National and Kapodistrian University of Athens

Inter-Institutional Postgraduate Course in Oceanography and Management of the Marine Environment

MASTER THESIS

RESPONSE STRATEGIES OF MICROALGAE UNDER THE EFFECT OF ALLELOPATHIC CHEMICALS FROM COMPETITOR SPECIES

Natalia G. Apostolopoulou

Registration code: 21809

Master Committee:

Dr. Athena Economou-Amilli, Professor at National and Kapodistrian University of Athens
Supervisor

Dr. Sofie Spatharis, Lecturer in the Department of Biodiversity and Associate in the Department of Animal Health & Comparative Medicine at University of Glasgow

Dr. Dionysios E. Raitsos, Assistant Professor at National and Kapodistrian University of Athens

ATHENS
October 2020

ABSTRACT

Studies on microalgae biomass suppression, have so far focused either on nutrient competition or potential toxicity due to allelochemical substances i.e. interference competition. Although certain microalgae species can be characterized as 'storage strategists' (i.e. store excess P in P-rich environments), increased nutrient storage has not been explicitly attributed to a response to interference competition by any empirical study to date. The aim of this research is to investigate the impact of interspecific chemical interactions of target species on shifts in their survival strategy and re-allocation of resources from growth to nutrient storage.

The allelopathic effect of harmful microalgae (i.e. toxin producers) has been thoroughly investigated on natural plankton communities. However, the drivers affecting coexistence and productivity beyond harmful species are still unknown. In this study, four different phytoplankton species. i.e. *Thalassiosira* sp., *Phaeocystis* sp., *Tetraselmis* sp. and *Heterosigma akashiwo*, were cultivated in monocultures. In each monoculture, only the cell-free filtrate from the other species was added. The response of each species to the filtrate of the other was examined by measuring cell number, chlorophyll-a (Chl-a), residual phosphorus (PO_4) in the medium and intracellular P. A supplementary analysis of excreted metabolites (i.e. exometabolites) from the control species' filtrate was carried out in order to interpret the cause of the indirect interactions among species.

Significantly higher Chl-a production was observed in *H. akashiwo* and *Tetraselmis* sp. under the effect of *Phaeocystis* sp. and *Thalassiosira* sp. filtrate and this was attributed to the greater Myo-inositol content found in their cell-free filtrate (i.e. excreted metabolites). Furthermore, a consistently positive and significant impact of *H. akashiwo* filtrate on intracellular phosphorus of all other species was detected, despite the fact that this filtrate had no higher P concentration than the other species. The only metabolite detected in *H. akashiwo* filtrate in higher concentration than all other species was Thiamine. However, Thiamin is an essential vitamin of algal growth and from our results it is inconclusive that this substance affected the other species' growth. A combination of metabolomics and microalgal physiological experiments is an integrated approach to changes in microalgal biomass due to allelopathy and nutrient storage simultaneously enabling a mechanistic understanding of what controls microalgae biomass. In this way, high-value applications can be achieved within priority areas related to ecosystem and human health such as bioenergy and biotechnology.

KEYWORDS: microalgae, allelopathy, exo-metabolites, nutrient storage, intracellular phosphorus

ΠΕΡΙΛΗΨΗ

Μέχρι στιγμής οι μελέτες που αφορούν την καταστολή της βιομάζας των μικροφυκών έχουν επικεντρωθεί είτε στον ανταγωνισμό για θρεπτικά συστατικά είτε στην πιθανή τοξικότητα που προκαλείται λόγω έκκρισης αλληλοχημικών ουσιών από άλλα μικροφύκη (δηλαδή στον ανταγωνισμό παρεμβολής). Αν και ορισμένα είδη μικροφυκών μπορούν να χαρακτηριστούν ότι ακολουθούν τη στρατηγική αποθήκευσης θρεπτικών (storage strategists) (δηλαδή ότι έχουν την ικανότητα να αποθηκεύουν περίσσεια φωσφόρου σε περιβάλλοντα που υπάρχουν επαρκείς ποσότητες), αυτή η περαιτέρω αποθήκευση θρεπτικών ουσιών σε ένα κύτταρο δεν έχει αποδοθεί επαρκώς από καμία εμπειρική μελέτη μέχρι σήμερα ως μια αντίδραση στον ανταγωνισμό μεταξύ των μικροφυκών. Στόχος αυτής της έρευνας είναι να διερευνηθούν οι επιπτώσεις των χημικών αλληλεπιδράσεων διαφόρων ειδών μικροφυκών στις μεταβολές της στρατηγικής επιβίωσής τους και της ανακατανομής των πόρων από την ανάπτυξη στην αποθήκευση των θρεπτικών ουσιών.

Η αλληλοπαθητική επίδραση των επιβλαβών μικροφυκών (δηλαδή των μικροφυκών που παράγουν τοξίνες) σε φυσικές πλαγκτικές κοινωνίες έχει διερευνηθεί διεξοδικά. Ωστόσο, οι κινητήριες δυνάμεις που επηρεάζουν τη συνύπαρξη και την παραγωγικότητα των κοινωνιών, πέραν των επιβλαβών μικροφυκών, παραμένουν ακόμα άγνωστες. Στη μελέτη αυτή, χρησιμοποιήθηκαν τέσσερα διαφορετικά φυτοπλαγκτικά είδη. Τα *Thalassiosira* sp., *Phaeocystis* sp., *Tetraselmis* sp. και *Heterosigma akashiwo,* τα οποία καλλιεργήθηκαν σε μονοκαλλιέργειες. Σε κάθε μονοκαλλιέργεια έγινε προσθήκη μόνο του διήθηματος (χωρίς κύτταρα) από τα άλλα είδη. Η απόκριση του κάθε είδους στο διήθημα του άλλου εξετάστηκε με τη μέτρηση της συγκέντρωσης των κυττάρων, της χλωροφύλλης-α (Chl-a), του υπολειπόμενου φωσφόρου (PO_4) στο μέσο και τον ενδοκυτταρικό φώσφορο. Επίσης, πραγματοποιήθηκε και μια επιπλέον ανάλυση των εκκρινόμενων μεταβολιτών (εξω-μεταβολιτών) από το διήθημα των ελεγχόμενων καλλιεργειών όλων των ειδών προκειμένου να ερμηνευθούν οι έμμεσες αλληλεπιδράσεις μεταξύ των ειδών.

Παρατηρήθηκε σημαντικά υψηλότερη παραγωγή Chl-a στα είδη *H. akashiwo* και *Tetraselmis* sp. υπό την επίδραση των διηθημάτων των ειδών *Phaeocystis* sp. και *Thalassiosira* sp. Αυτό το αποτέλεσμα αποδόθηκε στη μεγαλύτερη συγκέντρωση Μυο-ινοσιτόλης στα διηθήματα αυτά (δηλ. στους εκκρινόμενους μεταβολίτες). Επιπλέον, παρατηρήθηκε μια σημαντική και συνεχής θετική επίδραση στον ενδοκυτταρικό P όλων των ειδών λόγω της προσθήκης του διηθήματος *H. akashiwo,* παρά το γεγονός ότι στο συγκεκριμένο διήθημα δεν υπήρχε μεγαλύτερη ποσότητα P από τα άλλα είδη. Ο μόνος μεταβολίτης που ανιχνεύθηκε στο διήθημα της ελεγχόμενης καλλιέργειας του είδους *H. akashiwo* σε μεγαλύτερη συγκέντρωση από τα άλλα είδη, ήταν η Θειαμίνη. Η Θειαμίνη είναι μια απαραίτητη βιταμίνη για την ανάπτυξη των φυκών, όμως από τα αποτελέσματά μας δεν μπορεί να προκύψει το συμπέρασμα για καμία σημαντική επίδραση του *H. akashiwo* στην ανάπτυξη των άλλων ειδών. Πειράματα που συνδυάζουν τη μελέτη των μεταβολιτών και τη φυσιολογία των μικροφυκών αποτελούν μια ολοκληρωμένη προσέγγιση των

αλλαγών που μπορεί να επέλθουν στη βιομάζα των μικροφυκών λόγω αλληλοπάθειας και παράλληλα αποθήκευσης των θρεπτικών ουσιών παρέχοντας τη μηχανιστική κατανόηση για τον έλεγχο της βιομάζας των μικροφυκών. Αυτή η γνώση μπορεί να οδηγήσει σε εφαρμογές υψηλής αξίας σε τομείς προτεραιότητας που σχετίζονται με το οικοσύστημα και την ανθρώπινη υγεία όπως είναι η βιοενέργεια και η βιοτεχνολογία.

ΛΕΞΕΙΣ ΚΛΕΙΔΙΑ: μικροφύκη, αλληλοπάθεια, εξω-μεταβολίτες, αποθήκευση θρεπτικών, ενδοκυτταρικός φώσφορος

Table of Contents

INTRODUCTION

Allelopathy is the most widely characterized interspecific chemical interaction in aquatic microbial ecology, occurring when an organism produces chemicals. The effects of these chemicals on growth, survival and reproduction of other species either inhibit or stimulate their growth (Rice, 1984; Gantar *et al*. 2008; Granéli *et al*. 2008; Macías *et al*. 2008; Long *et al*. 2018). Most of the attention has so far been given to negative effects of toxin-producing algal species that form HABs on many phytoplankton species and other marine organisms that can be harmful to higher trophic levels including shellfish, fish and humans (Granéli & Johansson, 2003; Fistarol *et al*. 2004; Al-Ghelani *et al*. 2005; Granéli *et al*. 2006; Long *et al*. 2018; Poulin *et al*. 2018a; Poulin *et al*. 2018b). An understanding of how chemical interactions other than allelopathy extend beyond specific taxonomic groups and harmful algae is a key to revealing competition processes driving the structure and biomass of aquatic primary producers as well as cultured microalgae species.

