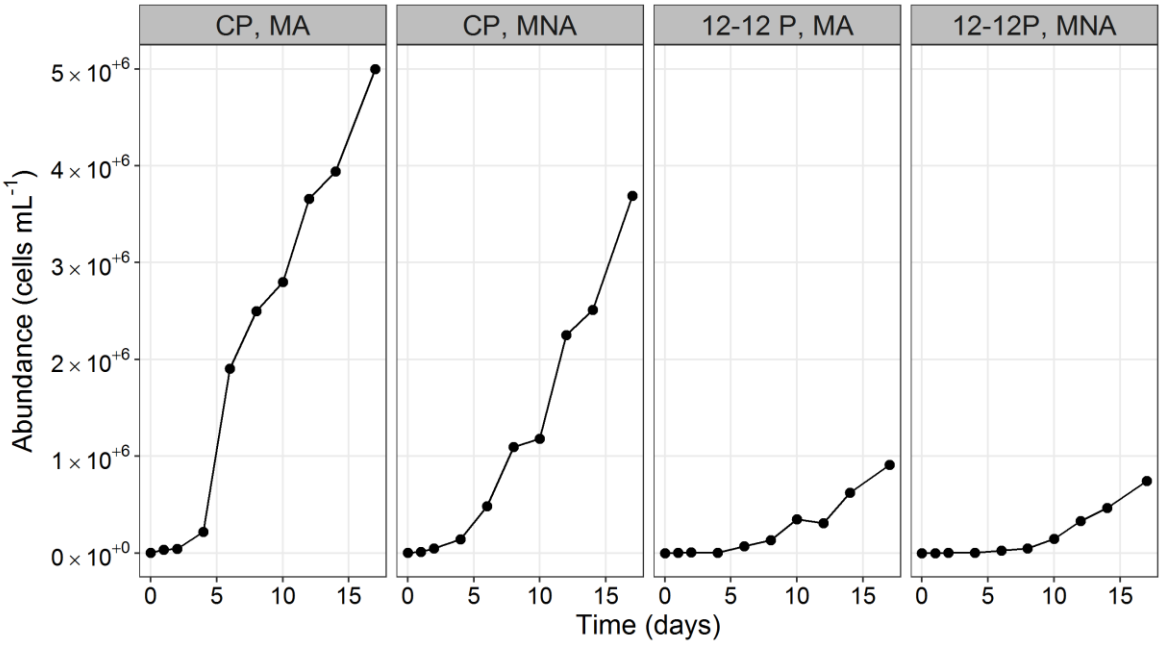

Fig. 12 Abundance of *P. tricornutum* along the experiment carried out to check the effects of the photoperiod and the autoclaving process. Each panel represent a different culture. Treatment and replicate are indicated at the top of each panel. CP, MA: Continuous photoperiod, medium autoclaved. CP, MNA: Continuous photoperiod, medium not autoclaved. 12-12 P, MA: 12-12 Photoperiod, medium autoclaved. 12-12 P, MNA: 12-12 Photoperiod, medium not autoclaved.

Significance and future prospects

This project provided one of the first set of traits related to nutrient acquisition measured for a phytoplankton specie growing at different nutrient conditions. Part of this information has been presented in a talk at the annual MASTS meeting.

We are currently developing models to explain our (somewhat counterintuitive) results, and using some of the obtained data to parameterize phytoplankton models including acclimation responses (Bonachela et al. 2011), which could be useful to understand the effect of short-, medium-, and long-term environmental changes on phytoplankton, and, consequently on upper trophic levels. In addition, the information and experience acquired with this investigation will be really useful in planning more ambitious projects.

Acknowledgements

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