Allelochemicals (i.e. secondary metabolites) excreted from microalgae can produce a negative response on other species' growth by causing damage on structure, photosynthesis and enzyme activity of cells. This reaction can eventually inhibit their growth by shifting their respiration, protein synthesis and gene expression (Tan *et al*. 2019). A major challenge in establishing these responses is the presence of nutrient competition among species which can be affected by species' reproductive strategy (i.e. r-strategists indicate fast growing species, while K-strategists indicate slow growing species) (Papanikolopoulou *et al*. 2018). Granéli & Hansen (2006) and Gross (2009) suggested using only the cell-free filtrate from the species to disentangle the effect of resource competition (i.e. the way microalgae utilize sources of energy to grow and reproduce) from any inferences regarding the pure allelopathic effect. An unresolved question in marine ecosystems is the effect of chemical cues from non-harmful species on other competitor species physiology under the exclusion of nutrient competition.

A well-documented process of algal cells is luxurious phosphorus (P) uptake which is the ability of a cell to accumulate more P than required for its growth in a P sufficient environment (Watanabe *et al*. 1988, Singh *et al*. 2018; Solovchenko *et al*. 2019) whenever P becomes bioavailable (i.e. inorganic orthophosphate (Pi)) (Solovchenko *et al*. 2019); since P is a worldwide limited nutrient. It constitutes a multifunctional procedure in order to accumulate Pi (mostly as polyphosphate) and provide the necessary quantities not only to the metabolic operations but also to its storage processes (i.e. for future requirements) (Solovchenko *et al*. 2019). Microalgae can easily adapt to nutrient fluctuations by modifying their resources uptake and metabolism, while maximize their acquisition of nutrients under P pulses (Cáceres *et al*. 2019) and P deficiency (Cáceres *et al*. 2019; Solovchenko *et al*. 2019). Luxurious phosphorus uptake might either lead other species, whose P uptake is slower, to starvation, or to store more P

than they need for supporting other potential defensive functions (Solovchenko *et al*. 2019). A very useful application of microalgae's luxurious P uptake is its capability to remove high P concentration from wastewater (i.e. P-rich substrate) (Powell *et al*. 2008; Powell *et al*. 2009; Powell *et al*. 2011). Understanding whether microalgae can accumulate and store more P than they need for their growth (i.e. luxurious P uptake and storage) as a response of sensing chemical cues from competitor species, can provide industries of wastewater treatment with favorable insights of sewage remediation.

Metabolomics is an advantageous biochemical approach for revealing molecular phenotypes (Sardans *et al*. 2011; Bujak *et al*. 2015). Metabolites are intermediate products of metabolism which are classified as primary (i.e. essential substances for growth and reproduction of a cell) and secondary (i.e. not fundamental substances for algal growth, mostly participate in the defensive and protective mechanisms of the cell). Metabolomics analysis refers to the quantification of the intracellular (endo-metabolome) or extracellular (exo-metabolome) metabolites which originating from cellular activity in response to changes in growth environment (Tugizimana *et al*. 2013; Romano *et al*. 2014). Poulson-Ellestad *et al*. (2014) and Poulin *et al*. (2018a) reported that a significant role of this analysis is to investigate the physiology of a cell while interacts with other harmful species allelochemicals. Many substances in algae's exometabolome are capable to create interspecific allelopathic effect on their cells metabolism (Kirpenko *et al*. 2012). Although evidence of using metabolites as an indicator of cellular physiology has been reported, the field of identifying the metabolic compounds that trigger interspecific competition in microalgae beyond harmful algal species is still under investigation.

Understanding the effect of excreted metabolites on phytoplankton community could provide knowledge which would be useful not only from an ecological aspect but also in many industrial sectors. Worldwide biotechnological interest has focused on microalgae's biomass as feedstock for producing various bioproducts as feed supplements, pharmaceuticals and biofuels (Leflaive *et al*. 2007; Markou & Nerantzis, 2013; Bacellar Mendes & Vermelho 2013; Skjånes *et al*. 2013; Wijffels *et al*. 2013; Sathasivam *et al*. 2019). Since allelopathy can affect the growth of surrounding organisms (Tan *et al*. 2019) it has the potential to be used as a technique to control algal growth (Bacellar Mendes & Vermelho, 2013; Tan *et al*. 2019). Many phytoplankton species grow by using essential nutrients and vitamins from other species and organisms ('beneficial symbiosis') as indicated by Croft *et al*. (2006). Consequently, it is interesting to investigate whether allelopathy between different species can affect positively microalgae's growth and presenting a more eco-friendly method of biomass productivity maximization.

The aim of this study was to investigate the response of microalgal species of different taxonomic groups to the presence of metabolites excreted by other microalgal species. To separate the effect of allelopathy from resource competition, only the culture extract (void of

cells) from the competitor species was introduced in the target species culture. The specific objectives of the study were to determine the effect of the excreted metabolites on the biomass of the target species (**objective 1**), the nutrient storage of the target species (**objective 2**) and then to associate these to excreted metabolites that characterize a specific species (**objective 3**).

METHODS

Experimental design

To address the research questions, we used four marine phytoplankton species belonging to four different taxonomic groups, i.e. *Heterosigma akashiwo* (Ochrophyta*), Phaeocystis* sp. (Haptophyta), *Tetraselmis* sp. (Chlorophyta) and *Thalassiosira* sp. (Bacillariophyta). A high volume (2L) monoculture of each species was used for the purpose of obtaining a cell-free filtrate of each species. These were referred to as mother cultures (MCs). The treatment cultures of each species comprised of three replicate monocultures (200 ml/flask) where each mother culture filtrate with essential nutrients was added (Figure 1) and a control treatment where plain seawater with essential nutrients was added. Thus, our experimental design comprised of 4 species x 4 treatments x 3 replicates = 60 samples. This was sampled at day 0 just before the mother culture's filtrate addition. Sampling and filtrate addition occurred at the same day (i.e. day 0). Samples were obtained prior the filtrate addition. This was repeated two more times, at days 2 and 4.

The growth stage of treatment and mother cultures was determined prior to the initiation of the experiment (ref. Appendix I). The experiment was initiated when mother cultures and treatments were in the stationary phase because allelochemicals from microalgae are higher than in the exponential phase (Arzul *et al*. 1999; Rengefors & Legrand 2001). MCs and treatments were cultured under P-deficiency (i.e. grown in medium F/2 Guillard (1975) with P/24, i.e. 3μM P) because allelopathy is known to be higher under nutrient-limited conditions (von Elert & Jüttner, 1997; Reigosa *et al*. 1999; Rengefors & Legrand, 2001; Granéli & Johansson, 2003; Fistarol *et al*. 2005; Granéli & Hansen, 2006; Rengefors & Legrand 2007; Macías *et al*. 2008).

Treatments and control cultures were diluted every second day by removing 30% of the volume (i.e. 60 ml) and replacing it with the same volume of mother culture filtrate or salt water, respectively. Prior to addition, essential nutrients were added into bioactivity treatments and control cultures (i.e. F/2 with P/24) to minimize any effects of residual nutrients in the mother culture on the growth of treatment cultures. MCs were not enriched with P-limited essential nutrients because they were intended to be P deprived in order to maximize the excreted metabolites produced due to stress conditions. We tested the response of allelopathic effects between species on three different time-points (i.e. day 0, day 2 and day 4). Consequently, each addition's response was detected at the next (Figure 1).

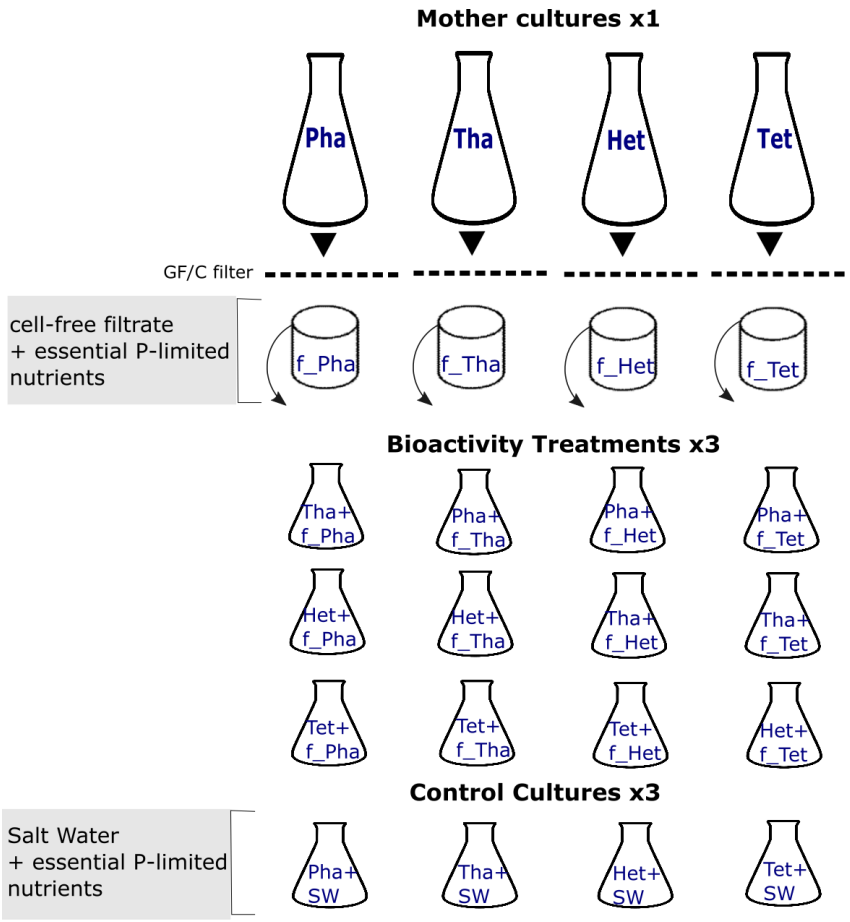

Figure 1: Experimental design testing for the effect of species excreted metabolites on the biomass and luxurious uptake response of four phytoplankton species: *H. akashiwo*, *Phaeocystis* sp., *Tetraselmis* sp. and *Thalassiosira* sp. Treatment cultures of each species comprised of three replicate monocultures where each mother culture filtrate with nutrients (f_species) was added and a control treatment where plain seawater with nutrients (SW) was added.

Experimental conditions & procedure

Phytoplankton cultures of *Phaeocystis* sp. and *Thalassiosira* sp. were obtained from the algal collection of the Hellenic Centre for Marine Research (HCMR) while cultures of *Tetraselmis* sp. and *H. akashiwo* from the algal collection of aquatic ecology laboratory of University of Glasgow. Specifically, *Tetraselmis* sp. was collected from a phytodisk cultured by Florida Aqua Farms (352-567-0226).

All cultures were incubated at 21°C, in a 24-hour continuous photoperiod under fluorescent light and in artificial ultrapure autoclaved seawater with salinity 35‰. Mother cultures had a continuous ventilation system while in the case of the treatments and control cultures there was no ventilation system. Treatment and control cultures were manually stirred before each sampling.

To achieve synchronization in the growth of the treatment cultures, all cultures were inoculated with a concentration of 5,000 cells ml^{-1} apart from *Phaeocystis* sp. which was inoculated with 10,000 cells ml^{-1} due to the much higher population carrying capacity of this species (fig. 5).

Filtrates from the 2L mother cultures were obtained by filtration through GF/C glass microfiber filters. This filtrate of a species was then added into the cell culture of the other three species in triplicates (i.e. bioactivity treatments) following methods indicated by Fistarol *et al*. (2003). Control cultures were also used for each species in triplicates and they were made by adding seawater instead of filtrate.

Sampling & analysis

At each time point (i.e. day 0, day 2 and day 4), 10 ml were removed from each monoculture (5ml for cell counting and 5ml for metabolomic analysis). Cell counting was used for estimating algal biomass of each species. Metabolomics analysis focused on the metabolic profile of each species in the control cultures, so as to detect the unique metabolites that each species excrete. We did not use MCs metabolites due to its uniqueness (i.e. no replicability). Consequently, the indirect effect of control cultures excreted metabolites among species pairs was tested. Samples for cell counting were preserved with a drop of Lugol solution and stored in the refrigerator (4°C) whereas samples for metabolomics were stored at -80^{o}C.

Cell counting was carried out under an optical Leica microscope at 200x magnification using "Fast-read" disposable counting slides (immune systems). Samples for metabolomics analysis (5 ml) were quenched by rapidly cooling cells in ice for 10 min; all the samples were cooled for the same time. The cells were removed by centrifugation for 10 minutes at 3000g at 4^{o}C and 25 μl of supernatant was taken from all the samples. In each sample 1 ml of Chloroform/Methanol/Water (1:3:1 ratio) was added, the samples were vortexed for 1 minute and centrifuged again for 3 minutes at 10000 rpm at 4^{o}C. Finally, 300μl of supernatant was added in cryovials (three times from each sample to create back-up technical replicates) and stored at -80^{o}C. To control for any substances present at the growth medium, a sample was taken (i.e. artificial saltwater with f/2, P/24 nutrients) during the last sampling time point. For quality analysis purposes, a pooled sample of all the samples at each sampling time point was also used for a metabolomics analysis. To determine the metabolic profiles of our cultures, a non-targeted metabolomics approach was applied, by using a combined ZIC-pHILIC (Hydrophilic Interaction Liquid Chromatography) / C18 (reversed phase) platform, coupled to ultra-high mass accuracy Orbitrap mass spectrometry for general metabolomics. Metabolomics analysis and quantification were performed by the Metabolomics Facility in Glasgow Polyomics.

Additional 50 ml aliquots of each culture was collected and from this, 25ml was filtered onto 25 mm Whatman GF/C glass microfiber filters for chlorophyll-a (Chl-a) analysis and the other 25ml was filtered for intracellular P analysis, while the 50 ml of the filtrate was used to evaluate the residual P in the medium. Chl-a and intracellular P filters were placed in aluminum foil and were frozen at -20°C. The samples for nutrients ($PO4^{-}$) were also preserved at -20°C.
Chl-a and residual phosphorus in the medium were quantified according to Parsons *et al*. (1984). For the determination of intracellular P the method described in Caceres *et al*. (2019) was followed. For the digestion of organic phosphorus, the filters were placed in glass vials with 2 ml of concentrated (70%) nitric acid. Glass loose caps were placed on each vial and they were deposited on a hotplate at a gentle boil (i.e. ~121^{o}C) for one hour. When the samples cooled down the filters were rinsed four times with 10ml of ultra pure water. The 10 ml of aliquots were transferred carefully to a volumetric flask and flasks were filled up to a total volume of 70 ml

with ultra pure water. Afterwards, the solution was neutralized (i.e. pH=7) by adding 5ml 5M sodium hydroxide and 2 drops of p-nitrophenol indicator (0.1% solution). Further drops of 5M sodium hydroxide were added until the indicator turned yellow. Thereafter, 0.5M sulphuric acid were added dropwise until the indicator turned colorless. Intracellular P were quantified following the same procedure as for the residual P in the medium (i.e. see above). Finally, P quota (umol $cell^{-1}$) was estimated from the inorganic P concentration measured in the digested solution according to the following equation (Eq. 1):

$$Q = \frac{(P_{ds} - P_{ds\_blank}) * Vol_{flask}}{N * Vol_f} \quad (1)$$

Where:
P_{ds}= Digested solution, Vol_{flask} = Volume of the volumetric flask (i.e. 100 ml) in L, N = Cell concentration, cells/L, Vol_f = Volume of culture filtered (i.e. 25 ml) in L, P_{ds_blank} = Phosphorus concentration measured in the digested solution for the blank

Data analysis

To test for the effect of the excreted metabolites, we focused on the difference between control cultures of the target species and the cultures of the same target species where the filtrate of competitor species was added (i.e. treatments). Thus, our response variable was the difference between the control and the treatment for each one of these variables: cell number, Chl-a, intracellular P and residual PO_4. For each of these response variables we tested the effect of treatment (i.e. species filtrate addition), species (i.e. what species are; which microalgae species were used) and their interaction using Generalized Linear Models (GLMs). Only data from the second addition (i.e. day 4) were used, due to some missing values of Chl-a in the previous time point (i.e. day 2) and because effects were expected to be more amplified after the previous addition.

To ensure that the difference between control and treatment cultures was not affected by any variation in initial conditions, we controlled for this effect in an initial model before calculating the aforementioned differences between controls and treatments. Specifically, we run GLMs, where each response variable at the tested time point (cell number, Chl-a, intracellular P and residual PO_4 in the medium at day 4) depended on its values at day 0 and the species name (e.g. Chl-a at day 4~Chl-a at day 0*species).

The extracted fitted values of the three replicates of control cultures were averaged and then subtracted from each replicate of the respective value of the same species treatment culture (e.g. $(Tha+f\_Pha)_A$- Tha_control average). Based on t-test analysis, this difference (from now on referred to as Δ) indicated a significant positive effect level of metabolites on the tested variables when >0 and a significant negative effect of metabolites when <0.

To further test for the effect of treatment, species name and their interaction (e.g. ΔChl-a~ treatment*species) on each response variable (ΔChl-a, Δcell number, Δintracellular P, Δresidual PO_4) we run GLMs.

Metabolomics analysis and reporting was performed by Glasgow Metabolomics department by pairwise analysis using t-tests of the standardization (through z-scores) of the measured peak values between the control treatments. Significant differences between treatments in the concentration of metabolites would indicate potential substances that might drive the observed differences in responses in the physiological level (i.e. biomass production, nutrient storage).

Statistics were performed using R programming language version 3.5.3 (2019-03-11) (R Core Team, 2019) in the software R-Studio Desktop. Packages ggplot2 (Wickham, 2016), magrittr (Bache & Wickham, 2014) and ggpubr (Kassambara, 2020) were used for plotting, packages

dplyr (Wickham *et al*. 2020) and tidyverse (Wickham, 2017) were used for the t-tests and package RColorBrewer (Neuwirth, 2014) was used to display colorblind-friendly palette to test difference between control and treatment of each response variable. Packages lmerTest (Kuznetsova *et al*. 2017), nlme (Pinheiro *et al*. 2020), ggplot2 (Wickham, 2016), plyr (Wickham *et al*. 2011), broom (Robinson & Hayes, 2018), emmeans (Lenth, 2020), dplyr (Wickham *et al*. 2020), scales (Wickham, 2018), reshape2 (Wickham, 2007), purr (Henry & Wickham, 2020), ggpubr (Kassambara, 2020), tidyr (Wickham & Henry, 2020), tidyverse (Wickham, 2017), ggdendro (de Vries & Ripley, 2020) and grid (R Core Team, 2019) were used for the metabolomic analysis.

RESULTS

Effect of excreted metabolites of other species on the cell number of the target species

Phaeocystis sp. was the only species which cell number was affected by the filtrate addition, presenting higher cell number when filtrate of *H. akashiwo* was added (Δcell number>0, t-test, $p<0.05$) and lower when filtrate of *Thalassiosira* sp. was added (Δcell number<0, t-test, $p<0.05$) (Figure 2). Overall, in regards to Δcell number, filtrate addition did not have an effect ($F_{3,24}$= 1.876, P= 0.161) and this was consistent between the different species (Species x Treatment interaction: $F_{5,24}$= 1.574, P= 0.205).

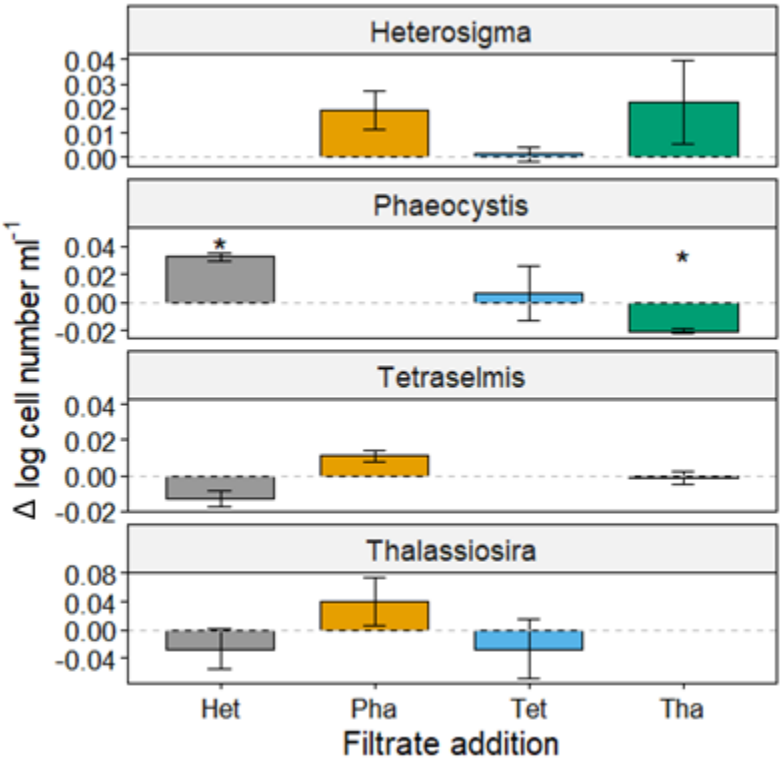

Figure 2. Effect of excreted metabolites of other species on the cell number of the target species. Δcell number indicates the difference between treatment and control cultures. Filtrate addition shows which species was used as a medium to another species culture. Each facet shows how a species react with the secondary metabolites of the other three species. The star (*) shows the statistical significance from 0.

Effect of excreted metabolites of other species on the chlorophyll-a of the target species

The chlorophyll of *H. akashiwo* and *Tetraselmis* sp. significantly increased when filtrate of *Phaeocystis* sp. and *Thalassiosira* sp. was added (ΔChl-a>0, t-test, p<0.05), although the effect on *H. akashiwo* was much greater than the effect on *Tetraselmis* sp. In contrast, Chl-a in *Thalassiosira* sp. decreased when filtrate of *H. akashiwo* was added (ΔChl-a<0, t-test, p<0.05) (Figure 3). Treatment had a significant effect on ΔChl-a concentration between treatment and control ($F_{3,24}$=70.214, P<0.001); however this effect was not consistent across the different species (Species x Treatment interaction: $F_{5,24}$= 41.952, P <0.001).

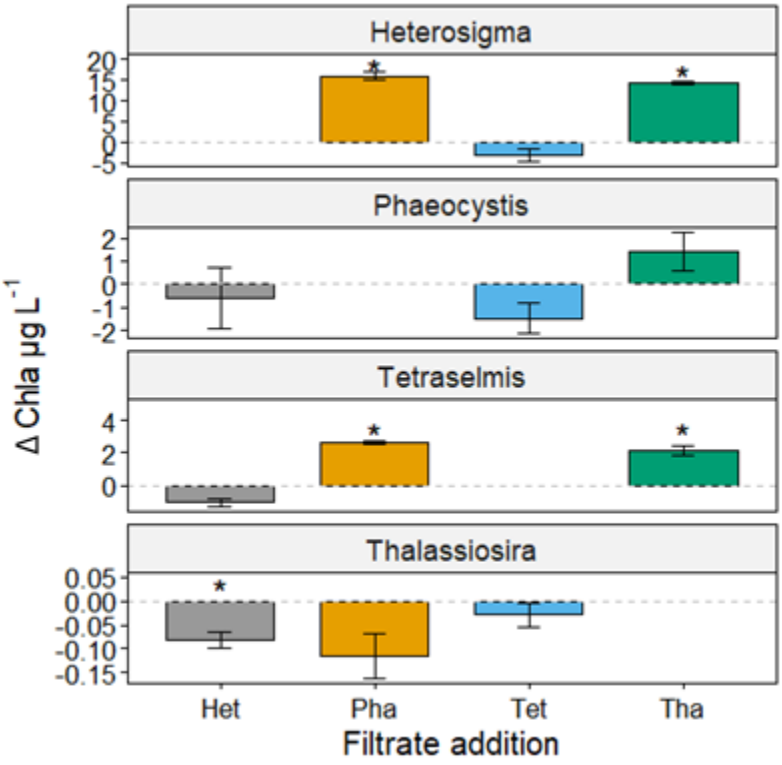

Figure 3. Effect of excreted metabolites of other species on the chlorophyll-a of the target species. ΔChl-a indicates the difference between treatment and control cultures. Filtrate addition shows which species was used as a medium to another species culture. Each facet shows how a species react with the secondary metabolites of the other three species. The star (*) shows the statistical significance from 0.

Effect of excreted metabolites of other species on the intracellular P of the target species

The addition of the filtrate of *H. akashiwo* significantly increased intracellular phosphorus of all species (Δintracellular P>0, t-test, p<0.05). This effect was more pronounced on *Thalassiosira* sp. Additionally, *Thalassiosira* sp. also seems to be positively affected by *Tetraselmis* sp. (t-test, p<0.05) (Figure 4). Species did not have an effect on the amount of intracellular phosphorous ($F_{3,24}$= 2.298, P =0.103). The filtrate addition also did not have a significant effect ($F_{3,24}$= 0.93, P= 0.441) and this was consistent across the different species (Species x Treatment interaction: $F_{5,24}$= 0.939, P= 0.474).

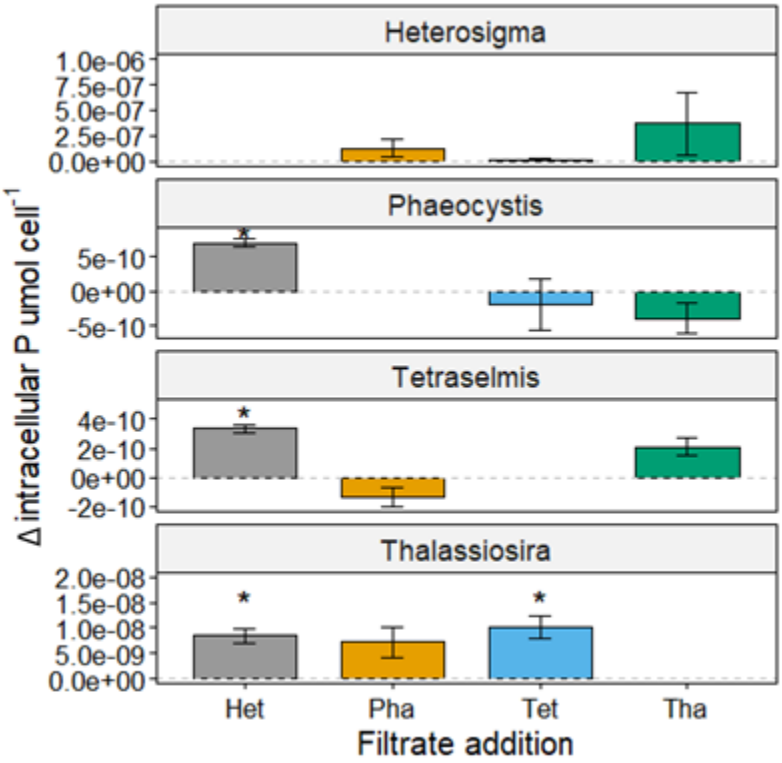

Figure 4. Effect of excreted metabolites of other species on the intracellular P of the target species. Δintracellular P indicates the difference between treatment and control cultures. Filtrate addition shows which species was used as a medium to another species culture. Each facet shows how a species react with the secondary metabolites of the other three species. The star (*) shows the statistical significance from 0.

Effect of excreted metabolites of other species on the residual phosphorus of the target species

Residual P in the medium of *H. akashiwo* was significantly higher when filtrate of all other species was added (ΔPO_4 medium >0, t-test, $p<0.05$) whereas filtrate addition of *H. akashiwo* increased residual P only in *Phaeocystis* sp. cultures (Δ PO_4 medium >0, t-test, $p<0.05$). Additionally, P in the medium of *Thalassiosira* sp. was higher when filtrate from *Phaeocystis* sp. and *Tetraselmis* sp. was added (ΔPO_4 medium >0, t-test, $p<0.05$). On the other hand, the filtrate of *Phaeocystis* sp. and *Thalassiosira* sp. decreased the residual phosphorus of *Tetraselmis* sp. (ΔPO_4 medium <0, t-test, $p<0.05$) (Figure 5). The amount of residual phosphorus differed significantly between the different species ($F_{3,24}$= 11.707, P<0.001). On the other hand, treatment code ($F_{3,24}$= 0.165, P= 0.918) and interaction between species and treatment code ($F_{5,24}$= 0.588, P= 0.709) showed no significant relationship with extracellular phosphorus.

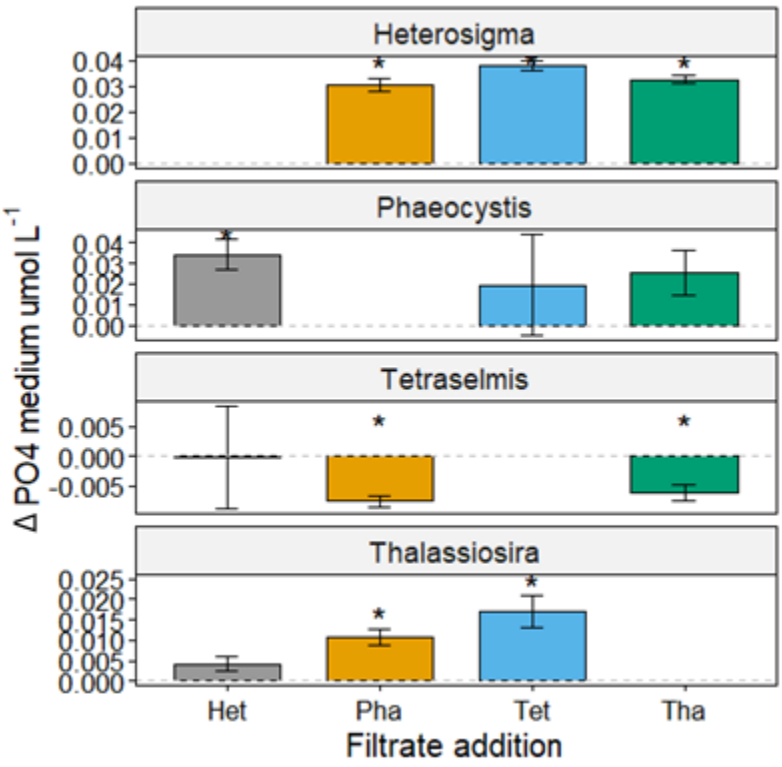

Figure 5. Effect of excreted metabolites of other species on the residual phosphorus of the target species. ΔPO_4 medium indicates the difference between treatment and control cultures. Filtrate addition shows which species was used as a medium to another species culture. Each facet shows how a species react with the secondary metabolites of the other three species. The star (*) shows the statistical significance from 0.

Species-specific excreted metabolites

Diacetyl was the only metabolite that appeared in significantly higher concentrations in *H. akashiwo* (Figure 6), although it cannot be the metabolite which affects growth of competitor species because it is also shared by all other species. Furthermore, Thiamin of *H. akashiwo* was detected at a greater concentration compared to other species. L-Histidinal was detected only in *Tetraselmis* sp. (Figure 6). Another metabolite assigned in *Tetraselmis* sp. in lower concentrations than Histidine was D-Ribose ().

Phaeocystis sp. seems to have the most distinct metabolic profile, yet it shares many of its metabolites with other species. Specifically, Myo-Inositol was detected in all four species, however in significantly higher concentrations in *Phaeocystis* sp. and *Thalassiosira* sp. (Figure 6). Additionally, the tetrapeptide Asp-Leu-Lys-Gln was found only in *Phaeocystis* sp. and *Thalassiosira* sp. Finally, 4-Methylpentanal was detected only in *Thalassiosira* sp. (Figure 6).

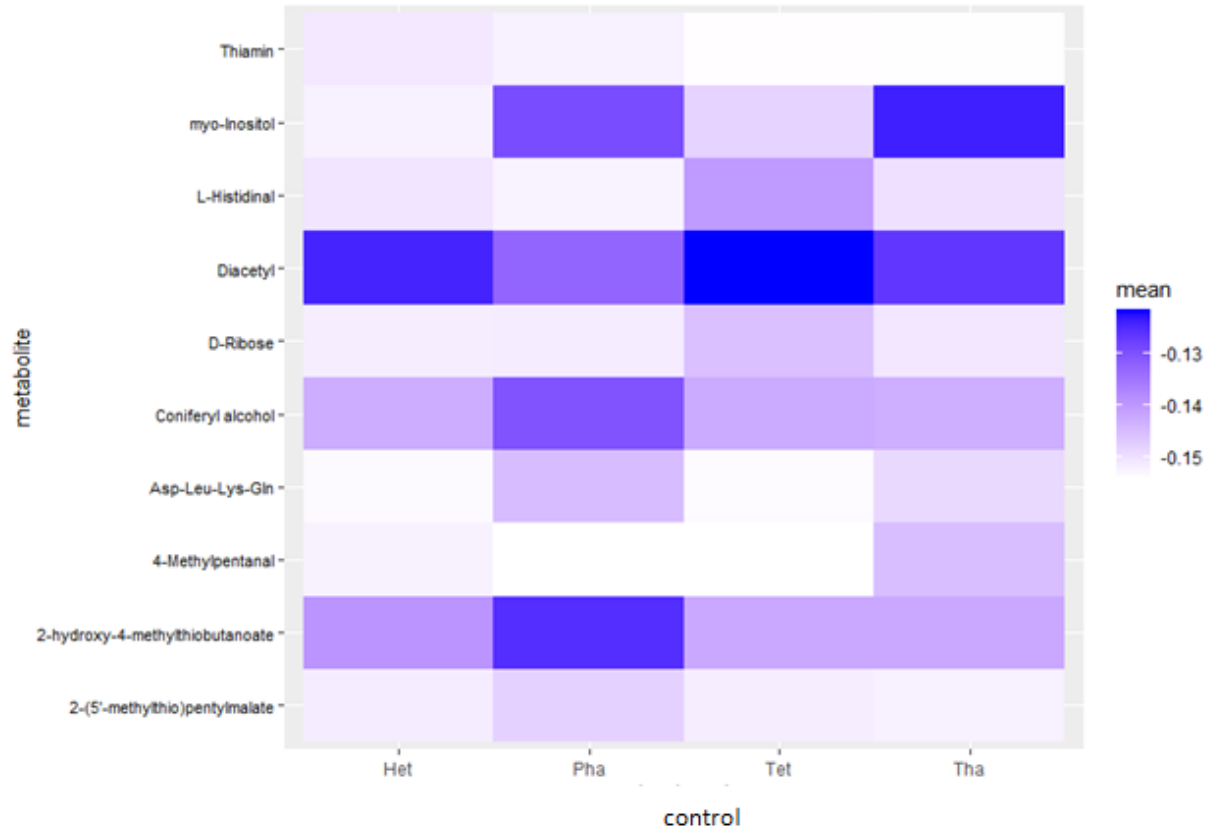

Figure 6. Significant differences of excreted metabolites between the control cultures of the four species, therefore: Het= Control culture of *H. akashiwo*, Pha= Control culture of *Phaeocystis* sp., Tet= Control culture of *Tetraselmis* sp., Tha= Control culture of *Thalassiosira* sp.

DISCUSSION

Based on our hypotheses, when filtrate with excreted metabolites from a competitor species was added to the culture of a target species then its growth would be affected negatively and intracellular phosphorus could increase as a growth strategy shift to the presence of a competitor's metabolites. Some of our results regarding cell growth show an opposite response of our original prediction. Specifically, the filtrate from *Phaeocystis* sp. and *Thalassiosira* sp. cultures had a positive effect on *H. akashiwo* and *Tetraselmis* sp. photosynthetic biomass (i.e. Chl-a) although no effects were observed in cell number. This response might be affected by the high concentration of Myo-Inositol from *Phaeocystis* sp. and *Thalassiosira* sp.

Myo-Inositol is one stereoisomer of a C6 sugar alcohol (Unfer *et al*. 2017) and is the most ordinary Inositol isomer in eukaryotic cells (Gillapsy, 2011) and has been found to support the growth of microalgae (Loewus and Murthy, 2000; Cho *et al*. 2015; Erbil *et al*. 2018). Specifically, the more quantity of Myo-inositol is added in the medium, the most photosynthetic biomass is produced as indicated by Cho *et al*. (2015). This can be explained as Myo-Inositol biosynthesis plays an important role in many metabolic and signal transduction pathways (Gillapsy, 2011; Basu *et al*. 2019) and helps eukaryotic organisms to survive by enhancing their defensive mechanism and adapting in stressful environments (Valluru & Van den Ende, 2011; Basu *et al*. 2019). Our findings also indicate that the addition of extra Myo-Inositol from *Phaeocystis* sp. and *Thalassiosira* sp. caused of a more pronounced effect on *H. akashiwo* than *Tetraselmis* sp. growth due to the limited ability of *H. akashiwo* to produce Myo-Inositol by itself. Consequently, Myo-Inositol can have a key role as growth enhancer compound.

Another important finding of this study was that the addition of *H. akashiwo* filtrate caused a consistent increase of intracellular P observed to all the other three species. Thiamin was the only metabolite which was present in the filtrate of *H. akashiwo* in higher amount than other species. Thiamin is a water-soluble B vitamin, known as B1 vitamin. Its synthesis occurs by certain microalgae (Helliwell, 2017; Alam *et al*. 2020) and is required for persistence and growth of many phytoplankton species (Gobler *et al*. 2007; Helliwell, 2017; Tandon *et al*. 2017; Higgins *et al*. 2018). Also, it has been proposed that thiamin has a vital role in the growth of chlorophyta and cryptophyta (Croft *et al*. 2006). In our study, despite the fact that Thiamin is required for growth, the addition of *H. akashiwo* filtrate seems to contribute to the accumulation of intracellular P in all the other species. Our analysis also showed that *H. akashiwo* control cultures did not have a higher residual P compared to the other species, thus it is unlikely that this rise in luxurious P can be attributed to higher incoming P from the mother cultures of *H. akashiwo*. On the other hand, it is possible that the response is triggered by unique metabolites that are present in such low abundances that are below the detection capacity of the method or due to labile trait of some toxins (Granéli & Hansen, 2006) that might not be detected.

H. akashiwo appears to increase its Chl-a content when metabolites from *Phaeocystis* sp. and *Thalassiosira* sp. were present, while leaving a significant amount of P remained unused in the medium. This suggests that *H. akashiwo* has the potential to produce higher photosynthetic biomass using lower P concentration in the presence of other species excreted metabolites. Due to the good relationship between Chl-a and total lipids (Rossi S. & Fiorillo I, 2010) as well as the ability of high lipid content of microalgae to be exploited as a renewable source for biodiesel production (Schenk *et al*. 2008; Huang *et al*. 2010; Mata *et al*. 2010; Chen *et al*. 2018) *H. akashiwo* can be considered as a promising species for sustainable biodiesel production when grown under certain conditions.

Another eco-friendly industrial process currently receiving a lot of attention (known as phycoremediation or else bioremediation (Gupta and Bux, 2019)) is the beneficial operation of P removal from wastewaters via microalgae (Powell *et al*. 2008; Powell *et al*. 2009; Powell *et al*. 2011; Rawat *et al*. 2011; Fathi *et al*. 2013; de Morais *et al*. 2015; Koutra *et al*. 2017; Chen *et al*. 2018; Gupta and Bux, 2019) as well as the exploitation of their biomass for biodiesel production (Rawat *et al*. 2011; Fathi *et al*. 2013; de Morais *et al*. 2015; Koutra *et al*. 2017; Chen *et al*. 2018; Gupta and Bux, 2019) and the further use (i.e. after the extraction of lipids) of biomass as feedstock for the production of animal feed or fertilizer (Rawat *et al*. 2011). Our findings indicate *H. akashiwo* is not a favorable choice for wastewater treatment due to the high concentration of remaining P under the effect of all the other species. On the other hand, *Tetraselmis* sp. seems to accumulate more Chl-a as a response of *Phaeocystis* sp. and *Thalassiosira* sp. filtrate, while reducing P in the medium. Consequently, *Tetraselmis* sp. is a promising species for P removal from wastewaters with the produced biomass generating additional benefits in biotechnological products.

In conclusion, our findings suggest that Myo-Inositol should be considered as natural growth promoter of microalgae. Furthermore, Thiamin in *H. akashiwo* detected at a greater concentration than in all other species' control cultures and might be the compound that led to a consistent increase of intracellular P of all species. Also *H. akashiwo* might be a favourable choice for the production of biodiesel due to the high concentration of Chl-a with low requirements in P. However, the large quantity of remaining phosphorus does not indicate it as a recommended option for water remediation. Our results indicate that by using excreted metabolites from one species to another species cell-culture can provide us not only with knowledge of ecological interest but also with various future ecological upgrades in biotechnological applications such as P-removal from wastewater and biodiesel production.

REFERENCES

Al-Ghelani H. M., AlKindi A. Y. A., Al-Akhzami Y. K. (2005). Harmful Algal Blooms: Physiology, Behavior, Population Dynamics and Global Impacts- A Review. Journal of Science. 10. 1-30. 10.24200/squjs.vol10iss0pp1-30.

Alam M. A., Xu J. L., Wang Z. (2020). Microalgae Biotechnology for Food, Health and High Value Products. Springer Nature Singapore Pte Ltd. Springer Singapore. 10.1007/978-981-15-0169-2.

Arzul G., Seguel M., Guzman I., and Erard le Denn E. (1999). Comparison of allelopathic properties in three toxic Alexandrium species. J. Exp. Mar. Bio. Ecol. 232, 285-295.

Bacellar Mendes L. B. & Vermelho A. B. (2013). Allelopathy as a potential strategy to improve microalgae cultivation. Biotechnology for biofuels, 6(1), 152.

Bache S. M. & Wickham H. (2014). magrittr: A Forward-Pipe Operator for R. R package version 1.5. https://CRAN.R-project.org/package=magrittr.

Bujak R., Struck-Lewicka W., Markuszewski M. J., Kaliszan R. (2015). Metabolomics for laboratory diagnostics. Journal of Pharmaceutical and Biomedical Analysis. 113. 108-120. 10.1016/j.jpba.2014.12.017.

Basu S., Basak A., Mahanty D. S., Bhattacharjee S., Adhikari J. (2019). Biosynthesis of myo-inositol in chloroplasts of salinity-stressed marine macroalga Ulva lactuca. Botanica, 25(1). 32–40.

Cáceres, C., Spatharis, S., Kaiserli, E. *et al*. (2019). Temporal phosphate gradients reveal diverse acclimation responses in phytoplankton phosphate uptake. ISME Journal. 13. 2834–2845. 10.1038/s41396-019-0473-1.

Chen J., Li J., Dong W., Zhang X., Tyagi R. D., Drogui P., Surampalli R. Y. (2018). The potential of microalgae in biodiesel production. Renewable and Sustainable Energy Reviews. 90. 336–346.

Cho K., Kim K., Lim N., Kim M., Ha J., Shin H. H., Kim M., Roh S.W., Kim D., Oda T. (2015). Enhanced biomass and lipid production by supplement of myo-inositol with oceanic microalga Dunaliella salina. Biomass & Bioenergy. 72. 1-7.

Croft M. T., Warren M. J., Smith A. J. (2006). Algae Need Their Vitamins. Eukaryotic Cell. 5 (8). 1175-1183. 10.1128/EC.00097-06.

Demirkaya C., Demirel Z., Imamoglu E., Dalay M., Demirci N. (2016). Investigation of the malic acid concentration on extremophilic red microalga galdieria sulphuraria.Deu Muhendislik Fakultesi Fen ve Muhendislik. 18. 101-101. 10.21205/deufmd.20165217550.

de Morais M. G. , Vaz B. da S., de Morais E. G., Costa J. A. (2015). Biologically Active Metabolites Synthesized by Microalgae. Biomed Res Int 2015. 835761. 10.1155/2015/835761.

de Vries A. & Ripley B. D. (2020). ggdendro: Create Dendrograms and Tree Diagrams Using 'ggplot2'. R package version 0.1.21. https://CRAN.R-project.org/package=ggdendro.

Erbil G. Ç., Durmaz Y., Tamtürk F., Konar N. (2018). Effect of Myo-inositol Concentration on the Growth of Marine Porphyridium Cruentum. Journal of Advanced Agricultural Technologies. 5:4. 289-292. 10.18178/joaat.5.4.289-292.

Fathi A., Azooz M., Al-Fredan M. (2013). Phycoremediation and the potential of sustainable algal biofuel production using watewater. Journal of Applied Sciences. 10. 189-194. 10.3844/ajassp.2013.189.194.

Fattal-Valevski A. (2011). Thiamine (Vitamin B1). Journal of Evidence-Based Complementary & Alternative Medicine. 16(1). 12-20. 10.1177/1533210110392941.

Fistarol G.O., Legrand C., Granéli E. (2003). Allelopathic effect of Prymnesium parvum on a natural plankton community. Marine Ecology Progress Series. 255. 115-125. 10.3354/meps255115.

Fistarol G., Legrand C., Selander E., Hummert C., Stolte W., Granéli E. (2004). Allelopathy in Alexandrium spp.: Effect on a Natural Plankton Community and on Algal Monocultures. Aquatic Microbial Ecology. 35. 45-56. 10.3354/ame035045.

Fistarol G., Legrand C., Granéli E. (2005). Allelopathic Effect on a Nutrient-Limited Phytoplankton Species. Aquatic Microbial Ecology. 41. 153-161. 10.3354/ame041153.

Fridolfsson E., Lindehoff E., Legrand C., Hylander S. (2020). Species-specific content of thiamin (vitamin B_1) in phytoplankton and the transfer to copepods. Journal of plankton research. 42(3). 274–285. 10.1093/plankt/fbaa015.

Gantar M., Berry J. P., Thomas S., Wang M., Perez R., Rein K. S. (2008). Allelopathic activity among Cyanobacteria and microalgae isolated from Florida freshwater habitats. FEMS microbiology ecology. 64(1). 55–64. 10.1111/j.1574-6941.2008.00439.x

Gillaspy G. E. (2011). The cellular language of myo-inositol signaling. New Phytologist. 192(4). 823-839. 10.1111/j.1469-8137.2011.03939.x

Gobler C. J., Norman C., Panzeca C., Taylor G. T., Sañudo-Wilhelmy S. A. (2007) Effect of B-vitamins (B1, B12) and inorganic nutrients on algal bloom dynamics in a coastal ecosystem. Aquat Microb Ecol. 49:181-194. 10.3354/ame01132.

Granéli E. & Johansson N. (2003). Increase in the Production of Allelopathic Substances by Prymnesium parvum Cells Grown Under N- or P-Deficient Conditions. Harmful Algae. 2. 135-145. 10.1016/S1568-9883(03)00006-4.

Granéli E. & Hansen P. (2006). Allelopathy in Harmful Algae: A Mechanism to Compete for Resources?. 10.1007/978-3-540-32210-8_15.

Granéli E., Salomon P., Fistarol G. (2008). The Role of Allelopathy for Harmful Algae Bloom Formation. 10.1007/978-1-4020-8480-5_5.

Gross E. M. (2009). Allelochemical Reactions. Encyclopedia of Inland Waters.

Guillard R.R.L. (1975). Culture of phytoplankton for feeding marine invertebrates. pp 26-60. In Smith W.L. and Chanley M.H (Eds.) Culture of Marine Invertebrate Animals. Plenum Press, New York, USA.

Gupta S. K., Bux F. (2019). Application of Microalgae in Wastewater Treatment Volume 1: Domestic and Industrial Wastewater Treatment. Springer Nature Switzerland AG.

Helliwell K. E. (2017). The roles of B vitamins in phytoplankton nutrition: new perspectives and prospects. New Phytol. 216(1). 62-68. 10.1111/nph.14669.

Henry L. & Wickham H. (2020). purrr: Functional Programming Tools. R package version 0.3.4. https://CRAN.R-project.org/package=purrr.

Higgins B. T., Wang Q., Du S., Hennebelle M., Taha A. Y., Fiehn O., VanderGheynst J. S. (2018). Impact of thiamine metabolites and spent medium from Chlorella sorokiniana on metabolism in the green algae Auxenochlorella protothecioides. Algal Research. 33(Algal Res. 6 2014). 197-208. 10.1016/j.algal.2018.05.019.

Huang G., Chen F., Wei D., Zhang X., Chen G. (2010). Biodiesel production by microalgal biotechnology. Applied Energy. 87. 38-46. 10.1016/j.apenergy.2009.06.016.

Hutchinson G. E. (1961). The Paradox of the Plankton. The American Naturalist. 95: 137-145

Inderjit, Dakshini, K.M.M. (1994). Algal allelopathy. The Botanical Review. 60. 182–196. 10.1007/BF02856576

Ingle R. A. (2011). Histidine biosynthesis. The arabidopsis book. 9. e0141. 10.1199/tab.0141.

IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN). (1984). Nomenclature and symbolism for amino acids and peptides. Biochem J. 219(2). 345-373. 10.1042/bj2190345.

Kassambara A. (2020). ggpubr: 'ggplot2' Based Publication Ready Plots. R package version 0.3.0. https://CRAN.R-project.org/package=ggpubr.

Kim H.G. (2006) Mitigation and Controls of HABs. In: Granéli E., Turner J.T. (eds) Ecology of Harmful Algae. Ecological Studies (Analysis and Synthesis), vol 189. Springer, Berlin, Heidelberg.

Kirpenko N. I., Kurashov Y. A., & Krylova Y. V. (2012). Component Composition of Exometabolites in Cultures of Some Algae. Hydrobiological Journal. 48(3). 59–70. 10.1615/hydrobj.v48.i3.60.

Klassen A., Faccio A. T., Canuto G. A., da Cruz P. L. R., 2, Ribeiro H. C., Tavares M. F. M., Sussulini A. (2017). Metabolomics: Definitions and Significance in Systems Biology. Advances in Experimental Medicine and Biology. 965. 3-17. 10.1007/978-3-319-47656-8_1.

Koutra E., Grammatikopoulos G., Kornaros M. (2017). Microalgal post-treatment of anaerobically digested agro-industrial wastes for nutrient removal and lipids production. Bioresource Technology. 224. 473–480.

Kuznetsova A., Brockhoff P.B. and Christensen R.H.B. (2017). "lmerTest Package: Tests in Linear Mixed Effects Models." Journal of Statistical Software, 82(13), pp. 1–26. doi: 10.18637/jss.v082.i13.

Leflaive J., Loïc Ten-Hage. (2007). Algal and cyanobacterial secondary metabolites in freshwaters: a comparison of allelopathic compounds and toxins. Freshwater Biology. 52. 199–214.

Legrand C., Rengefors K., Fistarol G., Granéli E. (2003). Allelopathy in phytoplankton - Biochemical, ecological and evolutionary aspects. Phycologia. 42. 406-419. 10.2216/i0031-8884-42-4-406.1.

Lenth R. (2020). emmeans: Estimated Marginal Means, aka Least-Squares Means. R package version 1.5.0. https://CRAN.R-project.org/package=emmeans.

Lin S., Litaker R.W., Sunda W.G. (2016). Phosphorus physiological ecology and molecular mechanisms in marine phytoplankton. J Phycol. 52(1). 10-36. 10.1111/jpy.12365.

Liu D. (2008). Phytoplankton diversity and ecology in estuaries of southeastern NSW, Australia. PhD thesis. University of Wollongong. Northfields Ave Wollongong, Australia.

Loewus F.A., Murthy P.P.N. (2000). Myo-inositol metabolism in plants, Plant Science. 150. 1–19.

Long M., Tallec K., Soudant P., Le Grand F., Donval A., Lambert C., Sarthou G., Jolley D., Hégaret H. (2018). Allelochemicals from Alexandrium minutum induce rapid inhibition of metabolism and modify the membranes from Chaetoceros muelleri. Algal Research. 35. 508-518. 10.1016/j.algal.2018.09.023.

Macías F., Galindo J. L., García-Díaz M., Galindo J. (2008). Allelopathic agents from aquatic ecosystems: potential biopesticides models. Phytochem. Rev. 7. 155-178. 10.1007/s11101-007-9065-1.

Mahoney D. E., Hiebert J. B., Thimmesch A., Pierce J. T., Vacek J. L., Clancy R. L., Sauer A. J., Pierce J. D. (2018). Understanding D-Ribose and Mitochondrial Function. Adv Biosci Clin Med. 6(1). 1-5. 10.7575/aiac.abcmed.v.6n.1p.1. PMID: 29780691; PMCID: PMC5959283.

Markou G. & Nerantzis E. (2013). Microalgae for high-value compounds and biofuels production: A review with focus on cultivation under stress conditions. Biotechnology Advances. 31. 1532–1542. 10.1016/j.biotechadv.2013.07.011.

Martin P., Dyhrman S. T., Lomas M. W., Poulton N. J., Van Mooy B. A. (2014). Accumulation and enhanced cycling of polyphosphate by Sargasso Sea plankton in response to low phosphorus. Proceedings of the National Academy of Sciences of the United States of America. 111(22). 8089–8094. 10.1073/pnas.1321719111.

Mata T. M., Martins A. A., Caetano N. S. (2010). Microalgae for biodiesel production and other applications: A review. Renewable and Sustainable Energy Reviews. 14(1). 217–232.

National Center for Biotechnology Information. PubChem Database. 4-Methylpentanal, CID=129, https://pubchem.ncbi.nlm.nih.gov/compound/4-Methylpentanal (accessed on June 20, 2020).

Neuwirth E. (2014). RColorBrewer: ColorBrewer Palettes. R package version 1.1-2. https://CRAN.R-project.org/package=RColorBrewer.

Papanikolopoulou L, Roelke DL, Kokkoris G, Dimitrakopoulos P, Smeti E, Spatharis S. (2018). Interplay between r- and K-strategists leads to phytoplankton underyielding when the resources are pulsed. Oecologia. 186. 755-764.

Parsons T.R., Maita Y., Lalli C.M. (1984). A manual of Chemical and Biological Methods for Seawater Analysis (Oxford: Pergamon Press).

Pinheiro J., Bates D., DebRoy S., Sarkar D., R Core Team. (2020). nlme: Linear and Nonlinear Mixed Effects Models. R package version 3.1-149, https://CRAN.R-project.org/package=nlme.

Poulin R.X., Hogan S., Poulson-Ellestad K.L.,Brown E., Fernández F. M., Kubanek J. (2018a). Karenia brevis allelopathy compromises the lipidome, membrane integrity, and photosynthesis of competitors. Scientific Reports. 8. 9572. 10.1038/s41598-018-27845-9.

Poulin R. X., Poulson-Ellestad K.L., Roy J. S., Kubanek J. (2018b). Variable allelopathy among phytoplankton reflected in red tide metabolome. Harmful Algae. 71. 50-56. 10.1016/j.hal.2017.12.002.

Poulson-Ellestad K. L., Jones C. M., Roy J., Viant M. R., Fernández F. M., Kubanek J., Nunn B. L. (2014). Metabolomics and proteomics reveal impacts of chemically mediated competition on marine plankton. Proceedings of the National Academy of Sciences of the United States of America. 111(24). 9009-9014. 10.1073/pnas.1402130111.

Powell N., Shilton A. N., Pratt S., Chisti Y. (2008). Factors Influencing Luxury Uptake of Phosphorus by Microalgae in Waste Stabilization Ponds. Environmental Science & Technology. 42 (16). 5958-5962. 10.1021/es703118s.

Powell N., Shilton A., Chisti Y., Pratt S. (2009). Towards a luxury uptake process via microalgae – Defining the polyphosphate dynamics. Water Research. 43. 4207 – 4213.

Powell N, Shilton A, Pratt S, Chisti Y. (2011). Luxury uptake of phosphorus by microalgae in full-scale waste stabilization ponds. Water Sci Technol. 63(4). 704-709. 10.2166/wst.2011.116.

R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Rao N. N., Gómez-García M. R., Kornberg A. (2009). Inorganic Polyphosphate: Essential for Growth and Survival. Annual Review of Biochemistry. 78:1. 605-647.

Rawat I., Ranjith- Kumar R., Mutanda T., Bux F. (2011). Dual role of microalgae: Phycoremediation of domestic wastewater and biomass production for sustainable biofuels production. Applied Energy. 88. 3411–3424.

Reigosa M. J., Sanchez-Moreiras A., Gonzales L. (1999) Ecophysiological approach in allelopathy. Critical Reviews in Plant Sciences. 18. 577–608.

Rengefors K. & Legrand C. (2001). Toxicity in Peridinium aciculiferum - An adaptive strategy to outcompete other winter phytoplankton? Limnol. Oceanogr. 46. 1990–1997.

Rengefors K. & Legrand C. (2007). Broad allelopathic activity in Peridinium aciculiferum (Dinophyceae). Eur. J. Phycol. 42. 341–349.

Rice E. L. (1984). Allelopathy (Orlando, Florida: Academic Press).

Robinson D. & Hayes A. (2018). broom: Convert Statistical Analysis Objects into Tidy Tibbles. R package version 0.5.1. https://CRAN.R-project.org/package=broom.

Romano S., Dittmar T., Bondarev V., Weber R. J., Viant M. R., Schulz-Vogt H. N. (2014). Exo-metabolome of Pseudovibrio sp. FO-BEG1 analyzed by ultra-high resolution mass spectrometry and the effect of phosphate limitation. PloS one. 9(5). e96038. doi.org/10.1371/journal.pone.0096038.

Rossi S. & Fiorillo I. (2010). Biochemical features of a protoceratium reticulatum red tide in Chipana Bay (Northern Chile) in summer conditions. Scientia Marina. 74(4). 633–642. 10.3989/scimar.2010.74n4633.

Sardans, J., Peñuelas, J. & Rivas-Ubach, A. (2011). Ecological metabolomics: overview of current developments and future challenges. Chemoecology. 21. 191–225. 10.1007/s00049-011-0083-5.

Sathasivam R., Radhakrishnan R., Hashem A., Allah E. (2019). Microalgae metabolites: A rich source for food and medicine. Saudi Journal of Biological Sciences. 26. 709-722. 10.1016/j.sjbs.2017.11.003.

Schenk P.M., Thomas-Hall S.R., Stephens E., Marx U. C., Mussgnug J. H., Posten C., Kruse O., Hankamer B. (2008). Second Generation Biofuels: High-Efficiency Microalgae for Biodiesel Production. BioEnergy Research. 1. 20–43. 10.1007/s12155-008-9008-8.

Schmidtke A., Gaedke U., Weithoff G. (2010). A mechanistic basis for underyielding in phytoplankton communities. Ecology. 91. 212-21.

Shikata T., Yoshikawa S., Matsubara T., Tanoue W., Yamasaki Y., Shimasaki Y., Matsuyama Y., Oshima Y., Jenkinson I. R., Honjo T. (2008). Growth dynamics of Heterosigma akashiwo (Raphidophyceae) in Hakata Bay, Japan, European Journal of Phycology. 43:4. 395-411. 10.1080/09670260801979295.

Singh D., Nedbal L., Ebenhöh O. (2018). Modelling phosphorus uptake in microalgae. Biochemical Society Transactions.46. 483–490. 10.1042/BST20170262.

Skjånes K., Rebours C., Lindblad P. (2013). Potential for green microalgae to produce hydrogen, pharmaceuticals and other high value products in a combined process. Critical reviews in biotechnology. 33(2). 172–215. 10.3109/07388551.2012.681625.

Solovchenko A. E, Ismagulova T. T, Lukyanov A. A, Vasilieva S. G, Konyukhov I. V, Pogosyan S. I, Lobakova E. S, Gorelova O. A. (2019). Luxury phosphorus uptake in microalgae. Journal of applied phycology, 31, 2755-2770. 10.1007/s10811-019-01831-8.

Tan K., Huang Z., Ji R., Qiu, Y., Wang Z., Liu J. (2019). A review of allelopathy on microalgae. Microbiology. 165. 10.1099/mic.0.000776.

Tandon P., Jin Q., Huang L. (2017). A promising approach to enhance microalgae productivity by exogenous supply of vitamins. Microbial cell factories. 16(1). 219. 10.1186/s12934-017-0834-2

Thomas M. P., Mills S. J., Potter B. V. (2016). The "Other" Inositols and Their Phosphates: Synthesis, Biology, and Medicine (with Recent Advances in myo-Inositol Chemistry). Angewandte Chemie (International ed. in English). 55(5). 1614–1650. 10.1002/anie.201502227.

Tugizimana F., Piater L., Dubery I. (2013). Plant metabolomics: A new frontier in phytochemical analysis. South Africa Journal Science. 109(5/6), Art. #0005, 11 pages. 10.1590/ sajs.2013/20120005.

Unfer V., Facchinetti F., Orrù B., Giordani B., Nestle J. (2017). Myo-inositol effects in women with PCOS: a meta-analysis of randomized controlled trials. Endocrine connections. 6(8). 647–658. 10.1530/EC-17-0243.

Valluru R. & Van den Ende W. (2011). Myo-inositol and beyond-Emerging networks under stress. Plant Science. 181. 387– 400. 10.1016/j.plantsci.2011.07.009.

Von Elert E. & Jüttner E. (1997). Phosphorus limitation and not light controls the extracellular release of allelopathic compounds by Trichonormus doliulum (Cyanobacteria). Limn. Oceanogr. 42. 1796-1802.

Watanabe M., Kohata K., Kunugi M. (1988). Phospahte accumulation and metabolism by Heterosigma akashiwo (Raphodophycea) during diel vertical migration in astratified microcosm.Journal of Phycology. 24(1). 22-28. 10.1111/j.1529-8817.1988.tb04452.x.

Weissbach A. (2011). The role of allelopathy in microbial food webs. Doctoral dissertation, School of Natural Sciences, Linnaeus University.

Wickham H. (2007). Reshaping Data with the reshape Package. Journal of Statistical Software, 21(12), 1-20. URL http://www.jstatsoft.org/v21/i12/.

Wickham H. (2011). "The Split-Apply-Combine Strategy for Data Analysis." Journal of Statistical Software. 40(1). 1–29. http://www.jstatsoft.org/v40/i01/.

Wickham H. (2016). ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York.

Wickham H. (2017). tidyverse: Easily Install and Load the 'Tidyverse'. R package version 1.2.1. https://CRAN.R-project.org/package=tidyverse.

Wickham H. (2018). scales: Scale Functions for Visualization. R package version 1.0.0. https://CRAN.R-project.org/package=scales.

Wickham H. & Henry L. (2020). tidyr: Tidy Messy Data. R package version 1.0.2. https://CRAN.R-project.org/package=tidyr.

Wickham H., François R., Henry L. and Müller K. (2020). dplyr: A Grammar of Data Manipulation. R package version 0.8.5. https://CRAN.R-project.org/package=dplyr.

Wijffels R., Kruse O., Hellingwerf K. (2013). Potential of industrial biotechnology with cyanobacteria and eukaryotic microalgae. Current opinion in biotechnology. 24. 10.1016/j.copbio.2013.04.004.

Zhang Y., Wang J., Tan L. (2019). Characterization of allelochemicals of the diatom Chaetoceros curvisetus and the effects on the growth of Skeletonema costatum. Science of The Total Environment. 660. 10.1016/j.scitotenv.2019.01.056.

Network

https://www.algaebase.org/

APPENDIX

Appendix I: Pre-experimental procedure

A pre-experimental monitoring of microalgae's growth rates occurred in HCMR in order to gain an understanding of the capability of selected microalgae strains to grow under an intense P deficiency (i.e. F/2 medium with P/24). Before the experiment started we had monitored growth rate of pilot cultures by measuring cell concentration according to Eq. (2) as a function of time to define the appropriate time to start the experiment. It was noticed that *Thalassiosira* sp. and *Phaeocystis* sp. lagged four days behind the other species. Experimental MCs and treatments were initiated at a different time points to ensure that they would all reach stationary phase simultaneously.

$$\text{Growth rate } (d^{-1}) = \frac{\ln(N_2 - N_1)}{(t_2 - t_1)} \quad (2)$$

Where N1 was the cell concentration (ml^{-1}) at time (i.e. in days) t1; at this point the culture had passed the lag phase and N2 was the cell concentration at time t2; when culture had reached its maximum cell concentration.

Appendix II

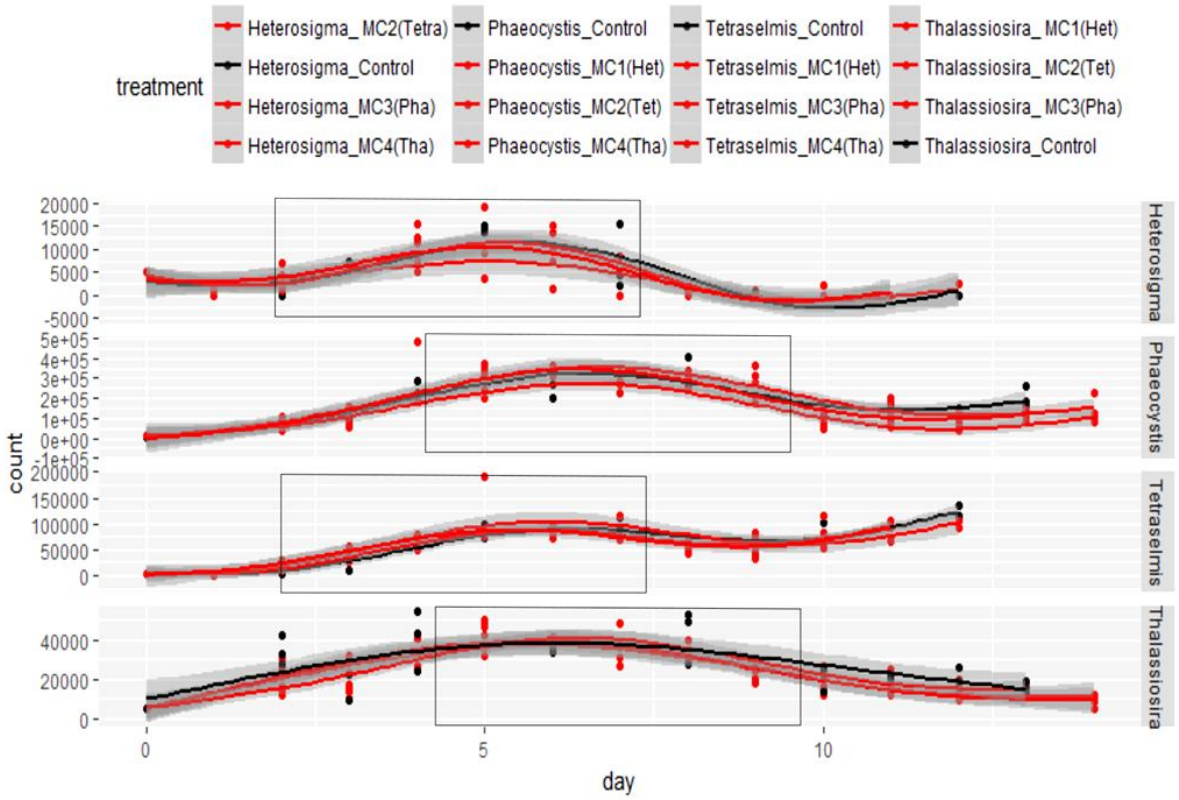

Figure 7. Cell count of bioactivity treatments (i.e. red lines) and control cultures (i.e. black lines) of all species. Boxes indicate when dilutions were started while cultures had reached the stationary phase. Fitted gam with 5 splines.

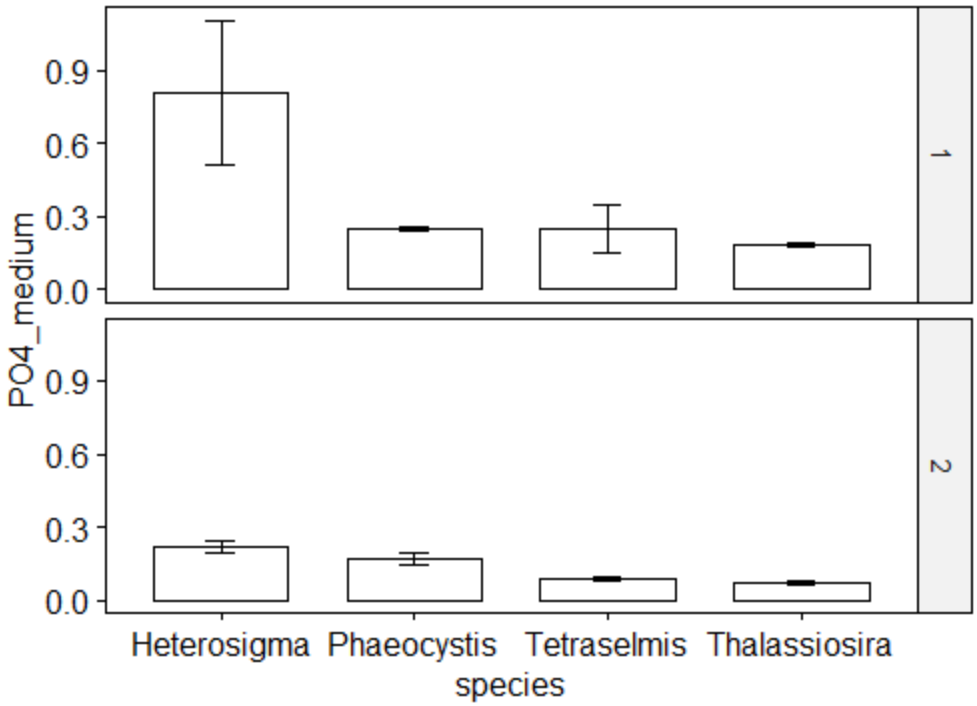

Figure 8. The residual P in the medium of the control cultures of time point 1 (day 0, i.e initial conditions) and time point 2 (day 2, i.e. the residual P from the medium which amplified the P of time point 3 or else day 4).

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to Dr. Sofie Spatharis for providing me guidance, freedom, advice and comments throughout the course of the project.

Also I am very thankful to Dr. Eva Smeti for guiding me through this research and her prompt and useful suggestions about my work.

I would like to express my sincere appreciation to Dr. Sofie Spatharis and Dr. Eva Smeti for helping me with the revision of my thesis.

I would also like to thank Dr. Athena Economou-Amilli and Dr. Dionysios Raitsos for providing me beneficial comments about the text of my thesis.

I sincerely want to thank Dr. Ioanna Varkitzi for constantly motivating me and for helping me in the pre-experimental design which occurred in HCMR's laboratory.

Last but not least, I want to thank all my fellow labmates and especially Marta Lamorgese for the excellent cooperation in the experimental procedure and her helpful observations through the whole experiment.